Heavy Metal(loid) Transformations by Bacteria Isolated from Extreme Environments

By

Chris Maltman

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Department of Microbiology
University of Manitoba
Winnipeg, Manitoba
Canada

Abstract

The research presented here studied bacteria from extreme environments possessing strong resistance to highly toxic oxyanions of Te, Se, and V. The impact of tellurite on cells of aerobic anoxygenic phototrophs and heterotrophs from freshwater and marine habitats was investigated. Physiological responses of cells to TeO_3^{2-} varied. In its presence, biomass either increased, remained similar or decreased, with ATP production following the same trend. Four detoxification strategies were observed: 1) Periplasmic based reduction; 2) Reduction needing an intact cytoplasmic membrane; 3) Reduction involving an undisturbed whole cell; and 4) Membrane associated reduction. The first three require *de novo* protein synthesis, while the last was constitutively expressed. We also investigated two enzymes responsible for tellurite reduction. The first came from the periplasm of deep-ocean hydrothermal vent strain ER-Te-48 associated with tube worms. The second was a membrane associated reductase from *Erythromonas ursincola,* KR99. Both could also use tellurate as a substrate. ER-Te-48 also has a second periplasmic enzyme which reduced selenite.

Additionally, we set out to find new organisms with the ability to resist and reduce Te, Se, and V oxyanions, as well as use them for anaerobic respiration. New strain CM-3, a Gram negative, rod shaped bacterium from gold mine tailings of the Central Mine in Nopiming Provincial Park, Canada, has very high level resistance and the capability to perform dissimilatory anaerobic reduction of tellurite, tellurate, and selenite. Its partial 16S rRNA gene sequencing revealed a 99.0% similarity to *Pseudomonas reactans*. We also discovered that the epibiotic bacterial community associated with tube worms living in the vicinity of deep sea hydrothermal vents of the Juan de Fuca Ridge in the Pacific Ocean can respire anaerobically on tellurite, tellurate, selenite, selenate, metavanadate and/or orthovanadate. Out of 107 isolates

tested, 106 were capable of respiration on one or more of the oxyanions. Based on partial 16S rRNA gene sequences, the bacterial community is phylogenetically and taxonomically highly diverse.

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♦ - No K_2TeO_3; ■ - 500 µg/mL K_2TeO_3. Error bars represent one standard
deviation.

List of abbreviations

ΔG_f^o	Gibbs free energy of formation
AAP	aerobic anoxygenic phototroph
ACN	acetonitrile
AMR	anaerobic metalloid respiration
ATP	adenosine triphosphate
AV	Axial volcano
CFU	colony forming unit
DHB	dihydroxybenzoic acid
DNA	deoxyribonucleic acid
E^o	standard electrode potential
ER	Explorer ridge
ETC	electron transport chain
$FADH_2$	flavin adenine dinucleotide
GETS	Graduate Enhancement of Tri-Council Stipends
K_m	Michaelis constant
MALDI	matrix-assisted laser desorption/ionization
MIC	minimum inhibitory concentration
MS	minimal salts
NADH	nicotinamide adenine dinucleotide
NCBI	National Center for Biotechnology Information
NSERC	National Science and Engineering Research Council
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PNSB	purple non-sulfur bacteria
ppm	parts per million
RNA	ribonucleic acid
RO	rich organic
ROPOS	remotely operated platform for ocean science
ROS	radical oxygen species
ROV	remote operated vehicle
R/V	research vessel
SDS	sodium dodecyl sulfate
TOFMS	time-of-flight mass spectrometry
V_{max}	maximal rate

Chapter 1.

Introduction

1.1. Bacterial metal(loid) interactions and their significance

Microorganisms possess a wide range of extraordinary abilities from production of bioactive molecules (Bhatnagar & Kim, 2010) to resistance and transformation of highly toxic compounds (Yurkov et al., 1996; Li et al., 2009; Van Agteren et al., 2013; Arenas et al., 2014). Of great interest are bacteria which can convert deleterious oxyanions of the metalloids tellurium (Te) and selenium (Se), and the transition metal vanadium (V), hereafter referred to as metal(loid)s, from one oxidation state to another through reduction. Currently, research into bacterial interactions with these elements has been lagging behind investigation of other metals such as nickel (Ni), molybdenum (Mo), tungsten (W), iron (Fe), and cobalt (Co). This is mainly due to the fact that the latter metals have long been known as essential for life through their involvement in many cellular activities (Schonheit et al, 1979; Waldron et al, 2009; Glass and Orphan, 2012). Bacterial interactions with the aforementioned Te, Se, and V have received attention (Turner, 2013; Zonaro et al., 2015; Romaidi & Ueki, 2016), but there is still much unknown about high level resistance. In recent years there has been more focus in this area due to increased environmental contamination from industrial and agricultural activities (Fujii et al., 1988; Macy et al., 1993; Prakash et al., 2001; Li et al., 2009; Yang et al., 2014). Microbial reduction of Te, Se, and V oxyanions, under both aerobic and anaerobic conditions, results in the detoxification of these harmful compounds (Yurkov & Beatty, 1998; Li et al., 2014; Javed et al., 2016). Therefore, heavy metal(loid) reducers have an important role in nature. Their removal of contaminants can allow many species to grow in environments with elevated toxic metal(loid) concentrations (Yurkov et al., 1999; Rathgeber et al., 2002; Csotonyi et al., 2006; Bajaj & Winter, 2014; Epelde et al., 2015). The details of interactions between microbes and high levels of metal(loid)s are still not well investigated, although some initial steps have already been done.

The increased environmental concentration of these toxins has led to the search for removal methods, which will not in turn cause more pollution. Some chemicals and resins have been used to neutralize and/or remove toxic oxyanions (Kim et al., 2004; Elwakeel et al., 2009), however, they can be expensive and result in increased release of xenobiotic compounds, which is problematic. More interest in biological methods of dealing with metal(loid)s has arisen as it would lead to a 'greener' environmentally friendly way for clean-up of pollutants (Gadd, 2010). Microbes reducing oxyanions from highly toxic states to less toxic elemental forms have been in the spotlight as a means to remediate contaminated locations. Bioremediation has been explored for removal of xenobiotics, metals, and radioactive compounds (Pieper & Reineke, 2000; Ruggiero et al., 2005; Shah & Nongkynrih, 2007; Gadd, 2010; Jadhav et al., 2010; Yong & Zhong, 2010; Dogan et al., 2011; Wasi et al., 2013), but little has been proposed regarding Te, Se, and V oxyanion treatments. The use of *Thauera selenatis* for removal of selenate/selenite from drainage water (Macy et al., 1993; Cantafio et al., 1996), and Te oxyanion clean-up from waste using *Pseudomonas mendocina*, strain MCM B-180 (Rajwade & Paknikar, 2003) and *Pseudoalteromonas* sp., EPR3 (Bonificio & Clarke, 2014) has been studied, along with using microbial communities for Se and Te remediation (Luek et al., 2014; Ramon-Ruiz et al., 2016). While all these approaches did result in removal of the majority of Se and Te contaminants, initial concentrations of the oxyanions were low, and in the case of *T. selenatis*, required over 120 days before significant remediation started to take place and many other factors needed to be adjusted and controlled (Cantafio et al., 1996). Therefore, currently proposed strains leave much room for improvement. Bacteria possessing greater resistance with the ability for faster reduction will prove to be more efficient and effective, allowing for feasible bioremediation.

Another area of great interest involving biological transformations of metal(loid)s is biometallurgy (Ilyas & Lee, 2014). This involves the use of microbes for retrieval, or 'mining', of the desired elements from ores. Currently, the costs associated with classic techniques of mining are increasing and efficiency is declining. Therefore, the use of biotic methods for recovery is gaining popularity (Rawlings & Silver, 1995; Zhuang et al., 2015). Some approaches are already employed for several applications. *Thiobacillus ferrooxidans* and *T. thiooxidans*, which are capable of Fe- and S-oxidation, respectively, have been used to disrupt the FeS_2 (pyrite) matrix of ores containing metals of interest. The biooxidation is 10^6 times faster than abiotic oxidation (Brierley & Brierley, 2002), highlighting the appeal. The process also results in the release of solubilized Te and V (Krebs et al., 1997), providing a further source of contamination. With Te, for example, a biological approach to recovery is highly sought after (Turner et al., 2012). The element is quite scarce in the Earth's crust, and the current methods for production and recycling are inefficient, with up to 90% of Te being lost (Claessens & White, 1993; Bonificio & Clarke, 2014). Currently, separation and purification is highly complex. One method used in the United States is a week long process, which involves reducing tellurium dioxide (TeO_2) to elemental tellurium (Te^0) through high pressure/temperature autoclaving in concentrated hydrochloric acid and sulfur dioxide (Bonificio & Clarke, 2014). Since this is tedious and involves dangerous chemicals, an alternative purification would be highly desirable from a safety and environmental perspective. Projections indicate that recycling of Te could virtually eliminate problems associated with its scarcity (Marwede & Reller, 2012), further fueling the search for bacteria with the potential for microbial reclamation of the highly critical and rare element. Not only would it provide a means of recovery, but also bioremediation, aiding in removal from the biosphere.

As one can see, there are significant reasons to search for microorganisms capable of interacting with very high levels of Te, Se, and V. Many microbes currently under investigation, such as *Citromicrobium bathyomarinum, Erythrobacter litoralis, Erythromicrobium ezovicum, E. hydrolyticum, E. ramosum, Erythromonas ursincola, Pseudoalteromonas spiralis, P. telluritireducens, Roseococcus thiosulfatophilus, Sandaracinobacter sibiricus,* and *Shewanella frigidimarina* relative, strain ER-Te-48 (Yurkov et al., 1996; Rathgeber et al., 2002; Csotonyi et al., 2006; Rathgeber et al., 2006), among others (Yurkov & Csotonyi, 2003; Arenas et al., 2014; Neethu et al., 2015), have come from extreme environments, making these locales hot-spots for study.

1.2. Extreme environments and extremophiles

Extreme environments are habitats where one would not expect to find life. Conditions there represent the limits at which organisms can survive. Hence, species diversity is low and some major taxonomic groups are not present (Brock, 1979). In order for microbes to exist in such abnormal areas, they must have undergone extensive adaptations, selected over time by nature. Microorganisms inhabiting harsh ecological niches that have optimal fitness only under such radical conditions are deemed extremophiles. Those which grow best at, or near to, conventionally 'anthropocentric' conditions, but can tolerate more extreme situations, are considered extremotolerant. They are classified based on the parameter which can be endured, such as pH, temperature, pressure, and water availability. Those which can withstand high levels of metal(loid)s have been referred to as metallophiles (Nies, 2000). Investigation of the microbial communities, which have managed to establish themselves under harsh conditions, has helped further our understanding of how life has evolved. Even though extreme environments have a low global distribution, they harbor a relatively high proportion of microbes considered valuable

to science and technology (Malik et al., 2013), such as antibiotic producing bacteria (Patel & Amaresan, 2014) and those used in biodegradation of pollutants (Yong & Zhong, 2010). These habitats contain representatives from many genera, providing a wealth of information on biodiversity and microbial physiology. As well, many of them possess the ability to resist and reduce very high levels of toxic metal(loid) compounds, which show great biotechnological potential, specifically in the areas of bioremediation and biometallurgy (Zannoni et al., 2008) discussed earlier.

1.2.1. Bacterial diversity in extreme environments

Microbial life can be found almost everywhere, no matter how sever the conditions are (Rothschild & Mancinelli, 2001). From the deepest seafloor to the top of the highest mountain or the hottest desert to the coldest Antarctic plain, microorganisms have been recovered. Extreme habitats can support diverse bacterial communities (Moyer et al., 1995; Teske et al., 2002; Huber et al., 2010; Campbell et al., 2013; Makhalanyane et al., 2013; Piubeli et al., 2015). While many genera are represented across different locations, diversity at individual sites can be limited. For example, acid mine drainage is usually dominated by iron-oxidizing, acidophilic genera, such as *Ferrovum* or *Acidothiobacillus* (Jones et al., 2015; Sun et al., 2015), while the high temperature and acidic environment at Yellowstone National Park, USA, gives rise to an endolithic community primarily comprised of *Mycobacterium* spp. (Walker et al., 2005). With metal contaminated locales, such as mine tailings or industrial effluent, microbial communities greatly differ from site to site (Roane & Kellogg, 1996; Sauvain et al., 2014; Brito et al., 2015; Epelde et al., 2015). The study of bacterial resistance and reduction of highly toxic metal(loid) oxyanions has been gaining momentum (Yurkov & Beatty, 1998; Malik et al., 2013; Zijuan et al., 2013). As extreme environments contain such microorganisms (Yurkov et al., 1996; Csotonyi et al., 2006;

Navarro et al., 2013; Arenas et al., 2014; Csotonyi et al., 2015), they provide us with an opportunity to study the interaction between microbes and metal(loid)s.

1.2.2. Deep sea hydrothermal vents

Considered to be the closest match to conditions present early in the Earth's development, deep sea hydrothermal vents are of great interest. However, our knowledge of these locales, including their actual abundance, is limited, with new discoveries emerging from each expedition (Rogers et al., 2012). Much of this is due to logistical problems associated with exploring areas of the deep ocean. The vents themselves are only present at areas of geological instability in the ocean floor, such as faults and spreading centers, like the Juan de Fuca Ridge in the eastern Pacific Ocean (Delaney et al., 1992). They result from the expulsion of sea water, which has percolated into the rock and sediment of the ocean floor and been superheated by subsurface magma (up to 400°C) and acidified (pH as low as 3.3). The water is then enriched with H_2S and various metal(loid)s, which have been leached from the basalt comprising the ocean floor (Kelley et al., 2002). Once released into the cold conditions of the deep ocean ($\sim 2^{o}$C), rapid cooling takes place resulting in the dissolved minerals precipitating out of solution, appearing like a cloud of black smoke, hence the name 'black smokers'. The process also leads to deposition of metal-sulfides around the plume, giving the appearance of a chimney (Fig. 1.1). Under the extreme pressure (250 ATM) and temperatures of vents, Te can replace S in the chimney walls (Butler & Nesbitt, 1999) increasing the concentration of the element. These unique locations present several extremes: pressure, temperature, acidity, and increased levels of metal(loid)s. This last feature is ideal for directing microbial evolution towards utilization of usually toxic compounds for biological processes, or at the very least, the ability to resist and/or
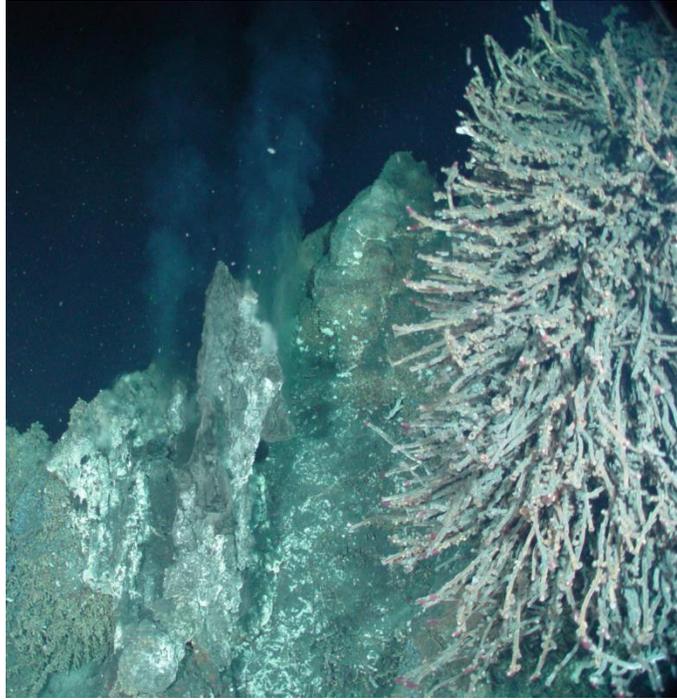
Fig 1.1. Photograph of deep ocean hydrothermal vent and associated vent worms at the Juan de Fuca Ridge Vent Field in the Pacific Ocean. (Archives of Dr. V. Yurkov laboratory).

reduce them. The harsh conditions suggest that life there should be scarce, however, numerous unique organisms call this ecological niche home (Van Dover, 2000). Of the biota inhabiting vents, sulfide and tube worms are of particular interest (Fig. 1.1). Due to their proximity to the plume waters, they and the associated microbes are in close contact with elevated levels of metal(loid)s (Van Dover, 2000). As a result, these animals harbor a community of metal(loid) resistant bacteria (Jeanthon & Prieur, 1990; Csotonyi et al., 2006; Rajasabapathy et al., 2014), indicating the microbial population has adapted to exposure. The worms also have a unique physiological trait, V enriched blood (Michibata et al., 2002; Ueki et al., 2015), which means any associated epibionts or symbiotic bacteria must have evolved a strategy to cope with increased concentrations. Black smokers have provided us with examples of bacteria possessing very high levels of metal(loid) oxyanion resistance (Yurkov et al., 1999; Rathgeber et al., 2002; Bonificio & Clarke, 2014). Vents also supply chemicals, including Te, Se, and V compounds, that are sufficiently reduced for energetically expensive biochemical reactions (Kelley et al., 2002), which lead to the discovery they can be used for survival and growth (Yurkova & Lyalikova, 1990; Csotonyi et al., 2006). This all confirms the environment provides conditions conducive for the evolution of resistance and biological reactions dependant on metal(loid)s.

1.2.3. Thermal springs

Similar to deep sea hydrothermal vents, thermal springs are produced by the emergence of geothermally heated groundwater from the Earth's crust (Fig. 1.2). They range from small ground seepage to flowing rivers and, if under pressure, fountains or geysers. They are found at many different locations globally and have a nearly ubiquitous association with faults and active hydrothermal outflow (Curewitz & Karson, 1997). Since these sites of geothermal heating can

Fig. 1.2. Sulfur Mountain thermal springs, Banff, Alberta, Canada. Outflow temperature is approximately 45°C. The microbial mat forming community is comprised primarily of *Spirulina* (green) and *Thiothrix* like (white) microorganisms. (Archives of Dr. V. Yurkov laboratory).

vary in temperature, so does the water that is expelled. Therefore, it has been under debate as to what water temperatures actually constitute a thermal spring. One definition states that the temperature must be greater than body temperature (36.7^oC), but it has been questioned as anthropocentric in nature and neglecting to consider the surrounding environment. Another suggests higher than the mean ambient air temperature (Pentecost et al., 2003), however, there is a problem with that definition. At locales of higher altitude and/or latitude, where temperatures never rise above 0^oC, water may be flowing at or near to freezing. Here, it would not be reasonable to consider the springs 'thermal'. In the context of this work, the first definition has been adopted.

Water emerging from these springs passes through bedrock before it is ejected at the surface, leading to enrichment in leached minerals, much like the waters expelled by deep sea hydrothermal vents. As a result, they are usually linked to increased concentrations of various metal(loid)s (Nelson & Giles, 1985; Ballantyne & Moore, 1988; Ebert & Rye, 1997; Bundschuh & Maity, 2015) and have provided examples of highly resistant bacteria (Chatziefthimiou et al., 2007; Zakeri et al., 2010; Masoudzadeh et al., 2011). Investigation of samples taken from the hot temperature springs of the Bolshaya river basin, Baykal Lake region and Kamchatka Island in Russia led to the discovery of freshwater aerobic anoxygenic phototrophic bacteria (AAP) capable of resisting and reducing very high levels of tellurite and selenite (Yurkov & Gorlenko, 1990; Yurkov et al., 1991; Yurkov & Gorlenko, 1992; Yurkov et al., 1992; Yurkov et al., 1994; Yurkov et al., 1996), discussed further in Section 1.3.6.

1.2.4. Mine tailings

The mechanical and chemical methods used in mining to obtain the desired product from ore produce waste, primarily ground rock and process effluents, creating tailings. The extraction process is neither 100% efficient nor is it ever possible to reclaim all reusable and expended processing reagents and chemicals. The unrecoverable and uneconomic metals, minerals, chemicals, organics and water are then discharged into the surrounding area (Fig. 1.3).

Te, Se, and/or V are often found in ores of more desirable metals. During extraction of coal, gold, silver, copper, and various others, they can be released as by-products (Cooper, 1971; Andseron, 2000; Siddique et al., 2007; Green, 2009; Sracek et al., 2014; Yang et al., 2014). Therefore, effluent from mining operations is enriched in these elements and their oxyanions, leading to an environment that exerts selective pressure on the microbial community to evolve survival mechanisms. This habitat, much like deep sea hydrothermal vents and thermal springs, has provided pure cultures of bacteria showing very high level resistance to Te and Se oxyanions (Siddique et al., 2007; Bautista-Hernandez et al., 2012). In the past, accidents and poor operating procedures have resulted in the release of vast quantities of toxic sludge (Grimalt et al., 1999; Kossoff et al., 2014; de Santiago-Martin et al., 2015), adding attention to the need for remediation of mine tailings. Microorganisms inhabiting these places may provide ecologically friendly approaches for clean-up as well as possible biorecovery of elements for use in industry.

1.3. Bacterial heavy metal(loid) resistance and transformations

Many different fields, including biogeochemistry, bioremediation, biotechnology, biometallurgy, and enzymology encompass microbial heavy metal(loid) interactions. As pollution from human activities increases, research has followed suit. However, our knowledge

Fig. 1.3. Gold mine tailings of the Central Mine, Nopiming Provincial Park, Manitoba, Canada. (Archives of Dr. V. Yurkov laboratory).

on this subject is still limited. Bacteria possess resistance mechanisms towards a wide variety of other harmful metal(loid)s (Nies, 1999; Lemire et al., 2013), but the focus here is on the relatively rare metalloids Te (section 1.3.1) and Se (section 1.3.2), and the transition metal V (section 1.3.3). The interaction between high levels of these elements, and their oxyanions, and bacteria has yet to be fully explored and many questions still remain unanswered.

1.3.1. Tellurium: Chemistry, abundance and microbial interactions

Tellurium is a metalloid element related to oxygen and sulfur in group 16 of the Periodic Table. It possesses stable oxidation states of VI (tellurate, TeO_4^{2-}), IV (tellurite, TeO_3^{2-}), 0 (elemental tellurium, Te^0), and II (telluride, Te^{2-}). Overall, Te has a very low global abundance (10^{-2} to 10^{-8} ppm) and its distribution is not homogenous (Cooper, 1971; Cox, 1989). As a result of such low natural concentrations, it has garnered little attention in regards to its effect on microbes (Ba et al., 2010; Belzile & Chen, 2015). However, levels can be elevated in certain locales. In gold mines it can be significantly concentrated (14.8 ppm) (Wray, 1998) and deep sea hydrothermal vent systems can also be enriched (Butler & Nesbitt, 1999). However, in recent years, use of this metalloid in industry has led to an increased environmental presence (Andseron, 2000; Prakash et al., 2001; Fuge, 2013; Mokmeli et al., 2016). Tellurite and tellurate are most common in the biosphere, while in the lithosphere it is found as tellurides of gold and silver (Cooper, 1971) and in copper ores (Andseron, 2000; Green, 2009). Tellurite is the most toxic form with levels as low as 1 µg/ml proving fatal to microorganisms (Yurkov et al., 1996). The details of toxicity are still debated, although it is most likely due to its strong oxidative properties (Taylor, 1999). The E^o for the conversion of tellurite to tellurium is 0.827 V (Csotonyi et al., 2006), supporting this idea. Exposure inhibits ATP production in aerobically grown non-resistant *E. coli* by disrupting the trans-membrane proton gradient, resulting in depletion of

intracellular ATP stores (Lohmeir-Vogel et al., 2004) and in murine hepatocarcinoma cells, a 80% drop in ATP is seen in its presence (Sandoval et al., 2010). The same detrimental effect is seen in regards to protein synthesis, specifically those containing reduced thiol groups (Turner et al., 1999; Chasteen et al., 2009; Burkholz & Jacob, 2013; Turner, 2013). However, these discoveries were made using bacteria with very low level resistance to Te oxyanions (as low as 0.5 - 25 µg/ml), when compared to microorganisms with very high level resistance (up to 4000 µg/ml (Pearion & Jablonski, 1999)). Previous work has identified several bacterial species that are highly resistant to tellurite, up to 2700µg/ml (Yurkov et al., 1996; Yurkov et al., 1999; Rathgeber et al., 2002; Etezad et al., 2009; Arenas et al., 2014), which have become the focus for study (Valkovicova et al., 2013; Ilyas & Lee, 2014; Plaza et al., 2016).

As oxyanions of Te are so toxic, it has long been believed they cannot have a significant role in biological processes. However, Te does share similar physical and electrochemical features with its Group 16 members Se and S, which can lead to its substitution in their place in proteins (Moroder, 2005). This has been observed in fungi, but the result was protein inactivity, suggesting erroneous inclusion (Ramadan et al., 1989). The same holds true for bacteria, with tellurocysteine and telluromethionine being identified, but just like in fungi, it is a result of erroneous incorporation (Boles et al., 1995; Budisa et al., 1995). However, in 2006, anaerobic respiration using Te oxyanions was discovered (Csotonyi et al., 2006), proving Te plays an important positive role in the life of some microbes.

Even though bacterial tellurite resistance/reduction has been known for almost 100 years (Fleming, 1932), research in the field of high level resistance is beginning to take off and any information is of great importance. The study of microbial strategies used to achieve this type of resistance, as well as the search for more species that use Te compounds as terminal electron

acceptors for anaerobic respiration (Discussed in Section 1.4), will shed new light on how bacteria carry out the process.

1.3.2. Selenium: Chemistry, abundance and microbial interactions

Selenium parallels Te in many features, but has received much more attention. It is also a group 16 element related to sulfur and oxygen with the same oxidation states of VI (selenate, SeO_4^{2-}), IV (selenite, SeO_3^{2-}), 0 (elemental selenium, Se^0), and II (selenide, Se^{2-}). Global abundance of Se, while very low, is still much higher than that of Te, around 0.05 ppm (Krauskopf, 1982) and like Te, it can be concentrated locally. The processing of sulfide ores of iron, nickel, copper, zinc and lead results in increased Se levels in mine tailings (Jones et al., 2015), and natural Se abundance in bedrock can lead to increased levels in soils (Haug et al., 2007). Selenite and selenate are the most prevalent forms, sometimes at levels so high that their toxic effect can be observed as selenosis in animals inhabiting those areas (Tinggi, 2005). Contamination has long been an issue in agricultural areas where Se compounds are leached from the soil and accumulated in higher than usual concentrations (Fujii et al., 1988; Mast et al., 2014). The toxicity of Se oxyanions is lower than that of Te, resulting in some microbial species having resistance in excess of 7000 µg/ml (Rathgeber et al., 2006), however, concentrations as low as 5 µg/ml are still lethal for many microbes (Rathgeber et al., 2002). Exposure to selenite also has a detrimental effect on protein production. In *Rhodobacter sphaeroides,* it causes a significant decrease in growth, and hence, protein synthesis (Bebien et al., 2001). It has also been shown to interfere with cellular respiration, enzyme activity, and DNA repair (Eustice et al., 1981; Turner et al., 1998; Dong et al., 2003). Because of this, it was long thought that Se was of no benefit to organisms and, therefore, little consideration was given to the study of its interactions with biological systems. However, it was later found Se was in fact essential for life,

as it composed the 21st amino acid selenocysteine (Cone et al., 1976) and is a component of many selenoenzymes (Labunskyy et al., 2014). Since then, it has been given more attention, which has included the study of the reduction of the toxic oxyanions selenate and selenite to elemental Se by microorganisms (Kieliszek & Blazejak, 2013). Even with added attention, there is still much to learn. Most of the focus has been on selenate, and more specifically, anaerobic resistance and reduction, which has shown us that like Te, Se oxyanions can be used as terminal electron acceptors during anaerobic respiration. This will be discussed further in Section 1.4.

1.3.3. Vanadium: Chemistry, abundance and microbial interactions

Vanadium is a transition metal, which can exist in many oxidation states: 0 (elemental, V^0), +II (vanadium oxide, V^{2+}), +III (vanadic oxide, V^{3+}), +IV (vanadyl, VO^{2+}), and +V (meta- or orthovanadate, VO_3^- or VO_4^{3-}). V is the 22nd most common element in the Earth's crust (150 ppm) (Habashi, 2002). Although not too abundant, it is omnipresent in nature, with a large amount (~1.8 ng/ml) being found in ocean surface water (Butler, 1998). This makes V the second most common transition metal in seawater after molybdenum (Butler, 1998). Organic vanadium compounds are also a well-known component of many crude oils and it is the most prominent trace element in the bitumen of the Athabasca Oil sands (150-170 ppm) (Jacob & Filby, 1983). It is further concentrated during the coking process, yielding 5-6 fold concentration (Zubot et al., 2012). As a result, one of the major sources is petroleum residues. V possesses some highly coveted features. High tensile strength, fatigue resistance, and hardness make it well suited for the manufacturing of ferrous and non-ferrous alloys (Moskalyk & Alfantazi, 2003; Arshad et al., 2014). The use and presence in two of the largest industries worldwide makes it an environmental pollutant above and beyond its natural abundance.

In humans, V compounds are known to inhibit glycolysis, Na^+/K^+ ATPase, acid phosphatase, alkaline phosphatase and adenylate kinase, among others (Cantley et al., 1978; Benabe et al., 1987; Nalewajko et al., 1995; Pessoa et al., 2015). They also negatively impact photosynthesis in phytoplankton (Nalewajko et al., 1995). Unwanted effects are correspondingly observed in bacteria. Vanadate can adopt a stable state analogous to phosphate, causing interference in cellular functions (Gresser & Tracey, 1990; Dieter, 2015), it impedes siderophore-mediated iron uptake in *Pseudomonas aeruginosa* (Baysse et al., 2000), it is known to inhibit methanogenesis in archea (Zhang et al., 2014), and vanadium pentoxide has been shown to prevent biofilm formation (Natalio et al., 2012). Due to this, one could assume V would not serve any purpose in biological systems. However, in reality it is not true. It has been long known that V(III) is essential in blood cells of ascidian worms (Frank et al., 2008). With microorganisms, V use is not common but it has been found essential for certain enzymes such as nitrogenase (Robson et al., 1986) and nitrate reductase from bacteria associated with ascidium worms (Antipov et al., 1998). As well, the cyanobacterium *Anabaena variabilis* possesses a high affinity vanadate transport system (Pratt & Thiel, 2006), which suggests this oxyanion could be of importance. Most of what we know about bacterial V interactions is related to anaerobic conditions, when V oxyanions are used as terminal electron acceptors during anaerobic growth of some organisms (Discussed in Section 1.4). Under aerobic conditions, very little is known about how oxyanions of this element impact bacteria. It has been suggested that vanadate resistant epibiotic microbes of ascidian worms from deep sea hydrothermal vents may actually gain some advantage from these compounds aerobically (Csotonyi et al., 2006). The idea is supported by discovery of strain EG13 from a hypersaline spring that does benefit from V exposure, showing a 4.5 fold increase in biomass in the presence of 7500 µg/ml $NaVO_3$.

However, no reduction is observed (Csotonyi et al., 2015). Such discoveries serve to raise our interest in the role of V for microbes.

V can be locally concentrated just like Te and Se. Non-biological approaches have been developed to help remediate high levels (Mandal et al., 2012; Bayo et al., 2014; Zhang et al., 2015), but similarly to strategies employed for Te and/or Se, they are less than ideal. A microbial approach would alleviate this issue (Zhang et al., 2014; Mirazimi et al., 2015), which makes study of bacterial interactions with V of paramount importance. Research in the area has led to the discovery of bacteria capable of resisting, reducing and respiring on meta- and orthovanadate (Csotonyi et al., 2006; Csotonyi et al., 2008; Csotonyi et al., 2015; Romaidi & Ueki, 2016), however, the field of V study and how bacteria interact with it is just beginning to expand.

1.3.4. Mechanisms of bacterial resistance and reduction of Te, Se, and V oxyanions

Many microorganisms possess mechanisms for resistance and reduction of Te, Se, and V oxyanions (Zannoni et al., 2008; Turner, 2013). Even though these elements are not relatively abundant globally, resistance is distributed among many different groups from phototrophs to heterotrophs under both aerobic and anaerobic conditions (Moore & Kaplan, 1992; Rathgeber et al., 2002; Csotonyi et al., 2006; Borsetti et al., 2009; Turner, 2013; Arenas et al., 2014). Resistance determinants across phylogenetically diverse taxa have a high degree of sequence similarity, suggesting they may be elements carried over from a common ancestor, evolved for survival in an ancient metal-rich environment (Agranoc & Krishna, 1998; Daubin et al., 2002) or through lateral gene transfer (Coombs & Barkay, 2003). Alternatively, metal resistance can be encoded for on plasmids, which are capable of being mobilized and transferred, conferring resistance to previously susceptible bacteria (Lebaron et al., 1994). Different mechanisms have

developed to deal with the presence of highly toxic oxyanions of Te, Se, and V, but the strategies to confer very high level resistance have yet to be investigated to any extent (Turner, 2013).

1.3.4.1. Aerobic resistance and reduction

Various strategies are used by bacteria in response to a metal(loid) challenge, however, the majority of these are the product of secondary reduction by non-specific enzymes. They do not result in the ability to resist and/or reduce very high levels of Te, Se, and V, only conferring basal, or at best, low level resistance. There have been a select few examples of specific enzymes for reduction, but there is still much more to discover. What is known mainly applies to low level resistance, while the mechanisms of high level resistance remain unsolved.

1.3.4.1.1. Tellurium oxyanions

Mechanisms of Te resistance/reduction under aerobic conditions are diverse. Mostly, reduction is carried out through non-specific reactions. Catalases, the key enzymatic defences against radical oxygen species (ROS), play a part in resistance to and reduction of TeO_3^{2-}. Once in the cell, tellurite can cause the formation of intracellular ROS (Perez et al., 2007; Tremaroli et al., 2007), resulting in significant damage. Also, because it most likely exerts its toxic effect through a high oxidizing ability, such enzymes are capable of using TeO_3^{2-} as a substrate, reducing it to Te^0 and minimizing the negative impact, as seen in *Staphylococcus epidermidis* (Calderon et al., 2006). In some microorganisms, such as *E. coli* and *R. sphaeroides*, periplasmic and membrane associated nitrate reductases reduce low levels (Avazeri et al., 1997; Sabaty et al., 2001). Several other enzymes have been shown to aid in resistance/reduction of small amounts of the oxyanion. The thiol:disulfide oxidoreductase of *R. capsulatus* acts as a conduit for electrons to pass from the metalloid oxyanion and the quinone pool in the membrane, resulting in

tellurite reduction (Borsetti et al., 2007). In *E. coli,* exposure causes the expression of gutS (Guzzo & Dubow, 2000). The true function of this protein has yet to be determined, however, it appears to be involved in transport of some kind, suggesting efflux of the oxyanion may be taking place. In other work, the product of the *cysK* and *cobA* genes of *Geobacillus stearothermophilis* V confer resistance in *E. coli* (Araya et al., 2009) and the dihydrolipoaminde dehydrogenase of *Aeromonas caviae* ST has NADH-dependant reducing activity (Castro et al., 2008). Three protein fractions from *Thermus thermophiles* HB8, as well as cell free extracts of *Mycobacterium avium*, also reduce TeO_3^{2-}, but again, the interaction is non-specific and concentrations are low (Terai et al., 1958; Chiong et al., 1988). Proteome work in *Halomonas* sp., strain MAM has shown a variety of proteins are over expressed in the presence of tellurite, but this is more of a general picture of whole proteome response (Kabiri et al., 2009). While all these enzymes do reduce Te compounds, many involve very low concentrations and it is not their primary function. Some higher level resistance determinants have been identified. The *ter* operon is a characteristic marker found on all but one incompatibility HI2 (IncHI2) and IncHII plasmids comprised of seven genes (*ZABCDEF)* (Taylor, 1999). It does confer high level resistance (minimum inhibitory concentration (MIC) of 1028 µg/ml), but the details are still under study. Work with the minimal resistance-conferring fragment (*terBCDE*) has shown resistance can only be achieved by the expression of all four components (Valkovicova et al., 2013). In this case, they each play an irreplaceable role, likely involving their mutual association at the inner membrane (Valkovicova et al., 2013). Also, some of the individual genes from the operon actually appear to be lethal when cloned alone, making them difficult to study (Whelan et al., 1997). While the operon clearly gives resistance, it is not its main function. *TerZABCDEF* provides resistance to phages and pore-forming colicins and it is also found in pathogenic

bacteria, which have no need for tellurite resistance. Therefore, it is believed to serve some unknown function, which increases fitness (Valkovicova et al., 2013). Also, TerD and TerE are involved in intracellular survival and proliferation of *Yersinia pestis* in macrophages (Ponnusamy et al., 2011). Other plasmid based resistance determinants have been identified, such as *kilAtelAB* from IncPα plasmid RK2 (Walter et al., 1991) and *arsR-DABC* from IncF1 plasmid R773 (Turner et al., 1992), but they do not confer very high level resistance. The elements can be chromosomally encoded as well, such as *tehAB* from *E. coli* (Taylor, 1999), *trgABcysK* and *telA* from *R. sphaeroides* (O'Gara et al., 1997), and the *tmp* gene from *Pseudomonas syringae* (Coumoyer et al., 1998). However, the molecular basis behind most of these systems is unclear (Valkovicova et al., 2013). As most research has focused on very low level resistance, where reduction is accomplished by non-specific enzymes and systems, identification of a specific reductase remained elusive. Then, in 2009, *Bacillus* sp., STG-83 was isolated from Neidasht spring in Iran (Soudi et al., 2009). This bacterium could reduce tellurite, selenate, and selenite at increased levels. It turned out that reduction of TeO_3^{2-} was accomplished by a specific cytoplasmic enzyme (Etezad et al., 2009), the first and only example of an isolated enzyme specific to the oxyanion.

Other strategies are employed for resistance, however, they do not involve direct enzymatic reduction. In *R. capsulatus*, it is a means of maintaining intracellular redox poise during photosynthetic growth (Moore & Kaplan, 1994). Decreased uptake is also suggested to play a role in this bacterium as an acetate transport system is responsible for ingress (Borghese & Zannoni, 2010). Therefore, even at low concentration (60 µg/ml), acetate competes with tellurite for entry into the cell limiting the toxicity (Borsetti et al., 2003). Recently, it has been found that transport of tellurite through this acetate permease is due to a 15-16 residue insert in *Rc*ActP2

between trans-membrane helices 6 and 7. The result is a conformational change which favors the binding and translocation of the oxyanion across the membrane (Borghese et al., 2016). A similar mechanism has also been identified in *E. coli*. Tellurite enters the cell through the PitA phosphate transporter (Elias et al., 2012), and mutation to the phosphate transport system (Thomas & Kay, 1986) or deletion of PitA (Elias et al., 2012) confers a higher level of resistance. Finally, some bacteria are capable of producing volatile organic Te compounds, such as dimethyltelluride (Belzile & Chen, 2015). While this is a proven way of detoxification, total amounts removed are negligible (Ollivier et al., 2008).

1.3.4.1.2. Selenium oxyanions

Aerobic high level resistance to Se oxyanions is not as commonly known as for those of Te, most likely due to their lower toxicity (Zannoni et al., 2008). However, they have a similar effect on cells and hence, similar enzymes can reduce them to elemental form. As Se compounds also have a high oxidizing ability, ROS can be generated (Seko et al., 1989; Spallholz, 1994). So, it is not surprising that just like with tellurite, catalase can be upregulated in their presence (Antonioli et al., 2007). Nitrate reductases can also reduce Se oxyanions (Sabaty et al., 2001; Hunter & Manter, 2009). Like for TeO_3^{2-}, many other non-specific enzymes are capable of reduction, such as hydrogenase I in *Clostridium pasteurianum* (Yanke et al., 1995), fumarate reductase in *Shewanella oneidensis,* MR-1 (Li et al., 2014), glutathione reductase and thioredoxin in *P. seleniipraecipitans* (Hunter, 2014), and the same inducible enzyme responsible for tellurite reduction in *E. coli*, gutS (Guzzo & Dubow, 2000). However, these are non-specific side reactions and not the result of action of a defined selenite or selenate reducing enzyme. Whole proteome analysis of *Halomonas* sp., strain MAM and *R. sphaeroides* in the presence of SeO_3^{2-} showed a variety of proteins are upregulated, but this represents a more general response

(Bebien et al., 2001; Kabiri et al., 2009). *R. sphaeroides* can also reduce large amounts to elemental Se under dark aerobic conditions (Bebien et al., 2001), but, just like with Te oxyanions, all these are non-specific reactions. There are examples of selenate reductases isolated from *T. selenatis* (Schroder et al., 1997) and *Bacillus selenatarsenatis* (Kuroda et al., 2011), which can reduce selenate to selenite and one from *Bacillus* sp., STG-83 that converts selenate and selenite to elemental Se (Etezad et al., 2009). There has yet to be a selenite specific reductase found. Our understanding of this family of enzymes is still in its infancy as many of those isolated have not been studied to any significant extent. Therefore, more work with bacteria that are strong reducers will expand our knowledge of how these enzymes work, their structure, as well as their abundance throughout the microbial world.

1.3.4.1.3. Vanadium oxyanions

Research on aerobic vanadium oxyanion resistance and reduction, specifically meta- and orthovanadate, is virtually non-existent. It has been known for several decades (Bautista & Alexander, 1972), but oddly has not received nearly as much attention as anaerobic reduction. As vanadate can resemble phosphate, altered uptake systems have been seen, which increase resistance in *Candida albicans* (Mahanty et al., 1991). Efflux has also been implicated. In *P. aeruginosa* the MexGHI-OpmD pump confers vanadium resistance (Aendekerk et al., 2002). Recently, aerobic reduction of vanadate was found to be accomplished by a membrane bound reductase in *Enterobacter cloacae*, strain EV-SA01 that is coupled to the oxidation of NADH (van Marwijk et al., 2009). Although the localization and identification of the enzyme was accomplished, it was not purified or characterized. With so little research in this area, it is difficult to make any conclusions about the true nature of bacterial interactions with V.

1.3.4.2. Anaerobic resistance and reduction

Even less is known concerning Te, Se, and V oxyanion resistance and reduction under anaerobic conditions compared to aerobic. Generally, resistance to metal(loid)s is decreased without oxygen present. In the phototrophic bacteria *R. sphaeroides* and *R. capsulatus*, the MIC for tellurite is much lower (4-8 fold decrease) under anoxic conditions compared to oxic (Moore & Kaplan, 1992; Borghese et al., 2004). Strain AV-V-25 is sensitive to tellurate anaerobically, but can resist/reduce it aerobically (Csotonyi et al., 2006) and anoxic growth of *Shewanella oneidensis*, MR-1 in the presence of 25 µg/ml selenite is decreased compared to little effect in the presence of oxygen (Klonowska et al., 2005). Strain ER-V-6 is not affected by high levels of selenite aerobically, but anaerobically no growth occurs (Csotonyi et al., 2006). The same trend is observed with V. Strain ER-Te-48 can resist meta- and orthovanadate aerobically but not anaerobically, and ER-Se-17L and ER-V-6 show the same response with metavanadate (Csotonyi et al., 2006). This could suggest that in the absence of oxygen, these compounds are of no use to microorganisms, however, it is untrue. A select few microbes use them for energy generation through dissimilatory reduction, which will be discussed later in Section 1.4.

1.3.4.3. Heterotrophic metal(loid) resistance

Basal resistance to metal(loid) oxyanions is reported in a wide variety of heterotrophic bacteria, which is mainly the result of secondary reduction by numerous enzymes as discussed above. With tellurite, low level resistance is present in a multitude of microbes, even *E. coli* can be considered resistant, and it has been used as a selective marker for some strains such as *E. coli*, O157 (Zadik et al., 1993) as well as other relevant human pathogens (Taylor, 1999; Kerangart et al., 2016). However, TeO_3^{2-} concentrations are extremely low (500 - 1000 fold

lower) when compared to very high level resistance (Rathgeber et al., 2002). Regardless of the extent of resistance, the end result is almost always the same, reduction of the oxyanion into black intracellular deposits of elemental Te (Rathgeber et al., 2002). These deposits can be present in the periplasm (Trutko et al., 2000), cytoplasm (Pearion & Jablonski, 1999), the cell surface (Baesman et al., 2007), or extracellularly (Bajaj & Winter, 2014; Borghese et al., 2016), most likely depending on the location of the enzyme responsible for reduction. In heterotrophic bacteria, accumulation of Te^0 appears to be primarily internal, suggesting reduction may be carried out by a cytoplasmic enzyme, as in *Bacillus* sp., STG-83 (Etezad et al., 2009). However, not enough is known about this process to make any conclusions. Se oxyanions parallel those of Te in many aspects and several pathways of reduction have been proposed (Nancharaiah & Lens, 2015). Levels of resistance vary from basal/low (5 µg/ml) to very high (7000 µg/ml) and resistance by reduction to elemental form also results in accumulation of red Se^0 particles. They can be found in the cytoplasm, in association with the cell surface (Blum et al., 1998; Debieux et al., 2011), and deposited extracellular as nanospheres, specifically in those bacteria capable of anaerobic respiration (Oremland et al., 2004). The cause is thought to be sloughing off of the particles attached to the cell surface (Debieux et al., 2011), or the secretion of internal Se^0 nanospheres (Butler et al., 2012). In the case of V oxyanions, their fate is somewhat different. While they are reduced to a lower oxidation state, accumulation of elemental V has not yet been observed. In most instances, colorless V(V) is reduced to blue/green/grey V(IV) (Csotonyi et al., 2006). The other path creates an accumulation of a brown precipitate, which has been shown to be comprised of V(IV) (Carpentier et al., 2005).

1.3.4.4. Aerobic anoxygenic phototrophs and metal(loid) resistance

While many heterotrophic bacteria possess low level resistance to the metal(loid) oxyanions, it is not fair to say that very high level resistance/reduction is a common trait. The same does not hold true for AAP, especially those isolated from extreme environments. With only a few exceptions, such as *Erythrobacter litoralis* strain T4 and *Hoeflea phototrophica* which can tolerate >1000 μg/ml tellurite (Yurkov et al., 1996; Biebl et al., 2006), the majority of AAP possessing high level metal(loid) resistance were isolated from extreme environments. Over half of taxonomically classified AAP hail from these types of locales and all can resist high levels of Te, Se, and/or V compounds (Yurkov & Csotonyi, 2009). The same trend is seen among yet unclassified AAP. Out of 15 isolates from the East German Creek hypersaline spring system in Manitoba, Canada, 8 could grow with at least 1000 μg/ml tellurite, selenite, and/or metavanadate, and all 15 showed resistance to at least one of them (Csotonyi et al., 2008). A correlation between AAP metal(loid) resistance and their habitat could be made, as extreme environments are often associated with increased metal(loid) levels, but any firm conclusion requires further investigation.

AAP primarily belong to the α-proteobacteria, with a few representatives in the β and γ subgroups. They are phylogenetically closely related to the physiological group of the purple non-sulfur bacteria (PNSB) (Yurkov & Csotonyi, 2009), which have also received some attention in regards to their interactions with metal(loid)s. In bacteria such as *R. sphaeroides, R. capsulatus, R. palustris* and *Rhodopseudomonas viridis,* these oxyanions can be used for disposal of excess reducing power generated during photosynthesis (Moore & Kaplan, 1992). AAP may employ a similar strategy (Sabaty et al., 1998). Research has indicated a mechanism analogous to re-oxidation of electron carriers during respiration rather than photosynthesis may take place aerobically in *Roseococcus thiosulfatophilus* (Yurkov et al., 1996). As AAP have much higher

levels of resistance than their PNSB relatives, it suggests they possess a different, or additional, mechanism(s). One proposed approach of resistance/reduction involves their highly elevated pool of carotenoids, which have been suggested to confer protection against photooxidative damage (Beatty, 2002). Therefore, they make good candidates for also protecting against oxidative metal(loid) species. Some, such as zeaxanthin and erythroxanthin sulfate, are especially good at quenching ROS (El-Agamey & McGarvey, 2008), and make up a large portion of those found in AAP like *Erythromicrobium ramosum* and *Erythrobacter litoralis* possessing very high level resistance (Yurkov et al., 1993). However, investigation of resistance in AAP has received insufficient attention to elucidate details, and these amazing microorganisms may possess unique novel pathways and enzymes. Therefore, research into this area is of high value. Whatever the strategy may be, the product of reduction is similar as for heterotrophic bacteria, formation of Te^0, Se^0, or $V(IV)$.

1.4. Anaerobic respiration with metal(loid) oxyanions as terminal electron acceptors

Microbial reduction of some metal(loid)s, such as iron (Fe), manganese (Mn), uranium (U), chromium (Cr), arsenic (As), and mercury (Hg), is well known to be coupled to the oxidation of organic or inorganic sources of energy in anaerobic respiration (Lovely, 1993; Stoltz & Oremland, 1999). However, dissimilatory electron transport (anaerobic respiration) with tellurite, tellurate, selenite, selenate, metavanadate, and/or orthovanadate is a rare phenomenon. To date, there are only 22 examples of bacteria that have been confirmed to utilize one or more of those as terminal electron acceptors during anaerobic growth (Table 1.1). However, their reduction potential, as compared to the SO_4^{2-}/HS^- redox couple, actually makes them very favorable as terminal electron acceptors for anaerobic respiration (Fig 1.4) (Myers et al., 2004; Bouroushian, 2010). Because sulfate reducing bacteria can conserve energy from the latter reaction, reduction

Table 1.1. Confirmed examples of bacterial anaerobic respiration using one or more Te, Se, or V oxyanions as a terminal electron acceptor for energy generation.

Organism	Metal(loid) Oxyanion					
	TeO_3^{2-}	TeO_4^{2-}	SeO_3^{2-}	SeO_4^{2-}	VO_3^{-}	VO_4^{3-}
Bacillus selenitireducens[1], *Bacillus beveridgei*[2]	+	+	+			
Sulfurospirillum barnesii[3]	+	+		+		
ER-Te-48[4]		+				
HGMK1[5], HGMK3[5] (*Aquificales*)			+			
Aeromonas hydrophila[6], *Bacillus arsenicoselenatis*[1], *Pseudomonas stutzeri* pn1[7], *Thauera selenatis*[8], *Sedimenticola selenatireducens*[9], Ke4OH1[10], S7[7], *Pelobacter seleniigens*[11], *Selenihalanaerobacter shriftii*[12]				+		
Desulfurispirillium indicum[13]			+	+		
P. isachenkovii[14], *P. vanadiumreductans*[14], *Shewanella oneidensis*[15], *Geobacter metallireducens*[16], ER-V-6[4]					+	
AV-V-25[4]						+

[1] (Baesman et al., 2007); [2] (Baesman & Kulp, 2009); [3] (Stoltz & Oremland, 1999); [4] (Csotonyi et al., 2006); [5] (Takai et al., 2002); [6] (Knight & Blakemore, 1998); [7] (Narasingarao & Haggblom, 2007); [8] (Macy et al., 1993); [9] (Narasingarao & Haggblom, 2006); [10] (Knight et al., 2002); [11] (Narasingarao & Haggblom, 2007a); [12] (Blum et al., 2001); [13] (Rauschenbach et al., 2011); [14] (Yurkova & Lyalikova, 1990); [15] (Carpentier et al., 2005); [16] (Ortiz-Bernard et al., 2004)

Redox couple	Reduction potential
$VO_2^+ \rightarrow VO^{2+}$	1.000
$TeO_4^{2-} \rightarrow TeO_3^{2-}$	0.897
$SeO_3^{2-} \rightarrow Se^0$	0.885
$TeO_3^{2-} \rightarrow Te^0$	0.827
$SO_4^{2-} \rightarrow HS^-$	- 0.217
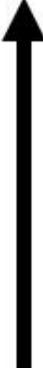
Increasing favorability of reaction

Fig. 1.4. Energetics of Te, Se, and V oxyanion redox couples showing they are more favorable for anaerobic respiration than the SO^{2-}/HS^- redox couple used by sulphate reducing bacteria.

of Te, Se, and V oxyanions should also have similar potential. Also, reduction of selenite to Se (ΔG_f^o = -132.4 kJ (mol electrons)$^{-1}$) (Blum et al., 1998) and tellurite to Te (ΔG_f^o = -71.3 kJ (mol electrons)$^{-1}$) (Baesman & Kulp, 2009) coupled to the oxidation of lactate is highly exergonic, providing abundant energy for growth (Nancharaiah & Lens, 2015). Likely factors contributing to the infrequent use of them for respiration is low global abundance and high toxicity. Nevertheless, respiration on other toxic oxyanions is known (Lovely, 1993; Stoltz & Oremland, 1999; Terry et al., 2015; Moroz et al., 2016), indicating toxicity to some species does not always prevent inclusion in metabolism of others. As mentioned previously, until recently Te was believed to serve no beneficial biological function. The first report of dissimilatory anaerobic use of tellurate was published for *Shewanella frigidimarina* relative, strain ER-Te-48 (Csotonyi et al., 2006), proving that Te has a role to play in some biological systems. Following this discovery, other examples were found. *Bacillus selenitireducens*, *B. beveridgei,* and *Sulfurospirillum barnesii* are capable of respiration anaerobically using tellurite and tellurate as terminal electron acceptors (Baesman et al., 2007; Baesman & Kulp, 2009). Some other bacteria, *S. oneidensis,* MR-1 and *Bacillus* sp.*,* STG-83, are suspected of having a similar capability, but it has not been confirmed (Klonowska et al., 2005; Etezad et al., 2009). There are more instances of Se compounds being used for energy production, but it is still an ability that has been rarely described for pure cultures. *Desulfurispirillium indicum* uses selenite and selenate reduction for respiration (Rauschenbach et al., 2011), two isolates (strains HGMK1 and HGMK3) utilize SeO_3^{2-} (Takai et al., 2002), and both *B. selenitireducens* and *B. beveridgei* can use selenite in addition to tellurite and tellurate (Baesman et al., 2007; Baesman & Kulp, 2009). Most research has focused on anaerobic selenate respiration, so it is not surprising that more examples of bacteria using it are known. A total of 9 isolates have been shown to respire on only selenate.

The first bacterium identified was *Thauera selenatis* (Macy et al., 1993), followed by the taxonomically classified species *Aeromonas hydrophila* (Knight & Blakemore, 1998)*, Bacillus arsenicoselenatis* (Blum et al., 1998)*, Selenihalanaerobacter shriftii* (Blum et al., 2001)*, Sedimenticola selenatireducens* (Narasingarao & Haggblom, 2006)*, Pelobacter seleniigens* (Narasingarao & Haggblom, 2007a)*, Pseudomonas stutzeri,* pn1 (Narasingarao & Haggblom, 2007)*,* and unclassified strains Ke4OH1 (Knight et al., 2002) and S7 (Narasingarao & Haggblom, 2007). There are also some cases of suspected Se oxyanion respiration that have yet to be confirmed, such as *Bacillus* sp., STG-83, which does reduce both selenite and selenate during anaerobic growth, but this has not been linked to energy generation to date (Etezad et al., 2009).

Use of V oxyanions is also not common, however, respiration on metavanadate has been known for many years. The first examples were *Pseudomonas isachenkovii* and *P. vanadiumreductans*, isolated from a tunicate worm and V containing effluent, respectively (Yurkova & Lyalikova, 1990). Since then, only three other cases were reported, including *Shewanella oneidensis*, MR-1 (Carpentier et al., 2005), *Geobacter metallireducens* (Ortiz-Bernard et al., 2004)*,* and strain ER-V-6 (Csotonyi et al., 2006). A single documented bacterium, AV-V-25, is capable of respiring using orthovanadate, suggesting that this may be a rare process in nature (Csotonyi et al., 2006).

1.4.1. Diversity of bacteria capable of metal(loid) oxyanion anaerobic respiration

The ability to respire on Te, Se, and/or V oxyanions does not appear to be confined to a single group, with four different phyla represented (Aquaficales, Chrysiogenetes, Firmicutes, and Proteobacteria). The 14 taxonomically classified examples are distributed among 10 genera, with

Bacillus spp. being most prevalent. Considering the unclassified strains, the same distribution is observed. The tube worm epibionts ER-Te-48 and ER-V-6 most likely belong to *Shewanella* (>99.3% 16S rRNA gene similarity to *Shewanella frigidimarina* (Csotonyi et al., 2006)), already known for its diverse metal(loid) respiring abilities, while AV-V-25 appears to be a member of genus *Vibrio* (99.8% 16S rRNA gene similarity to *Vibrio pomeroyi* (Csotonyi et al., 2006)), which is not well known for metal resistance, let alone anaerobic respiration. Two strains (HGMK1 and HGMK3) are members of the *Aquificales* (Takai et al., 2002), Ke4OH1 shares 98.3% 16S rRNA gene similarity with *Sedimenticola selenatireducens* (Knight et al., 2002), and strain S7 is about 98% similar to *Geovibrio ferrireducens* (Narasingarao & Haggblom, 2007). Based on this information, it would appear that the means of using metal(loid) oxyanions as terminal electron acceptors during anaerobic growth is a phylogenetically widespread phenomenon, evolved by various bacterial groups, or possibly a result of lateral gene transfer between members of multiple genera (Coombs & Barkay, 2003). One could also speculate it is a genetic element left over from ancient ancestors who developed the skill to survive on a primordial, metal-rich earth (Edwards et al., 2003). As species and genera diverged over time, the genes for the process remained.

1.5. Ongoing search for new metabolic abilities from extreme environments

Extreme environments and metal(loid)s go hand in hand (Butler & Nesbitt, 1999; Grimalt et al., 1999; Li et al., 2009). The result is a natural setting forcing microorganisms to evolve a means of surviving encounters with these highly toxic compounds. Therefore, the more sampling and analysis of extremophilic and extremotolerant bacteria taking place, the more we can increase our understanding of bacterial-metal(loid) interactions. As discussed in previous sections, these harsh habitats have provided us with many examples of metal(loid) resistant

bacteria (Yurkov et al., 1999; Rathgeber et al., 2002; Csotonyi et al., 2006; Csotonyi et al., 2008; Arenas et al., 2014; Epelde et al., 2015), as well as the first examples of Te, meta-, and ortho-vanadate being used as a terminal electron acceptor for anaerobic growth (Yurkova & Lyalikova, 1990; Csotonyi et al., 2006). Extreme environments have provided a wealth of information on bacterial metal(loid) resistance and new species with new abilities. Therefore, if we plan to better understand bacterial-metal(loid) interactions, these are the locales we must target for research.

1.6. Thesis objectives

As presented in the previous sections of this literature review, there is much to learn about bacterial interactions with high levels of oxyanions of the metalloids Te, Se, and V. Therefore, I set out to determine the physiological impact of tellurite on highly resistant bacteria, identify enzymes responsible for carrying out reduction, and investigate anaerobic respiration on Te, Se, and V oxyanions. My aim was two-fold. First, the effect of tellurite on bacteria from extreme environments possessing very high level resistance was investigated and the strategies/enzymes involved in reduction were identified. Also, as there is only a single example of a tellurite reductase from a resistant bacterium published to date, I wanted to determine if our highly resistant strains possessed enzymes of a similar nature, or if they resembled some of the better characterized selenite reductases, or possibly nitrate/nitrite reductases. Second, I endeavored to isolate new species from extreme environments, which could resist/reduce very high levels of Te, Se, and/or V oxyanions, and use them for anaerobic respiration to determine how unique this ability is. The specific goals were as follows:

(1) To investigate the effect of tellurite on microorganisms with very high level resistance, and the ability to reduce it to elemental form, and the strategies employed. Two groups of

bacteria were chosen: i) AAP and heterotrophs from marine habitats, including deep sea hydrothermal vents and hypersaline microbial mats (Chapter 2) and ii) AAP from freshwater thermal springs (Chapter 3). This was followed by identification, purification, and characterization of the enzymes responsible for tellurite reduction (Chapters 4 and 5).

(2) With so few microorganisms identified that can perform dissimilatory anaerobic respiration using Te, Se, and V oxyanions as terminal electron acceptors, I set out to find new bacteria with the ability to carry out this task from locales containing increased concentrations of Te, Se, and/or V (Chapters 6 and 7).

1.7. References

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Chapter 2.

The impact of tellurite on highly resistant marine bacteria and strategies for its reduction

Chris Maltman and Vladimir Yurkov

The first author was the major contributor to research presented.

2.1. Abstract

Five marine bacteria (*Pseudoalteromonas spiralis,* Te-2-2; *Pseudoalteromonas telluritireducens,* Se-1-2-red; *Erythrobacter litoralis,* T4; *Citromicrobium bathyomarinum,* JF1; and a *Shewanella frigidimarina* relative, strain ER-Te-48), possessing both very high level aerobic resistance and the ability to reduce TeO_3^{2-} to elemental Te were investigated to better understand their interaction with this metalloid oxyanion. It was found that reduction can be greatly influenced by several factors including carbon source, pH and aeration. The physiological and metabolic response of cells to tellurite differs between strains. In its presence, versus absence, cellular biomass varied, yielding relatively similar or decreased amounts of protein. ATP production was affected in the same manner, with similar and decreased levels in cells harvested from media containing TeO_3^{2-}. Three different strategies for tellurite reduction, which required de novo synthesis of a reductase, were observed. Strain ER-Te-48 employed a periplasmic reductase, while Te-2-2, T4 and JF1 required an intact cytoplasmic membrane for reduction. Lastly, Se-1-2-red was only able to reduce as an undisturbed whole cell culture.

Keywords: Tellurite, tellurite reduction, tellurium, bacterial metalloid resistance, marine bacteria.

2.2. Introduction

Tellurium (Te) is a rare metalloid element belonging to group 16 of the Periodic Table. It is related to sulphur and oxygen and possesses stable oxidation states of +VI (tellurate), +IV (tellurite), 0 (elemental Te), and –II (telluride). Most Te occurs as tellurate in the hydrosphere and as tellurides of gold and silver in the lithosphere (Cooper, 1971). Oxidized forms, specifically tellurite, are highly toxic to most microorganisms at concentrations as low as 1 µg/mL (Yurkov et al., 1996), but through reduction to elemental Te, certain species can resist up to 4,000 µg/mL (Pearion & Jablonski, 1999). The E^o for the conversion of TeO_3^{2-} to Te is 0.827 V (Lloyd et al., 2001), indicating a very strong ability to oxidize, leading to the idea that this property is a major reason for toxicity (Taylor, 1999; Turner et al., 1999). However, the direct impact on cells is still under debate.

Catalases, the key enzymatic defenses against radical oxygen species (ROS), play a part in resistance to and reduction of TeO_3^{2-}. Tellurite can cause the formation of intracellular ROS (Perez et al., 2007), possibly resulting in significant cellular damage, which can be neutralized by catalases. Also, because tellurite most likely exerts its toxic effect through a high oxidizing ability, they are capable of reducing the negative impact on the cell, as seen in *Staphylococcus epidermidis* (Calderon et al., 2006). It is believed that for some organisms, such as *E. coli*, reduction is accomplished by nitrate reductases (Avazeri et al., 1997; Sabaty et al., 2001), and is, therefore, non-specific. Several other enzymes, for example the thiol:disulfide oxidoreductase of *Rhodobacter capsulatus* (Borsetti et al., 2007) and GutS of *E. coli* (Guzzo & Dubow, 2000), among others (Chiong et al., 1988; Moscoso et al., 1998; Chiang et al., 2008; Kabiri et al., 2009), are involved in tellurite resistance and/or reduction, although activity was associated with moderate to low level resistance and is not their primary function. *R. capsulatus* employs

multiple approaches to deal with tellurite. First, reduction has been implicated as a means of maintaining intracellular redox poise during photosynthetic growth (Moore & Kaplan, 1994). Secondly, decreased uptake of tellurite was also suggested to play a role. An acetate transport system is responsible for uptake (Borghese & Zannoni, 2010), therefore, even at low concentration (1 µM), acetate competes with tellurite for entry into the cell (Borghese et al., 2008) limiting the toxicity, which results in enhanced resistance. A similar approach has also been found in *E. coli*. Mutation to a phosphate transport system conferred a higher level of resistance (Thomas & Kay, 1986), indicating that phosphate transport is responsible for TeO_3^{2-} ingress. Finally, some bacteria are capable of producing volatile organic Te compounds, such as dimethyltelluride. While this is a proven way of tellurite detoxification, total amounts removed are negligible (Ollivier et al., 2008).

The strategies described above are all utilized for TeO_3^{2-} reduction and resistance, however, none mentioned involves a specific tellurite reducing enzyme. Knowledge of how bacteria accomplish this task is limited. Unlike with selenate, where several specific reductases have been identified, such as the one from *Thauera selenatis* (Schroder et al., 1997), there is only a single example of an enzyme specific to tellurite reduction, found in *Bacillus* sp. STG-83 (Etezad et al., 2009). This bacterium is believed to be capable of dissimilatory anaerobic reduction, although it has not been proven, and therefore the enzyme may be respiratory in nature. Strain ER-Te-48 in our study is capable of anaerobic respiration on tellurate, using it as a terminal electron acceptor (Csotonyi et al., 2006). Research into reduction strategies among bacteria possessing very high level resistance is in its infancy. Recently, several extremely resistant marine microorganisms (up to 2700 µg/ml of tellurite) have been reported and characterized (Yurkov et al., 1999; Yurkov & Csotonyi, 2003; Csotonyi et al., 2006; Rathgeber

et al., 2006). The first group includes aerobic anoxygenic phototrophs: *Erythrobacter litoralis* (T4) isolated from littoral waters (Yurkov et al., 1994) and *Citromicrobium bathyomarinum* (JF1) recovered from the vicinity of black smokers at the Juan de Fuca Ridge (Yurkov et al., 1999). The second group is represented by non-phototrophic obligate aerobes: oceanic *Pseudoalteromonas spiralis* (Te-2-2) and *Pseudoalteromonas telluritireducens* (Se-1-2-red) (Rathgeber et al., 2006). Lastly, there is a facultative anaerobic bacterium (ER-Te-48), a close relative of *Shewanella frigidimarina*, which forms strong associations with the tube worm *Paralvinella sulfincola* around black smokers of the Pacific Ocean (Csotonyi et al., 2006). These strains were selected for our study based on their ability to resist very high levels of tellurite and effectively reduce it to elemental Te under aerobic conditions. We investigated the influence of tellurite on cells, factors affecting reduction and variations in the expression of the reductase/reduction systems.

2.3. Materials and Methods

2.3.1. Strains and growth conditions

Bacteria chosen for study included *Erythrobacter litoralis* (strain T4, ATCC 700002), *Citromicrobium bathyomarinum* (JF1), *Pseudoalteromonas spiralis* (Te-2-2, DSM 16099), *Pseudoalteromonas telluritireducens* (Se-1-2-red, DSM 16098), and a *Shewanella frigidimarina* relative (ER-Te-48) (Yurkov et al., 1994; Yurkov et al., 1999; Csotonyi et al., 2006; Rathgeber et al., 2006). They were grown aerobically at their optimal temperature ($28^{o}C$) on an incubator shaker (200 rpm) in liquid rich organic (RO) medium or liquid minimal salts (MS) medium (Yurkov et al., 1994) with 3.0 g/l glutamate, both containing 2% NaCl at pH 9.0, unless otherwise stated. All experimental results are averaged from three replicates.

2.3.2. Physiological and biochemical tests

Metalloid resistance, utilization of organic substrates, variation in pH, level of aeration, protein, and ATP production were all examined in the presence of K_2TeO_3. Resistance was confirmed in RO NaCl liquid medium containing varying concentrations of K_2TeO_3 (500, 750, 1000, 1500, 2000, and 2500 µg/mL). As growth of these strains with tellurite causes blackening of the culture due to reduction of the oxyanion, A_{600} is rendered ineffective for estimating growth. In order to alleviate the problem, A_{950} measurements can be used to overcome this darkening, which provides a means to determine growth coupled to the reduction of the oxyanion. Therefore, A_{950} is an established method for estimating growth in the presence of tellurite, as used by Yurkov et al. (Yurkov et al., 1996). All growth for subsequent experiments was monitored by A_{950} in the presence of 500 µg/ml K_2TeO_3, in liquid culture over 96 h, unless otherwise described. The effect of carbon source on cell growth and reduction of tellurite was investigated by transfer of actively growing cells to MS NaCl liquid medium (pH 7.8) with K_2TeO_3 containing one of: acetate, butyrate, citrate, ethanol, fructose, glucose, glutamate, L-glutamine, lactate, malate, pyruvate, or succinate at 1.5 or 3.0 g/l. Because the solubility of K_2TeO_3, and therefore the availability in solution, changes with pH (Redman & Harvey, 1967), the effect of pH (7.0, 8.0, 9.0, 10.0 and 11.0) on resistance and reduction was researched. As the addition of K_2TeO_3 to our growth medium caused the formation of precipitates under acidic conditions, pH below 7.0 was not tested. Liquid media was adjusted with 0.5 N NaOH to the desired pH and grown at $28^{o}C$ at 200 rpm. The role of oxygenation on growth and reduction was tested at 100 (low), 200 (average) or 300 (high) rpm in an incubator shaker at $28^{o}C$, pH 9.0. Once optimal conditions were established, strains were grown at those parameters with either 500 or 1000 µg/mL K_2TeO_3. To investigate the effect of tellurite on cellular protein and ATP levels, measurements were taken over 48 h in its presence and absence. Protein was measured by

the Bradford assay (Bradford, 1976). For ATP, 1 ml samples were taken and immediately centrifuged for 5 min at 10000 rpm to concentrate cells. The pellet was re-suspended in 1 ml cold 0.4 N perchloric acid and incubated for 5 min. to allow for cell lysis. The sample was then neutralized with 2 N K_2CO_3 and used for ATP determination with an ATP Bioluminescence Kit from Sigma-Aldrich (Conn et al., 1974; Maechler et al., 1998). Controls for both ATP and protein confirmed the presence of tellurite had no detectable effect on the readings.

2.3.3. Tellurite reductase expression, activity, and localization

To find if reductase activity needs *de novo* protein synthesis or is constitutively expressed, the influence of secondary K_2TeO_3 exposure, as well as the effect of tetracycline on reduction was carried out. Since minimum inhibitory concentration (MIC) values for tetracycline are not known for all strains used, several concentrations were tested and 25 µg/ml proved effective to halt protein synthesis without affecting cell viability. This amount, added prior to tellurite exposure, was used to confirm constitutive presence of a reductase or *de novo* synthesis (Carpentier et al., 2003) in the presence of K_2TeO_3. Reduction was monitored visually by blackening of the sample due to the production of elemental Te (Yurkov et al., 1996). For secondary exposure, strains were grown in their optimal liquid medium amended with K_2TeO_3 (primary exposure). This culture was then used to inoculate (5%) fresh medium of the same composition (secondary exposure).

For detection of reduction in cell extracts, strains grown for 12 h (mid to late log phase) without K_2TeO_3 were collected by centrifugation at 7000 rpm for 30 min at 4°C. Cells were resuspended in 2 ml of their respective growth medium followed by sonication on ice. Debris was removed by centrifugation at 15000 rpm for 10 min. The supernatant was collected, filtered with a 0.2 µm filter, brought to 5 mL with the proper sterile growth medium and 500 µg/ml

K_2TeO_3 were added with reduction monitored over 24 h under aseptic conditions. If none was observed, cellular exposure to tellurite is required to induce enzyme expression. Carbon sources in the growth medium acted as the electron donor(s).

Localization of reductase activity was achieved by cellular fractionation. Periplasm, spheroplast and spheroplast lysates were each studied for K_2TeO_3 reduction. Mid to late log phase liquid cultures (30 ml) were chilled on ice and cells harvested by centrifugation at 7000 rpm for 25 min and washed with 10 mM Tris HCl, pH 8.0. The periplasmic fraction was prepared by a modified method after Schroder et al (Schroder et al., 1997). The pellet was resuspended in 2 ml 30 mM Tris HCl, pH 8.0 and 0.75 M sucrose. Cells were gently shaken throughout the proceeding steps. Following 5 min incubation at 0^oC, 50 µl of a 5% lysozyme in distilled water solution was added and further incubated for 5 min on ice. Cold EDTA (4 ml) was added over 10 min followed by 10 min incubation on ice. After this, the solution was incubated at 37^oC for 10 min to allow for the formation of spheroplasts (confirmed by microscopy), which were removed by centrifugation at 15000 rpm for 30 min. The obtained clear supernatant contained the periplasmic components (confirmed by the absence of color from elemental Te or pigmented membranes as a result of lysed cells). The spheroplasts (confirmed by plating not to contain whole active cells) were collected to test for K_2TeO_3 reduction as well as subjected to sonication to observe activity in the lysate. Each fraction was exposed to 500 µg/ml K_2TeO_3 and monitored spectrophotometrically at 500 nm for reduction (Chiong et al., 1988; Moscoso et al., 1998; Molina et al., 2010). If no reduction was observed in any of the three fractions, experiments were repeated with cells previously exposed to tellurite. This would provide the chance for inducible reductase expression. The rate of reduction (1 unit equal to 1 µg tellurite reduced/µg protein/h) was calculated for each case. Protein was determined by Bradford assay

(Bradford, 1976) and K_2TeO_3 reduction by spectrophotometric readings at 500 nm based off standard curves in each media (Fig. S1) (Chiong et al., 1988; Moscoso et al., 1998; Molina et al., 2010).

2.4. Results and Discussion

2.4.1. Growth with tellurite

Exposure of strains to different K_2TeO_3 concentrations confirmed a wide range of resistance. In the RO medium, strain T4 was resistant up to 1500 µg/ml. The remaining bacteria (JF1, ER-Te-48, Se-1-2-red and Te-2-2) were all capable of tolerating up to 2000 µg/ml, and ER-Te-48 continued growth at the highest level tested, 2500 µg/ml (Table S1). In regard to growth medium, while many carbon sources were analyzed individually, and in combination, only two media compositions were preferred. Some strains (ER-Te-48, Se-1-2-red, and Te-2-2) performed best in a defined MS NaCl medium with glutamate at 3.0 g/l, while others (T4 and JF1) required a complex RO NaCl medium. Non-aerobic anoxygenic phototrophs appeared to prefer glutamate as the sole carbon source and there is a possible explanation. These microorganisms were isolated from a deep ocean hydrothermal vent habitat and were closely associated with sulfide worms (Csotonyi et al., 2006; Rathgeber et al., 2006). Certain species of sulfide worms, such as *Riftia pachyptila*, are provided with carbon by their symbiotic bacteria, predominantly in the form of glutamate (Felbeck & Jarchow, 1998). Therefore, higher than normal levels of the amino acid may exist in this habitat, leading to development of its preferential use. An alternative explanation could be based on the ability of bacteria to transform it into α-ketoglutarate (Commichau et al., 2008), potentially leading to its excess, which may be directed into the TCA cycle, increasing the energy generation and allowing the cell to cope with the presence of tellurite. All tested strains grew and reduced K_2TeO_3 optimally at pH 9.0. Again, the non-aerobic

anoxygenic phototrophs behaved similarly with Se-1-2-red, Te-2-2 and ER-Te-48, described above, showing strong growth up to pH 11.0 (Table 2.1). As for aeration, these heterotrophs grew and reduced well at all levels tested, while phototrophic JF1 and T4 preferred to grow at 200 rpm (Table 2.1). However, JF1 and T4 still reduced K_2TeO_3 at lower (100 rpm) and higher (300 rpm) levels of aeration, implying that the amount of oxygen does not significantly affect the process of reduction, but must be sufficient for respiration based cell growth and, therefore, allow metalloid reduction. Lastly, when grown in the presence of either 500 or 1000 µg/ml K_2TeO_3, optimal growth and reduction took place at 500 µg/ml. Hence, this concentration was chosen for all further experiments.

2.4.2. Effect of tellurite on protein and ATP production

While it has been shown that TeO_3^{2-} does damage proteins, specifically those containing reduced thiol groups (Turner et al., 1999), the direct effect in highly resistant bacteria is unknown. Since the majority of the published impacts of tellurite on cells are negative (Turner et al., 1999), with the exception of the select few species that can anaerobically respire on TeO_3^{2} (Csotonyi et al., 2006; Baesman et al., 2007; Baesman et al., 2009), we believed there would be reduced growth and, therefore, protein levels in its presence. The expected decrease was observed, though not in all cases. Strains JF1, Se-1-2-red, and Te-2-2 required a 48 h lag phase for adaptation while strain ER-Te-48 required only 24 h. We identified two separate responses to tellurite addition in terms of final protein production: 1) An initial lag phase followed by significant aftershock recovery, or 2) An initial lag with reduced recovery. Strains JF1, Te-2-2, and Se-1-2-red possessed the first reaction. Maximal protein production was similar (JF1, 69.4$\pm$1.9 vs. 67.5$\pm$0.6 µg/ml; Te-2-2, 64.7$\pm$6.4 vs. 65.8$\pm$2.0 µg/ml; Se-1-2-red, 57.7$\pm$2.1 vs. 55.3$\pm$2.7 µg/ml) compared to those in the absence of TeO_3^{2-} (Fig. 2.1 A, Fig. S2 B, C). The

Table 2.1. Effect of pH and aeration on growth in the presence of 500 µg/ml K_2TeO_3.

Strain	pH					Aeration (rpm)		
	7.0	8.0	9.0	10.0	11.0	100	200	300
JF1	78.0±1.8	85.4±2.9	100±2.1	NG	NG	13.7±4.6	100±3.3	16.2±1.8
T4	85.1±2.3	95.9±0.7	100±3.5	NG	NG	51.9±4.1	100±2.9	48.2±7.1
Te-2-2	69.0±4.1	71.5±1.1	100±5.3	85.7±4.8	84.1±3.7	92.2±3.8	100±4.7	76.9±2.2
Se-1-2-red	67.2±3.3	73.4±3.7	100±6.1	98.4±0.5	91.6±2.9	76.8±2.7	100±6.2	94.5±2.5
ER-Te-48	69.1±1.9	82.7±4.4	100±1.7	97.7±2.4	92.3±3.6	83.8±5.1	100±5.5	83.3±3.9

Growth estimated by A_{950} over 96 h and represented as percent maximal absorbance. NG-No growth, strains not capable of growth at these values.
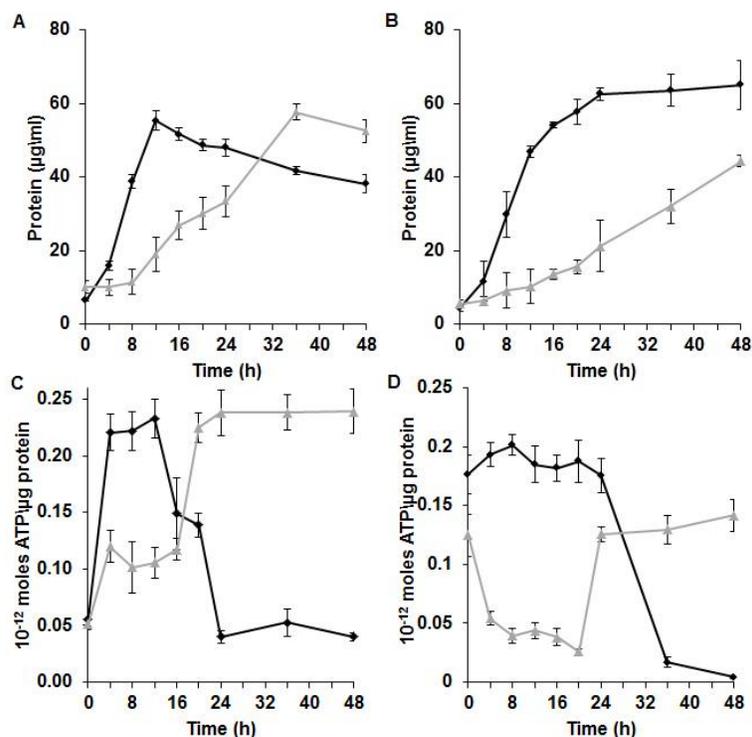
Figure 2.1. Protein and ATP production in presence versus absence of K_2TeO_3. A) Strain Se-1-2-red. The lag phase required with tellurite is not shown. Similar results for JF1 and Te-2-2 (Fig. S2 B, C). B) ER-Te-48. Similar results for T4 (Fig. S2 A). C) Se-1-2-red. The lag phase required with tellurite is not shown. D) ER-Te-48. The lag phase required with tellurite is not shown. Similar results for JF1, T4, and Te-2-2 (Fig. S2 D, E, F). ♦ − No K_2TeO_3; ▲ − 500 μg/mL K_2TeO_3. Error bars represent one standard deviation.

reason for the trend in JF1, Te-2-2, and Se-1-2-red is unknown, however, it is likely the initial toxicity shock from tellurite depresses protein synthesis as the cell tries to cope. Once the cells have adapted, growth and protein return to normal levels. It is also possible that the removal of tellurite helps reduce toxicity, thereby aiding in recovery.

Strains ER-Te-48 and T4 followed the second course. Maximal protein yield decreased over 48 hours (ER-Te-48, 44.4 ± 1.6 vs. 65.0 ± 6.7 µg/ml and T4, 63.9 ± 3.2 vs. 79.5 ± 2.9 µg/ml) (Fig. 2.1 B, Fig. S2 A) with tellurite compared to without. Possibly, in the presence of tellurite, these two bacteria are shifting electrons to facilitate tellurite reduction, rather than biomass production, resulting in the observed drop in protein. Or possibly, something related to the heat shock response in *E. coli* is taking place, resulting in a halt to protein synthesis to prevent damaging newly synthesized proteins (Arsene et al., 2000). Also when *E. coli* is exposed to tellurite, there is a 10 fold transcriptional increase of the *IbpA* gene (Perez et al., 2007). The IbpA protein has been associated with an increased resistance to oxidative stress, suggesting that a similar response may be taking place here, thereby increasing protein production per cell, but not overall biomass of the culture.

In murine hepatocarcinoma cells, exposure to tellurite causes an 80% drop in ATP (Sandoval et al., 2010) and in aerobically grown non-resistant *E. coli*, exposure to tellurite causes a loss of the transmembrane proton gradient and depletion of intracellular ATP in the short term, without affecting the intracellular concentration of glycolytic intermediates (Lohmeier-Vogel et al., 2004). This suggests components of the electron transport chain (ETC) could be targets of this oxyanion. As well, the generation of ROS by tellurite can lead to membrane damage (Storz & Imlay, 1999; Perez et al., 2007), further adding to the possibility of disruption of the ETC. Therefore, as the level of tellurite used here was significantly higher, and over a much longer

time frame, it was thought that ATP levels might be decreased for all studied strains in the presence of the oxyanion. Much like in protein experiments, the same 48 h or 24 h lag phases were needed for JF1, Se-1-2-red, Te-2-2 and ER-Te-48. Strain Se-1-2-red possessed comparable amounts (0.23 ± 0.02 vs. 0.22 ± 0.02 10^{-12} moles ATP/μg protein), although there was an initial lag phase due to the cells adapting to and overcoming the presence of tellurite (Fig. 2.1 C). Apparently, the cellular pool of ATP is not affected in this strain, which may possess a mechanism for protecting the transmembrane proton gradient during tellurite exposure and, therefore, preventing the depletion of the ATP pool. Strains ER-Te-48, JF1, T4, and Te-2-2 showed a maximal moles ATP/mg protein decrease (31.2%, 46.6%, 30.3% and, 32.0%, respectively) (Fig. 2.1 C and S2 D, E, F) as expected.

When both protein and ATP levels are compared, there are some contradictory results. While the response in tellurite exposed cells of ER-Te-48, T4, and Se-1-2-red is similar for both, the same is not true with the remaining bacteria. Strains JF1 and Te-2-2 had decreased ATP, while maintaining similar amounts of protein. It has been published that in a cell free protein synthesis system, *E. coli* is capable of maintaining protein production when ATP levels are reduced by using GTP (Jewett et al., 2009). GTP is rationed between metabolic processes, therefore allowing the cell to continue protein synthesis even though energy is depleted. In fact, it was found that an approximate 100 fold decrease in ATP delivers in only 50% decrease in protein production (Jewett et al., 2009). Most likely a similar strategy is being employed by JF1 and Te-2-2.

2.4.3. Characteristics of K_2TeO_3 reductase activity

In general, if a bacterium must induce expression of a protein to cope with the presence of a harmful substance, some lag phase will be observed during a primary exposure, while the

specific product/enzyme is being transcribed and translated (Rolfe et al., 2012). However, during a subsequent exposure of an already adapted culture, no lag phase is usually necessary, because everything required for synthesis is ready. Such phenomenon has been observed in experiments with metalloid oxyanions, including U(VI) (Spear et al., 1999). To determine if tellurite reduction involves *de novo* production of a specific enzyme(s), growth physiology during primary and secondary exposure was compared. If growth in both cases was similar, it is likely that the reducing enzyme is constitutively present. However, a lag phase detected during primary exposure, but absent in secondary, would imply the need for initiation of transcription. Strains JF1, T4, Se-1-2-red and Te-2-2 appear to have a significant advantage during a secondary exposure to TeO_3^{2-}, confirmed by the absence of a lag phase (Fig. 2.2 A, Fig. S3). Apparently, *de novo* protein synthesis is involved in establishing a TeO_3^{2-} reduction system. With ER-Te-48, similar rates of growth were observed in primary and secondary inoculations (Fig. 2.2 B), suggesting no need for initiation of transcription and, therefore, a constitutive reductase. A short lag phase, present during both primary and secondary exposure, is likely unrelated to enzyme expression and might be a normal growth physiology requirement. Based on these data, two distinct strategies can be proposed: The first depends on the constitutive presence of a reductase/reduction system, as seen in ER-Te-48 and the second involves *de novo* protein synthesis, as seen in JF1, T4, Se-1-2-red and Te-2-2. It is unclear yet whether both are specific to tellurite, however, since the second strategy requires cellular exposure to TeO_3^{2-}, this may be the case.

Upon halting protein synthesis with tetracycline, we found that organisms JF1, T4, Se-1-2-red and, Te-2-2 lost the ability to reduce TeO_3^{2-}. Coupled with results mentioned above, it provides confirmation that *de novo* enzyme synthesis is required for TeO_3^{2-} reduction. A similar
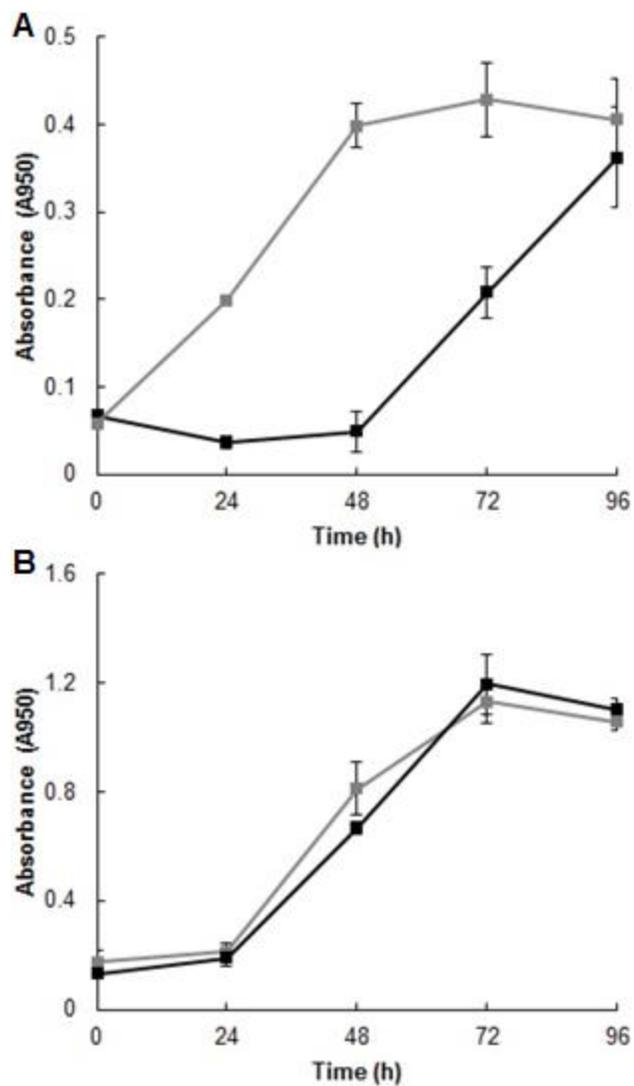
Figure 2.2. Growth during primary vs. secondary exposure to K_2TeO_3. A) Strain Se-1-2-red. Similar results for Te-2-2, JF1 and T4 (Fig. S3). B) Strain ER-Te-48. ■ - Primary exposure; ■ - Secondary exposure. Error bars represent one standard deviation.

effect was not expected for ER-Te-48 as prior experiments indicated no need for *de novo* synthesis. Surprisingly, it was also unable to reduce in the presence of tetracycline, indicating the need for some *de novo* protein preparations. Apparently the expression is a faster process, unlike in the other strains, demanding lag phases twice as long.

To further support the idea that specific enzymes must first be synthesized by introduction to tellurite, reduction in the cell lysates was analyzed. None of the strains were able to reduce TeO_3^{2-} in the lysate without prior exposure (Fig. 2.3 A), strongly supporting *de novo* synthesis. While such cell response could be predicted, whether or not reduction occurs in the lysate after exposure to TeO_3^{2-} was unpredictable without testing. Reduction appears to require an intact cell and cannot take place in disrupted fractions of JF1, T4, Se-1-2-red, and Te-2-2 (Fig. 2.3 C). Possibly, an operational membrane electron transport system involving cytochromes is needed, as previously reported in *Shewanella oneidensis* MR-1 for Fe(III), Mn(IV), and V(V) reduction (Meyers & Nealson, 1990; Meyers & Meyers, 2003; Borloo et al., 2007). In our study, only the reduction complex of ER-Te-48 was capable of function in the cell lysates, if cells were exposed to TeO_3^{2-} prior to lysis (Fig. 2.3 B). Indeed, ER-Te-48 is physiologically significantly different from other bacteria under study and possessed a reductase that can function free of whole cells.

2.4.4. Localization of reductase activity

We found that the reduction methods in all strains required *de novo* synthesis, however, the cellular location of the process was unknown. To get the answer, strains were fractionated and each fraction was monitored for reduction (Table 2.2). Reduction above control levels was not observed in the periplasm, spheroplast or spheroplast lysate of Se-1-2-red with or without prior exposure to TeO_3^{2-}, limiting function in intact cells only (Fig. 2.4 E, G, and H). However,

Table 2.2. Summary of cellular location of reductase activity.

Strain	Whole Cell Lysate	Periplasm	Spheroplast	Spheroplast Lysate
JF1	-	-	+	-
T4	-	-	+	-
Te-2-2	-	-	+	-
Se-1-2-red	-	-	-	-
ER-Te-48	+	+	-	-

+, reductase activity; -, no reductase activity
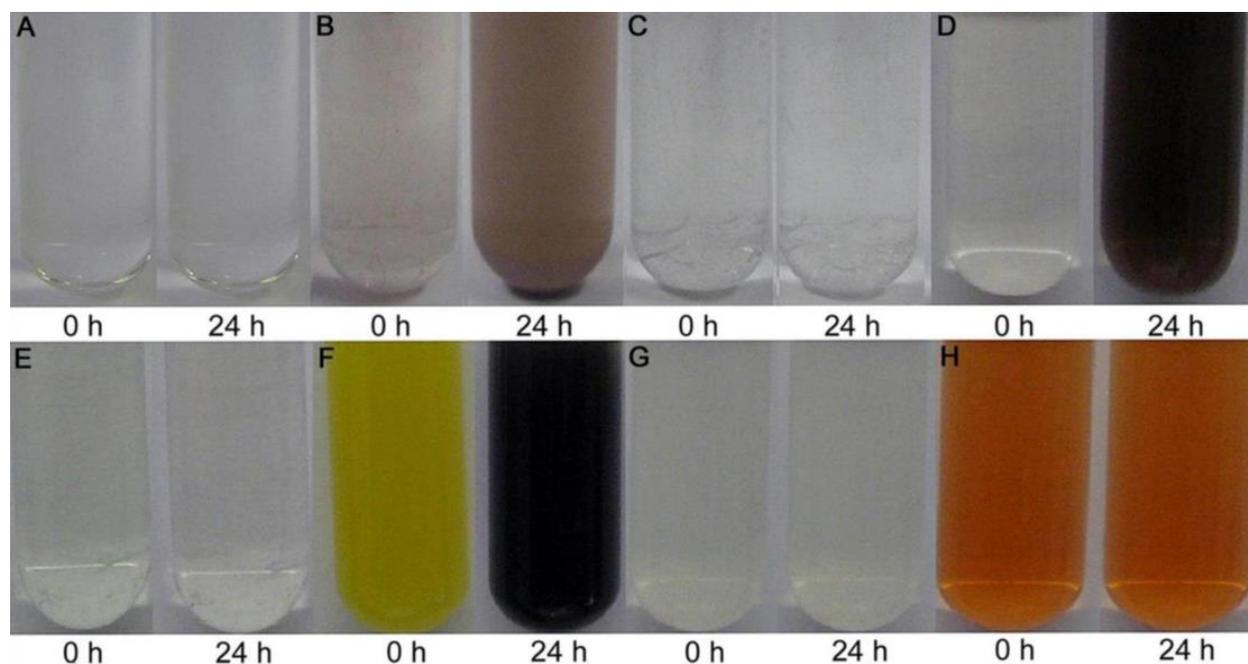
Figure 2.3. Reductase activity in cellular fractions. A) Cell lysate of strain ER-Te-48 grown without prior exposure to K_2TeO_3. Similar results for T4, JF1, Se-1-2-red, and Te-2-2. B) Strain ER-Te-48 lysate after cells exposure to K_2TeO_3. Initial darkening at 0 h is due to the trace presence of previously reduced K_2TeO_3 from exposure prior to lysis. C) Strain Te-2-2 cell lysate after K_2TeO_3 exposure. Similar results for Se-1-2-red, T4 and JF1. D) ER-Te-48 periplasmic fraction containing reductase activity following tellurite exposure. E) JF1 periplasmic fraction with and without prior tellurite exposure. No reductase activity observed. Similar results for Se-1-2-red, T4, and Te-2-2. F) JF1 spheroplast fraction containing reductase activity without prior exposure. T4 and Te-2-2 showed similar results. G) Se-1-2-red spheroplast fraction. No reduction observed with and without prior tellurite exposure. Similar results for ER-Te-48 H) T4 spheroplast lysate. No reductase activity with and without prior tellurite exposure. Similar results for JF1, Se-1-2-red, Te-2-2 and ER-Te-48.

spheroplasts of JF1, T4 and Te-2-2 were capable of reduction (Fig. 2.4 F). Although as shown above *de novo* enzyme synthesis is necessary, only an intact cytoplasmic membrane is for the complex to work, as a removal of the periplasm did not prevent the process.

ER-Te-48 behavior was the most interesting. Reducing activity was detected only in the periplasmic fraction of cells previously exposed to TeO_3^{2-} (Fig. 2.4 D). Clearly, the responsible enzyme is periplasmic in nature and remains active once removed from cells. This is much like the periplasmic selenate reductase isolated from *Thauera selenatis*, which also works free of cells (Schroder et al., 1997). *T. selenatis* anaerobically respires on selenate, while our strain ER-Te-48 is capable of anaerobic respiration on tellurate (Csotonyi et al., 2006), suggesting both processes may have commonality. Gram negative bacteria capable of anaerobic respiration could possess a similar periplasmic reductase, and future work may prove this hypothesis. In this study, we discovered three distinct strategies employed by bacteria for TeO_3^{2-} reduction, all requiring inducible *de novo* enzyme synthesis. However, the first, found in strain Se-1-2-red, needs a fully intact cell; the second present in JF1, T4, and, Te-2-2 requires an intact cytoplasmic membrane; and the third (strain ER-Te-48) involves a periplasmic enzyme for reduction.

To better understand the effectiveness of these distinct systems, the rate of TeO_3^{2-} reduction was estimated (Fig. 2.4). Strain ER-Te-48 has the most efficient system with a rate of 0.17 units (as defined in Methods) in the periplasm. The next best rates were in Te-2-2, JF1 and T4 (0.034, 0.027, 0.017 units in the spheroplast, respectively) and finally, Se-1-2-red with the lowest rate (0.006 units in periplasm). Therefore, ER-Te-48 possessed a highly effective and rapidly induced tellurite reductase.

2.5. Conclusion

Our understanding of tellurite reduction by marine bacteria is still incomplete and invites
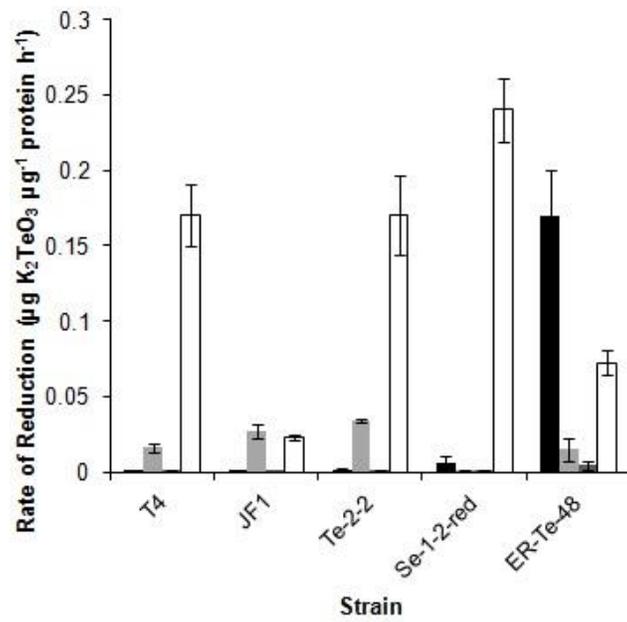
Figure 2.4. Rate of K_2TeO_3 reduction in cellular fractions. ■ Periplasm, ■ Spheroplast, ■ Spheroplast Lysate, □ Whole Cells. Error bars represent one standard deviation.

future work. However, we already established that among different species capable of high level TeO$_3^{2-}$ resistance and reduction, the strategies utilized vary. One bacterium, ER-Te-48, stands out from the analyzed group. This is the only organism that produces an inducible enzyme capable of TeO$_3^{2-}$ reduction not restricted to intact membranes or undamaged cells.

The strategies we identified were not completely unexpected and similarities exist between them and previously established physiological responses to metal(loid)s. The observed features of the periplasmic reductase from ER-Te-48 parallel those of the selenate reductase from *T. selenatis* (Schroder et al., 1997). Both can respire anaerobically on metalloid oxyanions and, therefore, may possess similar enzymes. In the case of JF1, T4, and Te-2-2, there is a precedent for needing an intact cytoplasmic membrane. One known tellurite reduction system is the *ter* determinant, which has an integral membrane component, TerC (Lloyd-Jones et al., 1994). This protein is believed to tap into the electron pool in the membrane to function, therefore, if the cytoplasmic membrane is intact, reduction can take place. A similar strategy may have evolved in our strains. Lastly, reduction in Se-1-2-red needs an intact cell. We know from an electron microscopy that Te(0) accumulation occurs in the cytoplasm in close proximity to the membrane (Rathgeber et al., 2002). Presumably, there are several integral components associated with the outer membrane, periplasm, and inner membrane, all involved in tellurite reduction, so an interruption of one results in loss of function. Arrangements such as this are typically seen in other bacterial responses to metals (Silver, 1996) and a similar complexity may be present in Se-1-2-red. We just touched on a few basic elements of this little understood pathway. Our investigation has provided a stepping stone for future research into the enzymes and cellular pathways involved and further study will aid in better understanding this process.

2.6. Acknowledgments

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Chapter 3.

The effect of tellurite on highly resistant freshwater aerobic anoxygenic phototrophs and their strategies for reduction

Chris Maltman and Vladimir Yurkov

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3.1. Abstract

Six fresh water aerobic anoxygenic phototrophs (*Erythromicrobium ezovicum*, strain E1; *Erythromicrobium hydrolyticum*, E4(1); *Erythromicrobium ramosum*, E5; *Erythromonas ursincola*, KR99; *Sandaracinobacter sibiricus*, RB 16-17; and *Roseococcus thiosulfatophilus*, RB3) possessing high level resistance to TeO_3^{2-} and the ability to reduce it to elemental Te were studied to understand their interaction with this highly toxic oxyanion. Tested organic carbon sources, pH, and level of aeration all had an impact on reduction. Physiological and metabolic responses of cells to tellurite varied among strains. In its presence, *versus* absence, cellular biomass either increased (KR99, 66.6% and E5, 21.2%) or decreased (RB3, 49.8%, E1, 57.8%, RB 16-17, 41.5%, and E4(1), 21.3%). The increase suggests a possible benefit from tellurite. Cellular ATP production was similarly affected, resulting in an increase (KR99, 15.2% and E5, 38.9%) or decrease (E4(1), 31.9%; RB 16-17, 48.7%; RB3, 55.9%; E1, 35.9%). Two distinct strategies to tellurite reduction were identified. The first, found in E4(1), requires *de novo* protein preparations as well as an undisturbed whole cell. The second strategy, in which reduction depended on a membrane associated constitutive reductase, was used by the remaining strains.

Keywords: tellurite; aerobic anoxygenic phototrophs; metalloid oxyanions; metalloid transformation; tellurite reduction

3.2. Introduction

Tellurium (Te) is a group 16 metalloid element related to sulphur and oxygen. It possesses stable oxidation states of +VI (tellurate), +IV (tellurite), 0 (elemental Te), and −II (telluride). The majority is found in the hydrosphere as tellurate and in the lithosphere as tellurides of gold and silver (Cooper, 1971). The most harmful forms of Te to microorganisms are the oxyanions, especially tellurite, with concentrations as low as 1 μg/mL being highly toxic (Yurkov et al., 1996). However, the ability to reduce it to the elemental form allows certain bacterial species to resist concentrations up to 4000 μg/ml (Pearion & Jablonski, 1999). The means by which this compound exerts its toxicity is still debated, however, the strong oxidative properties (Taylor, 1999), confirmed by an E° of 0.827 V for the TeO_3^{-2}/Te redox couple (Lloyd et al., 2001), are likely among the reasons.

Tellurite exposure can cause the formation of intracellular radical oxygen species (ROS) (Perez et al., 2007), leading to cellular damage. Catalases, the key enzymatic defence against ROS, play a role in mitigating the detrimental effects of the toxin. In *Staphylococcus epidermidis*, this family of enzymes is also capable of using TeO_3^{2-} as a substrate, reducing it to Te (Calderon et al., 2006), therefore minimizing the negative impact on cells. In *E. coli*, reduction of tellurite can occur through the actions of nitrate reductases, however, this is a non-specific reaction (Avazeri et al., 1997; Sabaty et al., 2001). Other enzymes, such as the thiol:disulfide oxidoreductase of *Rhodobacter capsulatus* (Borsetti et al., 2007), GutS from *E. coli* (Guzzo & Dubow, 2000), among others (Chiong et al., 1988; Moscoso et al., 1998; Chiang et al., 2008; Kabiri et al., 2009) have been implicated in tellurite resistance and/or reduction. Although they are associated with low to moderate levels of resistance, it is not their primary specific function. In the case of *R. capsulatus*, multiple approaches to dealing with tellurite have

been observed. One involves maintaining redox poise during photosynthetic growth through the reduction of tellurite (Moore & Kaplan, 1994), while another is based on reduced uptake of the tellurite oxyanion. With the latter, acetate permease is responsible for TeO_3^{2-} influx (Borghese & Zannoni, 2010) and competition between it and acetate for entry into the cell results in higher resistance. Even at low concentrations (60 ng/ml), acetate impacts tellurite entry (Borghese et al., 2008), limiting toxicity. A related approach has been identified in *E. coli*. Mutation to a phosphate transport system provided enhanced resistance (Thomas & Kay, 1986), which allowed tellurite ingress. Lastly, certain microorganisms can somewhat neutralize Te oxyanions by production of volatile organic telluride compounds, such as dimethyltelluride (Ollivier et al., 2008), however, such approach to detoxification delivers negligible removal.

While the aforementioned physiological reactions are utilized for TeO_3^{2-} resistance and/or reduction, none involves a specific tellurite reductase. Our understanding of how bacteria carry this out is limited. Unlike for selenate resistance/reduction, where specific reductases have been identified, such as in cells of *Thauera selenatis* (Schroder et al., 1997), only a single example of a tellurite specific reductase has been isolated to date from *Bacillus* sp. STG-83 (Etezad et al., 2009). Although not proven, this bacterium might be capable of dissimilatory anaerobic reduction, therefore, the enzyme is likely respiratory in nature. Investigation into the strategies for tellurite reduction has just begun to expand. Recently, several bacterial species have been isolated that are highly resistant to tellurite (up to 2700 µg/ml) (Yurkov et al., 1999; Rathgeber et al., 2006). Among bacteria possessing very high level resistance are aerobic anoxygenic phototrophs (AAP) isolated from extreme environments (Yurkov & Csotonyi, 2003). This group of bacteria seems to have evolved an inherent ability to deal with this oxyanion. Therefore, we set forth to investigate the physiological and metabolic effects of TeO_3^{2-} on cells,

factors affecting reduction, and differences in expression of a reducing system by AAP inhabiting extreme environments. Species chosen were *Erythromicrobium ezovicum* (strain E1), *Erythromicrobium hydrolyticum* (E4(1)), *Erythromicrobium ramosum* (E5), *Erythromonas ursincola* (KR99), *Sandaracinobacter sibiricus* (RB 16-17), and *Roseococcus thiosulfatophilus* (RB3). All are freshwater bacteria isolated from thermal springs on Kamchatka Island and the Baikal Lake region, in Russia (Yurkov & Gorlenko, 1990; Yurkov et al., 1991; Yurkov & Gorlenko, 1992; Yurkov et al., 1992; Yurkov et al., 1994). They reduce very high levels of tellurite to elemental Te under aerobic conditions (Yurkov et al., 1996).

3.3. Materials and Methods

3.3.1. Strains and growth conditions

Bacteria chosen for study include *Erythromicrobium ezovicum* (strain E1), *Erythromicrobium hydrolyticum* (E4(1)), *Erythromicrobium ramosum* (E5, ATCC 700003), *Erythromonas ursincola* (KR99, DSM 9006), *Sandaracinobacter sibiricus* (RB 16-17), and *Roseococcus thiosulfatophilus* (RB3, ATCC 700004) (Yurkov & Gorlenko, 1990; Yurkov et al., 1991; Yurkov & Gorlenko, 1992; Yurkov et al., 1992; Yurkov et al., 1994). They were grown aerobically in the dark at their optimal temperature (28°C) on an incubator shaker (200 rpm) in liquid rich organic (RO) or liquid minimal salts (MS) media (Drews, 1983; Yurkov et al., 1994) containing either glutamate, pyruvate, and malate or glutamate, pyruvate, and yeast extract each at 1.5 g/l, at pH 9.0 unless otherwise stated. All results are an average of three replicates.

3.3.2. Physiological and biochemical tests

Metalloid resistance, utilization of organic substrates, variation in pH, level of aeration, and protein and ATP production were all examined in the presence of K_2TeO_3. Resistance was confirmed in RO liquid medium with varying concentrations of K_2TeO_3 (100, 250, 500, 750,

1000, and 1500 µg/ml). Growth was monitored spectrophotometrically at A_{950}, an established method for estimating growth and reduction in the presence of tellurite (see section 2.3.2) (Yurkov et al., 1996). All growth for subsequent experiments was monitored at A_{950} with 500 µg/ml (strains E1, E4(1), E5, and KR99) or 100 µg/ml (RB3 and RB 16-17) K_2TeO_3 in liquid culture over 96 h, unless otherwise described. The effect of carbon sources on growth and reduction was investigated by transfer of actively growing cells to MS liquid medium, pH 7.8, with K_2TeO_3 containing one of: acetate, butyrate, citrate, ethanol, fructose, glucose, glutamate, L-glutamine, lactate, malate, pyruvate, or succinate at either 1.5 or 3.0 g/l. The solubility of K_2TeO_3, and therefore the availability in solution, changes with pH (Redman & Harvey, 1967) that is why the effect of pH on resistance and reduction was tested. As the addition of K_2TeO_3 to the growth medium caused the formation of precipitates under acidic conditions and strains could not grow beyond pH 9.5, only pH range 7.0 to 9.0 was considered. Liquid medium was adjusted with 0.5 N NaOH to the desired pH. The role of oxygenation was analyzed in an incubator shaker set to 100 (low), 200 (typical) or 300 (high) rpm. Once optimal conditions were established, strains were grown at those parameters with 500 or 1000 µg/ml (strains E1, E4(1), E5, and KR99) or 100 or 500 µg/ml (RB3 and RB 16-17) K_2TeO_3. To observe the effect of tellurite on cellular protein and ATP levels, measurements were taken in its presence and absence over 48 h. Protein was assayed by the Bradford method (Bradford, 1976) and ATP was monitored with an ATP Bioluminescence Kit from Sigma-Aldrich (see section 2.3.2).

3.3.3. Tellurite reductase expression, activity, and localization

Tellurite reductase expression experiments were carried out as recently published (Chapter 2), with one modification: 100 µg/ml chloramphenicol was used for strain RB 16-17 instead of tetracycline. Detection of TeO_3^{2-} reduction in cell extracts and localization of

reductase activity was performed as described (Chapter 2). Rate of reduction (1 unit equal to 1 µg tellurite reduced/µg protein/h) was calculated for each cellular fraction based of standard curves prepared in each media (Fig. S4). For isolation of membranes, cells were broken by French Press and centrifuged at 20,000 rpm for 1 h to remove debris. The supernatant was collected and ultracentrifuged at 60,000 rpm for 12 h. The membrane pellet was then washed with 10 mM Tris HCl, pH 8.0. Membranes were resuspended in their respective growth media containing K_2TeO_3.

3.4. Results

3.4.1. Growth with tellurite

Growth of AAP in the presence of different K_2TeO_3 concentrations confirmed these bacteria possess a high level resistance (Table S2). Strains appear to be similar, resisting and reducing up to 1500 µg/ml, however, optimal growth and reduction occurred at 500 µg/ml K_2TeO_3 for E1, E4(1), E5, and KR99, while 100 µg/ml K_2TeO_3 was best for RB3 and RB 16-17. With respect to media composition, we observed that it does have an effect, as has been previously reported (Yurkov et al., 1996). Although many different carbon sources and combinations were tested, only three media compositions gave optimal growth and reduction. Strains E4(1), RB3, and RB 16-17 performed best in complex RO medium, while E5 and KR99 preferred defined MS medium containing a combination of glutamate, malate, and pyruvate. E1 showed the best results in MS medium with glutamate and pyruvate, however, some yeast extract was still required, suggesting a need for the undefined component of this complex organic substrate. To determine if the specific combination, but not the increased organics, was responsible for increased growth and reduction with tellurite, each individual source was tested at 3.0 g/l. Single organic carbon sources at increased concentrations were not as good as the

combination. All tested strains grew and reduced K_2TeO_3 optimally at pH 9.0 and aeration was achieved at 200 rpm (Table 3.1). However, reduction still occurred at 100 and 300 rpm, albeit at much reduced levels.

3.4.2. Effect of tellurite on protein and ATP production

While it has been shown that proteins, specifically those containing reduced thiol groups, can be damaged by TeO_3^{2-} (Turner et al., 1999), its direct effect on highly resistant bacteria is unclear. With the majority of known impacts of tellurite on cells being negative (Turner et al., 1999), except for the select few species that can anaerobically respire on TeO_3^{2-} (Baesman et al., 2007; Baesman et al., 2009), we expected there would be reduced growth and, therefore, decreased protein in its presence. Indeed, strains E1, E4(1), RB3, and RB 16-17 showed a drop in protein production as expected (57.8%, 21.3%, 49.8%, and 41.5%, respectively) (Fig. 3.1 B, Fig. S3 B-D). However, KR99 and E5 had an increase in protein levels (66.6% and 21.2%, respectively) (Fig. 3.1 A, Fig. S5 A).

Exposure to lower levels of tellurite in murine hepatocarcinoma cells resulted in an 80% drop in ATP (Sandoval et al., 2010), and aerobically grown non-resistant *E. coli*, monitored over the short term, causes a loss of the transmembrane proton gradient leading to depletion of intracellular ATP, but does not affect intracellular concentrations of glycolytic intermediates (Lohmeier-Vogel et al., 2004), suggesting tellurite may target components of the electron transport chain (ETC). Also, this oxyanion generates ROS, which may lead to membrane damage (Storz & Imlay, 1999; Perez et al., 2007), possibility resulting in further disruption of the ETC. Therefore, as the concentration of tellurite used here was significantly higher and prior work showed even resistant strains can be negatively affected (Chapter 2), we predicted that ATP levels in our experiments might be decreased. As expected, this was observed for E1, E4(1),

Table 3.1. Effect of pH and aeration on growth with K_2TeO_3 estimated at A_{950} over 96 h and represented as percent of maximal absorbance.

Strain	pH			Aeration (rpm)		
	7.0	8.0	9.0	100	200	300
E1 [1]	78.4 ± 3.8	82.5 ± 4.8	100 ± 1.8	61.8 ± 5.9	100 ± 5.4	64.5 ± 4.9
E4(1) [1]	68.1 ± 5.1	77.6 ± 2.9	100 ± 0.7	54.2 ± 4.6	100 ± 2.2	67.4 ± 3.5
E5 [1]	62.8 ± 4.5	74.9 ± 2.4	100 ± 4.7	81.7 ± 3.7	100 ± 2.7	72.1 ± 2.9
KR99 [1]	77.7 ± 1.9	97.6 ± 1.1	100 ± 1.5	47.6 ± 2.2	100 ± 3.2	53.9 ± 4.4
RB 16-17 [2]	70.8 ± 3.3	71.4 ± 4.6	100 ± 4.3	55.9 ± 2.9	100 ± 3.7	66.8 ± 3.3
RB3 [2]	78.2 ± 3.6	76.6 ± 5.5	100 ± 3.2	59.7 ± 3.1	100 ± 4.3	62.1 ± 3.6

[1] 500 µg/ml tellurite added; [2] 100 µg/ml tellurite added.
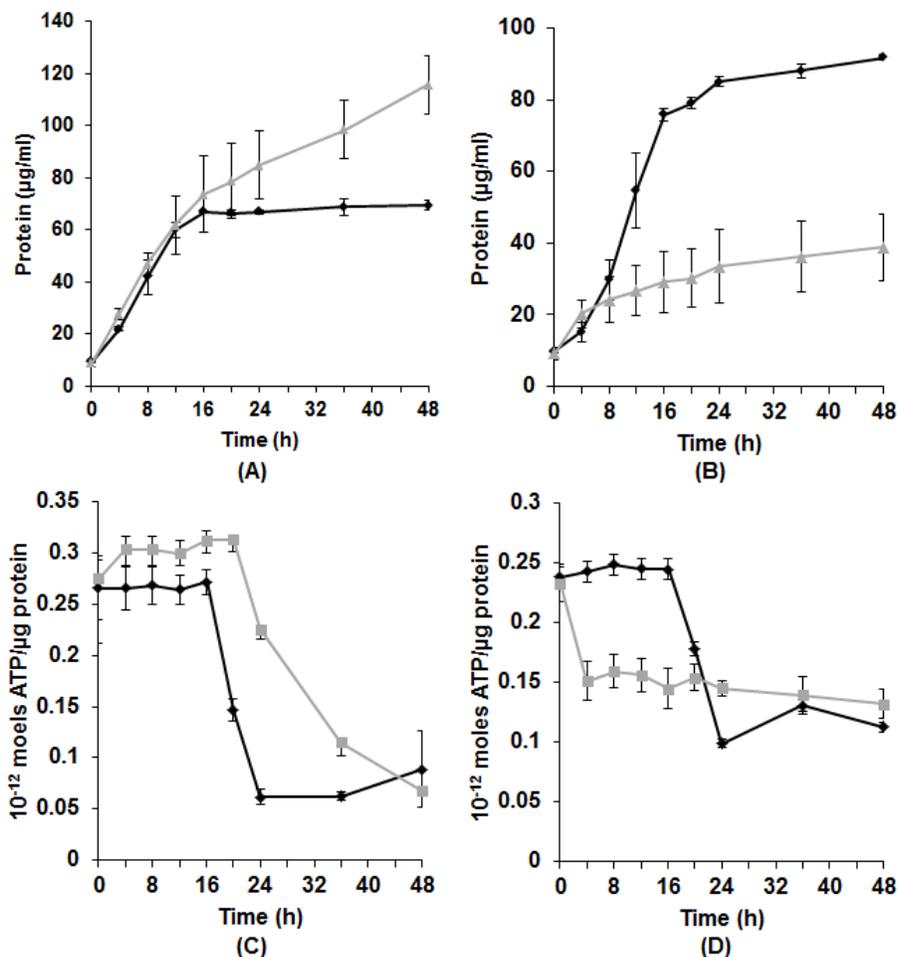
Figure 3.1. Protein and ATP production in the presence versus absence of K_2TeO_3. (**A**) Strain KR99. Similar results for E5 (Fig. S5 A); (**B**) Strain E1. Similar results for E4(1), RB3, and RB 16-17 (Fig. S5 B-D).♦ - No K_2TeO_3; ▲ - 500 µg/ml K_2TeO_3; (**C**) Strain KR99. Similar results for E5 (Fig. S5 E); (**D**) Strain E1. Similar results for E4(1), RB3, and RB 16-17 (Fig S5 F-H). ♦ - No K_2TeO_3; ■ - 500 µg/ml K_2TeO_3. Error bars represent one standard deviation.

RB3, and RB 16-17 (35.9%, 31.9%, 55.9%, and 48.7% decrease per unit protein, respectively) (Fig. 3.1 D, Fig. S5 F-H). However, unexpectedly, E5 and KR99 cells produced higher levels of ATP in the presence of tellurite (38.9% and 15.2% increase, respectively) (Fig. 3.1 C, Fig. S3 E). For these two strains, an increase was measured in both protein and ATP, and for E1, E4(1), RB3, and RB 16-17 a decrease in each case.

3.4.3. Characteristics of tellurite reductase activity

Generally, if a bacterium must induce expression of a protein to cope with the presence of a harmful substance, a lag phase will be observed during a primary exposure, while the specific product/enzyme is being transcribed and translated (Rolfe et al., 2012). However, during subsequent exposure, a lag phase is usually unnecessary, since cells have already adapted. Such phenomenon has been observed in experiments with tellurite (Chapter 2) as well as some other metal(loid) oxyanions, for example U(VI) (Spear et al., 1999). To determine if tellurite reduction involves *de novo* production of a specific enzyme, growth physiology during primary and secondary exposure was compared. If growth parameters in both cases were similar, it is likely that the reducing enzyme was constitutively present. However, a lag phase detected during primary exposure, but absent in secondary, would imply the need for initiation of transcription. Strains E1, E5, KR99, RB3, and RB 16-17 all possessed similar growth rates during both primary and secondary exposure (Fig. 3.2 A, Fig. S6), indicating a constitutive reductase. The remaining strain E4(1) was the exception. Growth was significantly hindered during secondary exposure (Fig. 3.2 B). As a result, it could not be determined if there was a lag phase during primary exposure.

Upon halting protein synthesis with tetracycline or chloramphenicol, we found strains E1, E5, KR99, RB3, and RB 16-17 were still capable of reducing tellurite, supporting a constitutive
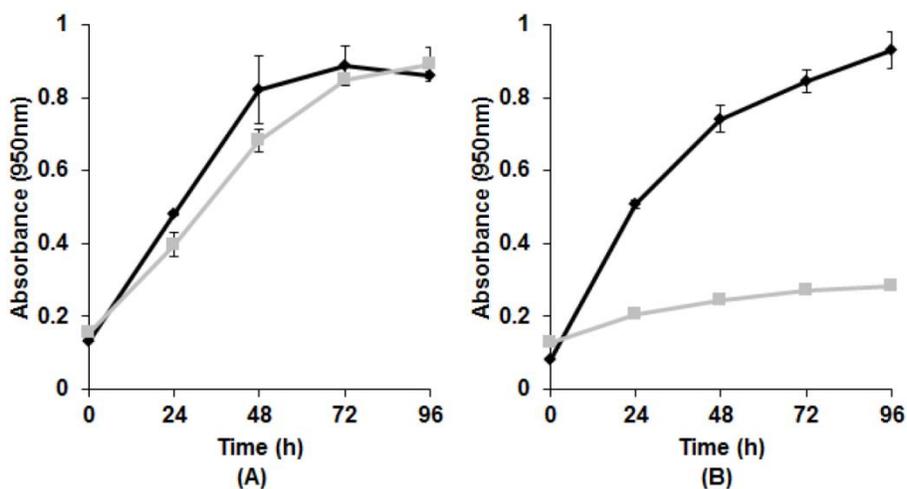
Figure 3.2. Growth and reduction during primary versus secondary exposure to K_2TeO_3. (**A**) Strain KR99. Similar results for E1, E5, RB3, and RB 16-17 (Fig. S6); (**B**) Strain E4(1). ♦ -Primary exposure; ■ - Secondary exposure. Error bars represent one standard deviation.

system. In cells of E4(1) reduction was inhibited, indicating *de novo* preparations are required. To further support the idea that the enzyme(s) responsible for reduction are constitutive, reduction in the cell lysates was analyzed (Fig. 3.3). Lysates of E1, E5, KR99, RB3, and RB 16-17 cells were capable of reducing K_2TeO_3 without prior exposure (Fig. 3.3 A), whereas those of E4(1) could not (Fig. 3.3 B), even following exposure (Fig. 3.3 C).

3.4.4. Localization of reductase activity

While all strains, with the exception of E4(1), possessed a constitutive tellurite reductase, its location was unknown. Therefore, strains were fractionated and each fraction monitored for reduction. No reductase activity was observed in the periplasm of any strain (Fig. 3.3 D), however, E1, E5, KR99, RB3, and RB 16-17 all possessed activity in the spheroplast and spheroplast lysate (Fig. 3.3 E, F). In the case of E4(1), no activity was observed in any fraction with or without prior exposure to TeO_3^{2-} (Fig. 3.3 G, H), indicating a possibility of reduction in intact cells only under the conditions tested. Upon separation of the membranes from the cytoplasmic contents, activity was detected for E1, E5, KR99, RB3, and RB 16-17. The rate of reduction was calculated for each fraction (Fig. 3.4). Strain KR99 possessed the highest rate of 0.284 units in the membranes. The strain with the second highest rate was E5 (0.159 units in the membranes), followed by E1 (0.056 units in the membranes), RB3 (0.038 units in spheroplast), and RB 16-17 (0.024 units in spheroplast).

3.5. Discussion

Our understanding of AAPs in general (Yurkov & Beatty, 1998; Yurkov & Hughes, 2013) and their interaction with tellurite in particular is still poor with many questions remaining. Obviously, the composition of the growth medium influences the level of resistance. Previous work has shown that in complex medium tellurite resistance can increase (Yurkov et al., 1996).
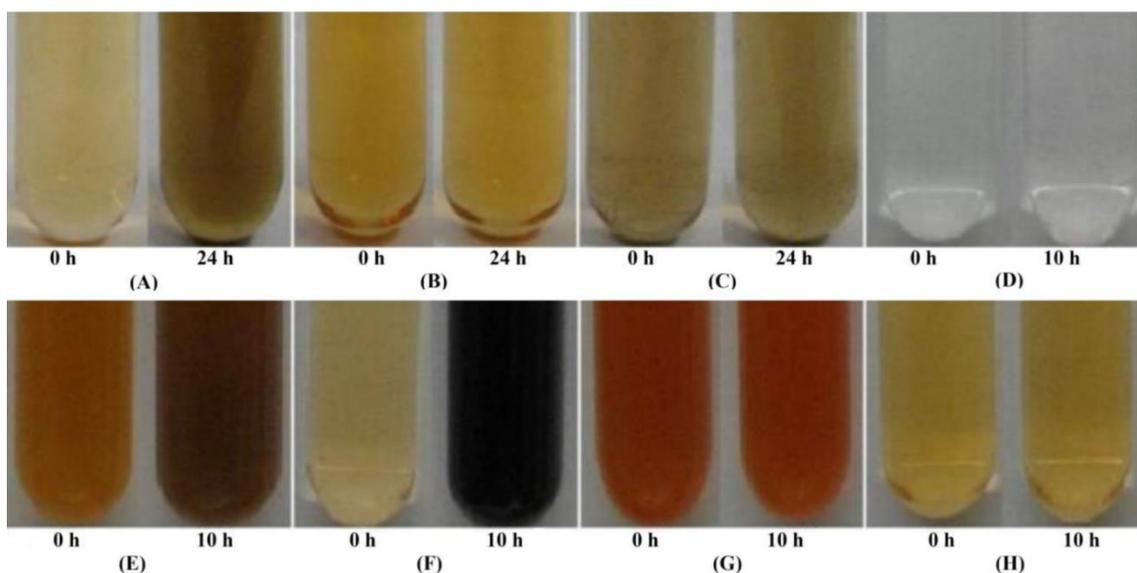
Figure 3.3. Reductase activity in cellular fractions. (**A**) Cell lysate of strain E1 grown without prior exposure to K_2TeO_3. Similar results were found for KR99, E5, RB3, and RB 16-17; (**B**) Lysate of strain E4(1) grown without prior exposure to K_2TeO_3; (**C**) Lysate of E4(1) cells grown with prior exposure to K_2TeO_3. Initial darkening at 0 h is due to the trace presence of previously reduced K_2TeO_3 from prior exposure; (**D**) Periplasmic fraction of KR99 without K_2TeO_3 exposure. No reductase activity observed. Similar results for E5, E4(1), E1, RB3 and RB 16-17; (**E**) Spheroplast fraction of E1 without prior K_2TeO_3 exposure containing reductase activity. Similar results for E5, KR99, RB3, and RB 16-17; (**F**) Spheroplast lysate of KR99 without prior K_2TeO_3 exposure containing reductase activity. Similar results for E5, E1, RB3, and RB 16-17; (**G**) E4(1) spheroplast fraction. No reductase activity observed with or without prior K_2TeO_3 exposure; (**H**) E4(1) spheroplast lysate. No reductase activity observed with or without prior K_2TeO_3 exposure.
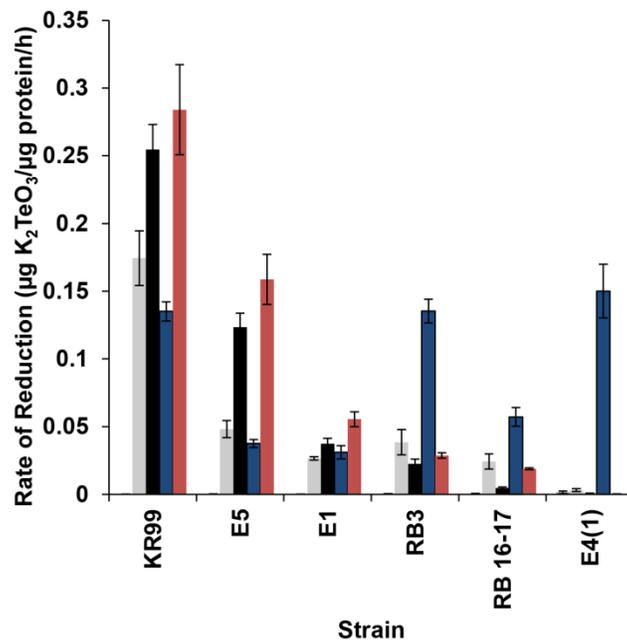
Figure 3.4. Rate of K_2TeO_3 reduction in cellular fractions. ■ Periplasm, ■ Spheroplast, ■ Spheroplast Lysate, ■ Whole Cells, ■ Membranes. Error bars represent one standard deviation.

In this study we found a similar trend, with E1, E4(1), RB3, and RB 16-17 favoring a complex medium. However, E5 and KR99 preferred a defined medium containing glutamate, pyruvate, and malate. We do not know why such a difference was seen. Perhaps as these carbon sources are involved with, or can be directly utilized by, the TCA cycle (Commichau et al., 2008), they are providing an advantage in energy generation under the stress of K_2TeO_3 pressure. Therefore, with conditions conducive to easier growth, the negative impact of tellurite can be overcome. It is also likely that these compounds are acting similar to how acetate does in *R. capsulatus*, competing with tellurite for cellular entry, thereby increasing resistance (Borghese et al., 2008; Borghese & Zannoni, 2010), especially in the case of those grown in RO medium, which does contain acetate.

The impact of TeO_3^{2-} on cellular proteins was negative for most of our strains. However, there were also some unexpected turns. Strains E5 and KR99 surprisingly produced more protein in the presence of tellurite. The reason for this increase is unclear, however, there is precedent, as the bacterium strain EG13 also has increased biomass production (4.5 fold) in the presence of other metalloid oxyanions ($NaVO_3$) compared to metal free medium (Csotonyi et al., 2015). It has been suggested that reduction of metal(loid) oxyanions can help dispose of excess electrons through the reoxidation of NADH, $FADH_2$, or quinones, therefore retaining optimal redox poise *in vivo* (Moore & Kaplan, 1992 and 1994; Csotonyi et al., 2015). This may result in optimal conditions for growth being maintained longer than in the absence of the oxyanion. Possibly, something similar is happening in KR99 and E5. An increase/decrease in biomass resulted in corresponding increased/decreased ATP, as one would expect.

Interestingly, strains E1, E5, KR99, RB3, and RB 16-17 appear to possess similar strategies for tellurite reduction, in both expression and location. While this may imply they are

alike, the ability of each strain to reduce and resist tellurite is different (Yurkov et al., 1996), which suggests they may actually have different sets of physiological reactions to interact with the oxyanion. Also, one can see reduction profiles in strains RB3 and RB 16-17 differ from the others, with whole cells having a higher rate than fractions even though activity is present in membranes. It is possible more than one mechanism is employed for reducing tellurite and/or some other cellular component is required for maximal effectiveness. Further research is needed to elucidate the exact approach utilized here. Strain E4(1) was the only one requiring a fully functional intact cell, as well as *de novo* protein preparations, for reduction. Other studied species capable of tellurite reduction/resistance at very high levels have been reported to possess a similar requirement (Chapter 2), and may share a comparable physiology. Possibly, E4(1) has an operational membrane electron transport system similar to *Shewanella oneidensis*, MR-1 developed for Fe(III), Mn(IV), and V(V) reduction (Borloo et al., 2007).

In this work, we have established that among different species of AAP, two strategies for tellurite reduction may be required. First, there is a constitutive membrane associated reduction pathway, as seen in E1, E5, KR99, RB3, and RB 16-17. The second, requires *de novo* protein synthesis and fully functional unbroken cells, found in strain E4(1). The identified approaches were not completely unexpected as similarities exist to previously established physiological responses to metal(loid) oxyanions. Membrane associated metal(loid) reduction has been previously observed in *R. capsulatus* and *Enterobacter cloacae* EV-SA01 (Moore & Kaplan, 1994; Van Marwijk et al., 2009). There is also precedent for the need of fully intact cells (Silver, 1996; Chapter 2). It is likely that there are several integral components associated with the outer membrane, periplasm, and inner membrane which are all involved in reducing tellurite. Hence,

removal of one component results in loss of function of the entire system. This complex coordinated arrangement is published for reduction of other metal oxyanions (Silver, 1996).

3.6. Conclusions

In summary, this paper broadens what we know about the strategies used by AAP for tellurite reduction and shows that more than one approach has evolved in this physiological group of bacteria to deal with this toxic compound. These strategies differ from the only known example of a tellurite specific reductase system, in Gram positive bacterium *Bacillus* STG-83 (Etezad et al., 2009). Our investigation has provided a stepping stone for future investigation of the key enzymes and pathways that provide the AAP capability to resist extremely high concentrations of toxic oxyanions.

3.7. Acknowledgments

3.8. Author Contributions

Chris Maltman and Vladimir Yurkov conceived and designed the microbiological experiments and analyzed and interpreted the data. Chris Maltman carried out the experiments and wrote the first draft of the paper. Vladimir Yurkov performed the critical revision and edited the final version of paper.

3.9. Conflicts of Interest

The authors declare no conflict of interest.

3.10. References

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Chapter 4.

Two distinct periplasmic enzymes are responsible for tellurite/tellurate and selenite

reduction by strain ER-Te-48 associated with the deep sea hydrothermal vent tube worms

at the Juan de Fuca Ridge black smokers

Chris Maltman, Lynda J. Donald, and Vladimir Yurkov

The first author was the major contributor to research presented.

Dr. L. Donald performed the mass spectrometry and preliminary protein fragmentation data interpretation.

4.1. Abstract

Strain ER-Te-48 isolated from deep-ocean hydrothermal vent tube worms is capable of resisting and reducing extremely high levels of tellurite, tellurate, and selenite, which are used for respiration anaerobically. Reduction is carried out by periplasmic enzymes that were purified and characterized. Tellurite and tellurate reduction is accomplished by an enzyme with a molecular weight of 215 kDa and comprised of 3 subunits of 74, 42, and 25 kDa in a 2:1:1 ratio. The optimum pH and temperature for activity is 8.0 and 35°C, respectively. Tellurite reduction had a V_{max} of 5.6 µmol/min/mg protein and a K_m of 3.9 mM. In the case of the tellurate reaction, V_{max} and K_m were 2.6 µmol/min/mg protein and 2.6 mM, respectively. Selenite reduction was carried out by a second distinct enzyme with V_{max} of 2.8 µmol/min/mg protein, K_m of 12.1 mM, and maximal activity at pH 6.0 and 38°C. The protein has a molecular weight of 165 kDa and is made up of 3 subunits with sizes of 98, 44, and 23 kDa in a 1:1:1 ratio. This is the first reported specific Te oxyanion reductase from a Gram negative organism and the only known reductase specific for selenite.

4.2. Introduction

Tellurium (Te) oxyanions, especially tellurite (TeO_3^{2-}), are toxic to many microorganisms at concentrations as low as 1 µg/ml (Yurkov et al., 1996). This toxicity is believed to be a consequence of its properties as a strong oxidant (Taylor, 1999; Lloyd et al., 2001). Reduction of the oxyanion to elemental Te is one strategy employed by bacteria for detoxification (Taylor et al., 1988; Lloyd-Jones et al., 1991; Moore & Kaplan, 1992). To date, there are very few known enzymes capable of reducing tellurite. In some organisms, such as *E. coli*, nitrate reductases carry out reduction (Avazeri et al., 1997; Sabaty et al., 2001) and in others (*Staphylococcus epidermidis*), catalases are implicated (Calderon et al., 2006). Several different enzymes, for example the thiol:disulfide oxidoreductase of *Rhodobacter capsulatus* (Borsetti et al., 2007) and GutS of *E. coli* (Guzzo & Dubow, 2000), among others (Chiong et al., 1988; Moscoso et al., 1998; Chiang et al., 2008; Kabiri et al., 2009), are involved in tellurite resistance and/or reduction. However, in all these cases the function is non-specific, not associated with their primary role, and yields low level resistance. Only one specific tellurite reductase, from cells of *Bacillus* sp., STG-83, has been published (Etezad et al., 2009). The cytoplasmic enzyme is 197 kDa, comprised of 3 subunits (66, 43, and 20 kDa), functions optimally at $35^{o}C$, pH 8.0, and has a K_m of 2.6 mM with a V_{max} of 5.2 µmol/min/mg protein (Etezad et al., 2009). *Bacillus* sp., STG-83 possesses increased resistance and might be capable of dissimilatory anaerobic reduction of tellurite. Although it has not been proven, the enzyme is most likely respiratory in nature (Etezad et al., 2009). Due to high toxicity, Te was long considered as a biologically unimportant element. However, it was recently discovered that certain bacteria could respire anaerobically on Te oxyanions (Csotonyi et al., 2006; Baesman et al., 2007; Baesman et al., 2009; Chapter 6; Chapter

7), establishing that in some habitats, Te does have a role to play in bacterial survival and energy generation.

Selenium (Se) oxyanions are also very toxic to most organisms at as low as 5µg/ml (Rathgeber et al., 2002). For a long time, Se was thought to be of no benefit to life, therefore, little attention was given to its interactions with biological systems. However, later Se was found to be biologically essential. It is important in the structure of the 21st amino acid, selenocysteine, and today we know many selenoenzymes (Cone et al., 1976). Just like Te, Se oxyanion reduction can often be carried out by nitrate reductases and catalases (Sabaty et al., 2001; Hunter & Manter, 2009), as well as some other non-specific enzymes (Yanke et al., 1995; Li et al., 2014). There are reports about specific reductases responsible for the transforming of selenate to selenite. The first was isolated from *Thauera selenatis* (Schroder et al., 1997). Since then, others have been identified, including a cytoplasmic enzyme in *Bacillus* sp., STG-83 (Etezad et al., 2009). It is 182 kDa, comprised of 4 subunits (70, 60, 50, and 25 kDa), and functions optimally at 38°C and pH 5.5-6.0. It also reduces selenite, with a K_m of 10.9 mM and a V_{max} of 1.6 µmol/min/mg protein (Etezad et al., 2009). That being said, a selenite specific reductase has yet to be discovered.

In both anaerobic and aerobic environments, many elemental transformations are a direct result of microbial enzymatic function (DeMoll-Decker & Macy, 1993; Laverman et al., 1995; Csotonyi et al., 2006). As so little is known about the metalloid oxyanion reducing enzymes, more work with bacteria capable of strong reduction is needed. This will enrich our knowledge of their importance, their structure and evolution, as well as abundance throughout the microbial world, and the role in biogeochemical cycling in nature. Since the industrial revolution, human activities have resulted in the release of enormous amounts of toxic chemicals, including Te and

Se oxyanions, into the biosphere, which contribute to serious pollution problems (Wood, 1974). Bioremediation of pollutants is a potentially attractive and ecologically sound removal method, which has been explored, but only to a very limited extent (Macy et al., 1993; Cantafio et al., 1996; Rajwade & Paknikar, 2003). Hence, the availability of specific enzymes capable of reducing aggressive oxyanions could aid in the development of environmentally friendly methods of remediation.

In this study, we investigated the periplasmic enzymes responsible for tellurite/tellurate and selenite reduction in the *Shewanella frigidimarina* phylogenetic relative, strain ER-Te-48 (Csotonyi et al., 2006; Chapter 2). This microorganism was isolated from the deep sea hydrothermal vent tube worm, *Paralvinella sulfincola*, inhabiting the fields of the Juan de Fuca Ridge black smokers in the Pacific Ocean. Strain ER-Te-48 is capable of resisting extreme levels of tellurite, tellurate, and selenite, as well as using them as terminal electron acceptors for anaerobic respiration (Csotonyi et al., 2006; Chapter 7).

4.3. Materials and Methods

4.3.1. Purification and characterization of tellurite and selenite reductases

Strain ER-Te-48 was grown under its optimal conditions (Chapter 2) in the presence of either tellurite or selenite. Periplasmic contents were collected, and reductase activity confirmed as described (Chapter 2). The fraction was then filtered through a 0.2 μm syringe filter to remove any large contaminants. Crude protein was separated into >100 kDa, 3-100 kDa, and <3 kDa fractions using centrifugal concentrators from Millipore. Purification of the desired reductase began with a Superdex S-200 gel filtration column. The >100 kDa periplasmic fraction was loaded onto the column previously equilibrated with 20 mM Tris HCl, pH 8.0. The flow rate was set to 1.0 ml/min and separate 2 ml fractions were collected (Fig. S7). The fractions exhibiting

tellurite or selenite reductase activity were pooled and concentrated with 100 kDa concentrators. Each sample was loaded onto a Source 15 Q anion exchange column equilibrated with 20 mM Tris HCl, pH 8.0 and eluted using a 0-1 M NaCl gradient. The flow rate was set to 3.0 ml/min and 2 ml fractions were collected (Fig. S8). Those exhibiting reductase activity were pooled and concentrated as above. Native PAGE (5%) was used to confirm the presence of only a single protein. Molecular weight was estimated with a 20 mM Tris HCl, pH 8.0 equilibrated Superdex S-200 gel filtration column. The protein standards used were: aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa), and aprotinin (6.5 kDa). The column void volume was determined with ferritin (440 kDa). Lawsone (2-hydroxy-naphthoquinone) and 9, 10-anthraquinone-2-sulfonic acid were assayed for replacement of the unknown periplasmic component required for reduction (Lovley et al., 1998). Controls were used without the addition of enzyme to confirm reduction was enzymatic and not chemical. The number and size of subunits was resolved by SDS PAGE as published (Laemmli, 1970) with a 5% stacking gel and a 12% running gel. Samples were denatured by boiling for 4 min in 1% (w/v) SDS in the presence of 2-mercaptoethanol. Gels were stained with Coomassie brilliant blue (Wilson, 1983). ImageJ software was used to determine the stoichiometry of subunits (Schneider et al., 2012).

4.3.2. Enzyme properties and kinetics

Glutamate, aspartate, pyruvate, succinate, malate, lactate, acetate, and NADH were assayed for the capacity of electron donors in the oxyanion reduction reactions. Multiple metal(loid) oxyanions (tellurite, tellurate, selenite, selenate, meta- and orthovanadate (250 µg/ml)) were tested as well as nitrate and nitrite (100 µg/ml) as possible substrates. Reduction of the metal(loid) compounds was monitored visually and nitrate/nitrite reduction

spectrophotometrically (Garcia-Robledo et al., 2014). To study the tellurite/tellurate reductase functional kinetics, the reaction mixture contained: 500 µl 20 mM Tris HCl, pH 8.0; 10 µl glutamate (10 mM stock); 25 µl lawsone (10 mM stock in 95% ethanol); 50 µl purified enzyme (100 µg protein/ml) and tellurite (0 to 14 mM) or tellurate (0 to 12 mM). Reduction was measured spectrophotometrically at 500 nm based of standard curves (Fig. S9 A, B) (Chiong et al., 1988; Moscoso et al., 1998; Molina et al., 2010). For selenite, the reaction mixture contained: 500 µl 20 mM PBS, pH 6.0; 10 µl glutamate (10 mM stock); 25 µl lawsone (10 mM stock in 95% ethanol); 50 µl purified enzyme (90 µg protein/ml); and selenite (0 to 45 mM). Reduction was monitored spectrophotometrically based off a standard curve created for selenite using the method for tellurite described by Molina et al. (Molina et al., 2010) at 590 nm (Fig. S9 C) (Dhanjal & Cameotra, 2010). Optimal pH for both enzymes was determined in PBS or Tris HCl buffer adjusted to pH 6.0, 7.0, 8.0, or 9.0 with 0.5 N NaOH or HCl. The reaction mixture was incubated at 20, 25, 28, 32, 35, 38, and 42°C to find the optimal temperature. The K_m of glutamate was determined in the same reaction mixture as for tellurite or selenite reduction, using varying concentrations of glutamate (0 to 500 µM) and 10 mM TeO_3^{2-} or 15 mM $SeO3^{2-}$. Enzymatic activity was defined as the amount of enzyme that catalyzes conversion of 1 µmol of substrate to product per minute per mg protein. Protein was measured by the Bradford method (Bradford, 1976). Controls containing all components but no enzyme were used to confirm no chemical reduction was taking place.

4.3.3. Mass spectrometry

Protein-containing slices were taken from native gels (Fig. 4.1 A, Lanes 4, 5) then reduced, alkylated, and digested with trypsin as described by Shevchenko et al (Shevchenko et al., 2006). Peptides were eluted from the gel slices, then mixed with an equal volume of DHB
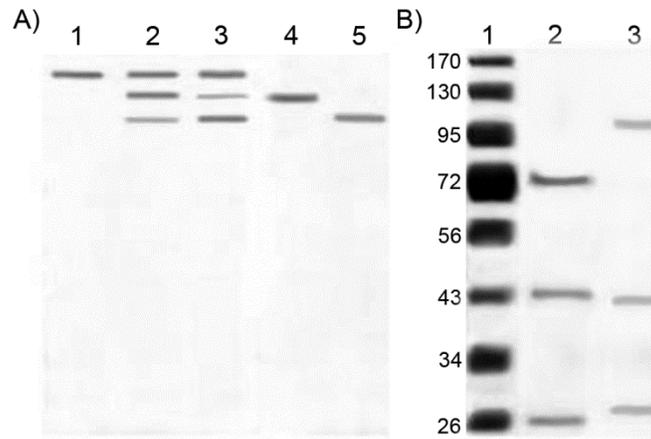
Figure 4.1. A) Native PAGE of > 100 kDa periplasmic fraction of strain ER-Te-48: Lane 1, No tellurite or selenite exposure; Lane 2, with tellurite exposure; Lane 3, with selenite exposure; Lane 4, Purified tellurite reductase; Lane 5, Purified selenite reductase. B) SDS-PAGE of purified enzymes: Lane 1, Molecular weight standards (in kDa); Lane 2, Tellurite reductase; Lane 3, Selenite reductase.

matrix (saturated 2,5-dihydroxybenzoic acid in 50% ACN, 2% formic acid) on a metal target. Some samples were further purified with Millipore C18 ZipTips™. Spectra were obtained using a MALDI quadrupole time-of-flight mass spectrometer built in the department of Physics and Astronomy, University of Manitoba (Loboda et al., 2000). Analysis of spectra was done with TOFMS and pTOOL, non-commercial software developed with the instruments. Ions were selected with a signal/noise of 2, and the list was sent to MASCOT for identification (Matrix Science Limited, 2014). All data were checked against the Swissprot and NCBI databases using a 30 ppm error level and limited to bacterial taxonomy. An open search was also done for contaminants. When data were adequate, tandem mass spectrometry was done to compare the fragmentation pattern of the observed ions to those expected from ions of the putative sequence (Shevchenko et al., 2000).

4.4. Results and Discussion

4.4.1. Physical characteristics of reductases

The periplasmic contents of ER-Te-48 were collected and the crude extract was divided based on protein size using 100 and 3 kDa membrane cut-off centrifugal concentrators. Tellurite or selenite reductase activity was assayed for in the >100 kDa and 3 - 100 kDa fractions, but none was observed. However, upon addition of the <3000 Da flow through to each tested sample, oxyanion reduction was restored in the >100 kDa mixture, indicating that some unknown periplasmic constituent is essential for reduction. Other similar examples are known where a reaction needs some kind of small but important molecule to proceed. For example, it has been found that humic acids can be reduced and then used for reduction of Fe(III) (Lovley et al., 1998). Spectrophotometric analysis of the <3000 Da fraction revealed an absorption peak at 274 nm (Fig. 4.2), which suggested some type of quinone-like compound (Morton & Earlam,
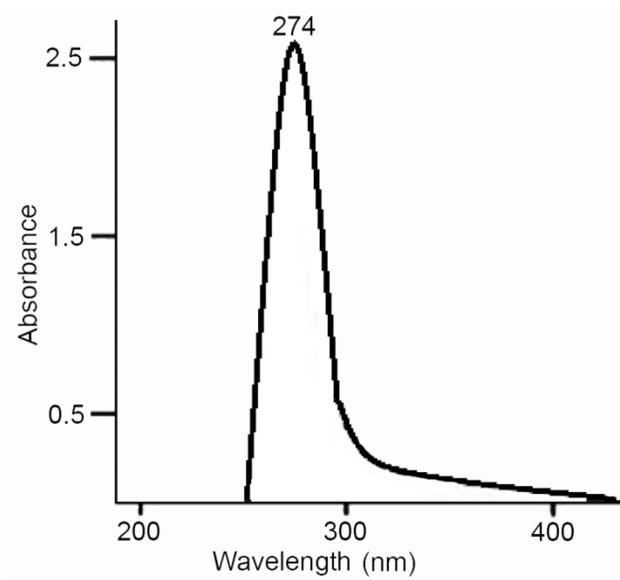
Figure 4.2. Spectrophotometric analysis of unidentified periplasmic molecule required for tellurite/tellurate and selenite reductase activity in strain ER-Te-48.

1941). Lawsone and 9, 10-anthraquinone-2-sulfonic acid, which are known to function as electron shuttles in bacterial metal reduction (Lovley et al., 1998), could be substituted in place of this unidentified factor, with the former being equally effective. Therefore, lawsone was used in all experiments.

Our previous work has shown that strain ER-Te-48 does not have a constitutively expressed reductase and requires *de novo* enzyme synthesis for reduction (Chapter 2). Native PAGE of the >100 kDa fraction revealed two protein bands, which were only present following exposure of cells to tellurite or selenite (Fig. 4.2 A, Lanes 1-3), further supporting *de novo* production. Therefore, two different proteins are present regardless of the oxyanion to which the bacterium is exposed. It suggests that either two distinct enzymes carry out reduction in tandem, or one reduces tellurite and the other selenite. Upon further purification of each protein individually, the larger reduced TeO_3^{2-} and the smaller SeO_3^{2-}. For the tellurite reductase, size exclusion chromatography followed by anion exchange chromatography resulted in the 148 fold purification (Table 4.1) of a single protein, which was confirmed by native PAGE (Fig. 4.2 A, Lane 4). The molecular weight was 215 kDa and SDS-PAGE showed 3 subunits of approximately 74, 42, and 25 kDa (Fig. 4.2 B, Lane 2) in a 2:1:1 ratio. Following the same procedure, a selenite reductase was purified 138 fold (Table 4.1) and native PAGE confirmed the presence of a single protein (Fig. 4.2 A, Lane 5). This enzyme was 165 kDa and contained 3 subunits of 98, 44, and 23 kDa (Fig. 4.2 B, Lane 3) in a 1:1:1 ratio.

The isolated proteins appear novel because they originate from the periplasm of a Gram negative bacterium and are specific to either Te oxyanions or selenite. There are no other published examples to draw comparisons. The known tellurite and selenite/selenate reductases from *Bacillus* sp., STG-83 (Etezad et al., 2009) are cytoplasmic but from a Gram positive

Table 4.1. Isolation of tellurite and selenite reductase from the periplasm of strain ER-Te-48.

Fraction	Activity (µM K$_2$TeO$_3$/min)	Total Protein (mg/l)	Specific Activity (µM K$_2$TeO$_3$/min/mg protein)	Yield (%)	Fold Purification
Cell Lysate	3.7 (2.8)*	209 (187)	0.018 (0.015)	100 (100)	1 (1)
Periplasm	2.9 (2.3)	36 (34)	0.081 (0.068)	78.4 (82.1)	4.5 (4.5)
>100 kDa proteins	2.6 (1.3)	8 (6)	0.325 (0.217)	70.2 (46.4)	18.1 (14.5)
S200 with reductase activity	1.4 (0.7)	0.67 (.38)	2.100 (1.842)	37.8 (25.0)	116.7 (122.8)
Ion Exchange with reductase activity	1.2 (0.6)	0.45 (.29)	2.667 (2.069)	32.4 (21.4)	148.2 (137.9)

*Values in parentheses for selenite reductase.

organism. The selenite reductase in our study is somewhat similar to the periplasmic selenate reductase from *Thauera selenatis*, which showed 3 subunits of 96, 40, and 23 kDa, however, it does not use selenite as a substrate (Schroder et al., 1997). Furthermore, later sequencing has since shown that *T. selenatis* selenate reductase appears to contain a fourth subunit (Krafft et al., 2000).

4.4.2. Biochemistry of reductases

Besides glutamate, which is a known electron donor for the reduction of tellurite by ER-Te-48 (Chapter 2), other compounds were tested as possible candidates (Table 4.2). Besides glutamate, only NADH could perform this function, but nowhere near maximal activity (Table 4.2), further pointing to glutamate as a natural electron donor. The idea is also supported by a low K_m of 37.8 µM and 33.5 µM for tellurite and selenite reduction respectively (Fig. 4.3, Fig. S10), (Mantsala & Nieme, 2009) and the fact this amino acid may be abundant in ER-Te-48's natural environment. This strain is an epibiont of tube worms inhabiting deep ocean hydrothermal vents (Csotonyi et al., 2006). Certain species of tube worms, such as *Riftia pachyptila*, are provided with organic carbon by symbiotic bacteria, predominantly in the form of glutamate (Felbeck & Jarchow, 1998). Hence, some of it could be used as an electron donor in biological tellurite and selenite reduction. Upon testing other metal(loid) oxyanions and nitrate/nitrite as substrates, the tellurite reductase could also reduce tellurate, but the selenite reductase was specific to only one oxyanion. As these enzymes could not reduce either nitrate or nitrite, it rules out the possibility they belong to this family of enzymes. Tellurite reductase had its highest activity at 35^oC and pH 8.0 (Fig. 4.4 A, B). The selenite reductase had a slightly higher optimal temperature of 38^oC, but a lower optimal pH of 6.0 (Fig. 4.4 C, D). From Lineweaver–Burk plots the K_m and V_{max} values were calculated for reduction of each oxyanion.

Table 4.2. Compounds tested as electron donors for tellurite/tellurate and selenite reduction.

Electron Donor	Reductase Activity (% Maximal)
Glutamate	100* (100)
Aspartate	0 (0)
Pyruvate	0 (0)
Succinate	0 (0)
Malate	0 (0)
Lactate	0 (0)
Acetate	0 (0)
NADH	18 (23)

*Activity with glutamate represents maximal reduction. Values in parentheses for selenite
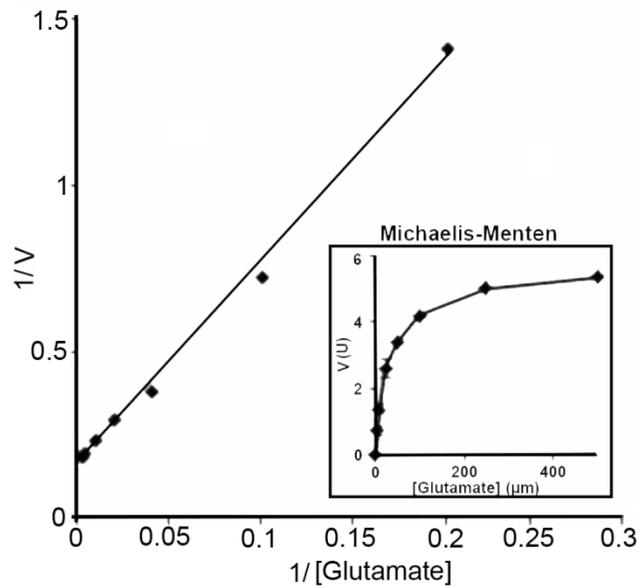
reductase.

Figure 4.3. Lineweaver–Burk plot of the electron donor glutamate for tellurite reduction. The insert shows the Michaelis–Menten plot. Error bars represent one standard deviation.
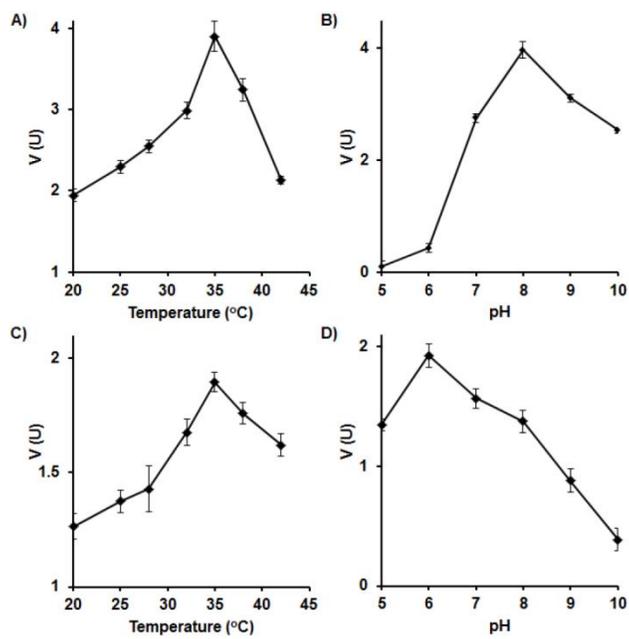
Figure 4.4. Effect of temperature on (A) Tellurite reductase (Same results for tellurate (Fig. S11 A)) and (C) Selenite reductase. Impact of pH on (B) Tellurite reductase (same results for tellurate (Fig. S11 B)) and (D) Selenite reductase. Error bars represent one standard deviation.

For tellurite reduction, values of 5.6 µmol/min/mg of protein and 3.9 mM were obtained for V_{max} and K_m, respectively (Fig. 4.5 A), whereas the V_{max} for tellurate reduction was 2.6 µmol/min/mg of protein with a K_m of 2.6 mM (Fig. 4.5 B). The slower rate for tellurate reduction could be attributed to steric hindrance due to the extra oxygen present or possibly the need for more electrons to transform it to Te^0 as compared to conversion of tellurite. Either may result in the need for more time to carry out reduction. In the case of selenite reduction, V_{max} was 2.8 µmol/min/mg of protein and the K_m was 12.1 mM (Fig. 4.5 C).

4.4.3. Mass spectrometry analysis

Our first goal was to test that the observed bands on the native gel were proteins. However, single bands from those gels did not yield enough material for analysis. Therefore, we used a pool of 5 slices of each protein, as shown in Fig. 4.2 A, lanes 4 and 5. For the ions from tellurite/tellurate reductase, the MASCOT search produced a reasonable identification to alkaline phosphatase of *Pseudomonas* sp., TKP (Ohtsubo et al., 2014) (Fig. 4.6). This enzyme is a periplasmic binding protein from the type 2 superfamily, and is likely part of the phosphate transport system in *Pseudomonas* species. Tandem mass spectrometry of some of the abundant ions did not verify the identification, although there were several instances of runs of matching amino acids (Fig. 4.7). The conserved sequence "VPSVATSVAIPFR" from the ion at *m/z* 1826 is similar to the actual phosphate binding region, and a BLASTn search (Altshul et al., 1997) using only this sequence identified the same alkaline phosphatase. However, because we have a mixture of protein subunits, and many ions not identified, but it may be part of a system where the phosphate binding is repurposed to tellurate or tellurite, similar to what has been observed in *E. coli*, where a phosphate transporter allows tellurite ingress (Thomas & Kay, 1986; Elias et al., 2012). The nearest match for the selenite reductase was the molecular chaperone GroEL,
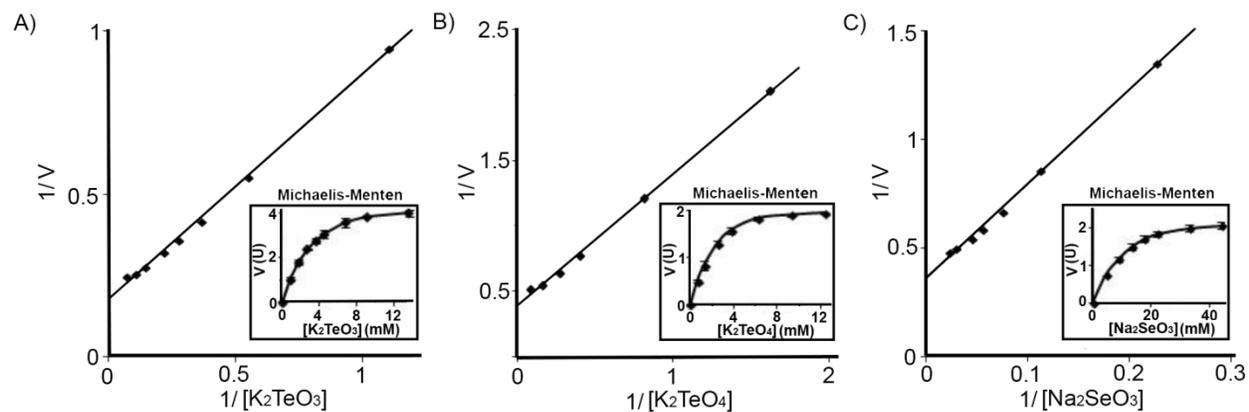
Figure 4.5. Lineweaver–Burk plots. Kinetics of reduction of (A) Tellurite, (B) Tellurate, and (C) Selenite. The insert shows the Michaelis–Menten plot. Error bars represent one standard deviation.

>gi|568200452|ref|WP_024074816.1| alkaline phosphatase [*Pseudomonas* sp. TKP]

MFKRNVLAVSMTLAALCSAQAAMADINGGGATLPQK<u>LYQTSGVLTAGFAPYIGVGSG</u>

<u>NGKAAFLTNDYTKFVAGVSNKNVHWAGSDSKLTATELSTYATNKQPTWGKLIQVPSV</u>

<u>ATSVAIPFRKSGANAVDLSVSELCGVFSGRITDWSGISGAGRTGPITVVYRSESSGTTELF</u>

<u>TR</u>FLNAKCTETGTFNITTTFGTSYTGGLPAGAVAATGSQGVMDAVNDTSVAEGR<u>ITYMS</u>

<u>PDFAASTLAGLDDATKVAR</u>VGKNTAKGIKGVSPAPSNVSDAIAQVLPPNDPSAPLDVTN

PDNWVPVFGKTGVAGVQPYPDSGYPILGFTNLIFSQCYADATQTSQVR<u>AFFTKHFGDTN</u>

<u>NNDDAITANRFVPLPDNWKTAITDNFVTASSALSIGKTNVCNGIGRPL</u>

Fig. 4.6. Mass spectrometry sequence analysis for the periplasmic tellurite/tellurate reductase compared to the nearest match, alkaline phosphatase from *Pseudomonas* sp., TKP from the NCBI database. Sequence identified by data matching is underlined.

| MALDI SPECTRUM | | VIRTUAL DIGEST OF ALKALINE PHOSPHASE of *Pseudomonas* sp strainTKP | | | | INFERRED FROM MSMS[1,2] |
OBSERVED IONS	INTENSITY	EXPECTED IONS	from	to	expected sequence	[1] L used here can be Leu or Ile [2] lower case are best fit for mass
		821.452	71	78	FVAGVSNK	
1005.573	14737	1005.573	157	165	TGPITVVYR	xxxxxVVYR
		1043.633	251	260	VARVGKNTAK	
		1100.512	79	88	NVHWAGSDSK	
1115.584	14353	1115.589	363	371	FVPLPDNWK	FvPLPDNWK
		1143.569	61	70	AAFLTNDYTK	
		1200.616	390	400	TNVCNGIGRPL	
1219.605	33878	1219.607	145	156	ITDWSGISGAGR	xxxxxxxGR
1276.628	4884					
1314.619	7332	1314.618	166	177	SESSGTTELFTR	too weak
		1412.727	89	101	LTATELSTYATNK	
1676.782	1883					
1698.005	6767	1697.995	108	123	LIQVPSVATSVAIPFR	xxxsvaTSvALPFR
1707.786	5081					
1774.803	4922	1774.775	347	362	HFGDTNNNDDAITANR	xxxxxDSTPAnR
1791.75	4782					
1795.936	4485	1795.944	372	389	TAITDNFVTASSALSIGK	too weak
1798.985	4279					
1826.096	27512	1826.09	108	124	LIQVPSVATSVAIPFRK	QxxVPsVATSVALPFRK
1902.931	4169	1902.946	71	88	FVAGVSNKNVHWAGSDSK	too weak
1946.002	4710	1946.002	61	78	AAFLTNDYTKFVAGVSNK	no breakage
2024.976	6996	2024.971	125	144	SGANAVDLSVSELCGVFSGR	xxxxAvDLSLVSELCGVFSGR
2110.083	8211	2110.082	89	107	LTATELSTYATNKQPTWGK	too weak
2145.042	3980					
		2153.066	124	144	KSGANAVDLSVSELCGVFSGR	
2185.043	3922					
		2188.048	230	250	ITYMSPDFAASTLAGLDDATK	
2204.052	4358					
2220.076	7496					
2225.142	5281					
2299.092	2552					
2357.181	3482	2357.214	37	60	LYQTSGVLTAGFAPYIGVGSGNGK	too weak
2369.093	7459	2369.091	342	362	AFFTKHFGDTNNNDDALTANR	xxxxxHFGDTNNNDDALxxxx
		2395.35	102	123	QPTWGKLIQVPSVATSVAIPFR	
2466.27	3579					
		2494.221	79	101	NVHWAGSDSKLTATELSTYATNK	
2514.292	12331	2514.255	230	253	ITYMSPDFAASTLAGLDDATKVAR	xxxxSPDFAASTLAGLDDATKxxxx
		2523.445	102	124	QPTWGKLIQVPSVATSVAIPFRK	
2530.251	4607					
2705.191	2098					
		2798.44	230	256	ITYMSPDFAASTLAGLDDATKVARVGK	
2977.524	953	2977.542	372	400	TAITDNFVTASSALSIGKTNVCNGIGRPL	too weak
		3144.586	5	36	NVLAVSMTLAALCSAQAAMADINGGGATLPQK	
3163.463	563					
		3191.576	79	107	NVHWAGSDSKLTATELSTYATNKQPTWGK	
3211.478	2158					
		3225.56	125	156	SGANAVDLSVSELCGVFSGRITDWSGISGAGR	
		3296.655	71	101	FVAGVSNKNVHWAGSDSKLTATELSTYATNK	
		3300.687	4	36	RNVLAVSMTLAALCSAQAAMADINGGGATLPQK	
		3353.655	124	156	KSGANAVDLSVSELCGVFSGRITDWSGISGAGR	
3509.807	2012					
		3789.059	89	123	LTATELSTYATNKQPTWGKLIQVPSVATSVAIPFR	
		3832.043	108	144	LIQVPSVATSVAIPFRKSGANAVDLSVSELCGVFSGR	
		4074.112	363	400	FVPLPDNWKTAITDNFVTASSALSIGKTNVCNGIGRPL	
4082.04	587	4082.051	264	303	GVSPAPSNVSDAIAQVLPPNDPSAPLDVTNPDNWVPVFGK	
4114.042	340					
		4121.992	304	341	TGVAGVQPYPDSGYPILGFTNLIFSQCYADATQTSQVR	
		4212.115	125	165	SGANAVDLSVSELCGVFSGRITDWSGISGAGRTGPITVVYR	
		4641.136	183	229	CTETGTFNITTTFGTSYTGGLPAGAVAATGSQGVMDAVNDTSVAEGR	
4972.967	369					

Figure 4.7. Comparison of ions from the tryptic digest of ER-Te-48 tellurite/tellurate reductase to those expected from a similar hypothetical digest of the alkaline phosphatase from *Pseudomonas* sp., strain TKP.

>gi|499958341|ref|WP_011639075.1| molecular chaperone GroEL [*Shewanella frigidimarina*]

M<u>AAKEVLFGNDARVKMLAGVNILANAVKVTLGPKGRNVVLDKSFGSPMITKDGVSVA</u>

<u>KEIELTDKFENMGAQMVK</u>EVASK<u>ANDAAGDGTTTATVLAQAIVVEGLK</u>AVAAGMNP

MDLKRGIDK<u>AVIAAVAELKLLSQECSDTKAIAQVGTISANSDESIGDIIATAMEKVGKEG</u>

<u>VITVEEGQALENELDVVEGMQFDR</u>GYLSPYFINKPETGSIELDSPFILLVDKK<u>VSNIRELL</u>

<u>PILEGLAKTGKPLLIVAEDVEGEALATLVVNNMR</u>GIVK<u>VAAVKAPGFGDRR</u>KAMLQDV

AILTGGTVIAEEIGLELEKATLEDLGTAKR<u>VIITKDNTTIIDGSGDQVQISAR</u>VSQIKQQVE

DSTSDYDKEKLQERMAKLAGGVAVIKVGAATEVEMKEKK<u>ARVEDALHATRAAVEEG</u>

<u>VVAGGGVALVRVASKIKDVEVLNEDQKHGVVIALRAMEAPLRQIATNAGQEASVVAN</u>

<u>TVKNGEGNFGYNAGNDTYGDMLEMGILDPTKVTR</u>SALQFAASIAGLMITTEAMIAEVP

QDSAPDMGGMGGMGGMGGMM

Figure 4.8. Mass spectrometry sequence analysis for the periplasmic selenite reductase compared to the nearest match, GroEL from *Shewanella frigidimarina* from the NCBI database. Sequence identified by data matching is underlined.

MALDI SPECTRUM		VIRTUAL DIGEST OF GroEL OF *Shewanella frigidimarina*				MSMS confirmation
OBSERVED IONS	INTENSITY	EXPECTED IONS	from	to	expected sequence	
		827.535	372	380	LAGGVAVIK	
		847.441	59	65	EIELTDK	
832.292	34061					
864.509	23009	864.542	438	445	HGVVIALR	
875.423	46071	875.449	278	285	APGFGDRR	
		967.492	43	51	SFGSPMITK	
		984.609	123	132	AVIAAVAELK	
1003.511	24078	1003.544	278	286	APGFGDRRK	
1011.484	18505	1011.522	396	404	VEDALHATR	
		1018.542	312	321	ATLEDLGTAK	
	74489	1020.511	5	13	EVLFGNDAR	
		1034.519	381	390	VGAATEVEMK	
1135.531	18292					
1153.547	19022					
		1154.534	66	75	FENMGAQMVK	
1165.707	17361					
		1180.552	133	142	LLSQECSDTK	
1187.623	37997	1187.654	273	284	VAAVKAPGFGDR	
		1188.575	428	437	DVEVLNEDQK	
1195.691	32819	1195.73	232	242	ELLPILEGLAK	
		1217.602	106	117	AVAAGMNPMDLK	
1238.621	17660	1238.661	394	404	ARVEDALHATR	
1247.643	82657	1247.675	5	15	EVLFGNDARVK	
1290.645	49515	1290.681	2	13	AAKEVLFGNDAR	
1313.7	20390	1313.762	16	28	MLAGVNILANAVK	
1343.716	51503	1343.755	273	285	VAAVKAPGFGDRR	
		1414.597	351	362	QQVEDSTSDYDK	
1428.623	33876					
1502.709	11580					
1517.796	24526					
1529.69	11891					
1535.797	12612					
1540.864	20197	1540.925	14	28	VKMLAGVNILANAVK	
1553.821	74799	1553.865	405	421	AAVEEGVVAGGGVALVR	yes
1562.797	14453					
1584.869	17486	1584.923	269	284	GIVKVAAVKAPGFGDR	
1587.839	18630					
1635.834	31605	1635.878	37	51	NVVLDKSFGSPMITK	
1651.824	17357					
1707.762	15749					
1765.01	42691	1765.059	227	242	VSNIRELLPILEGLAK	yes
1783.876	15204					
1800.913	25326	1800.946	453	470	QIATNAGQEASVVANTVK	
1814.916	12598	1814.986	422	437	VASKIKDVEVLNEDQK	
1838.893	12866					
1851.89	12964					
		1889.921	328	345	DNTTIIDGSGDQVQISAR	
1909.074	23711	1909.131	16	34	MLAGVNILANAVKVTLGPK	
1925.053	12744					
1939.033	15354	1939.098	405	425	AAVEEGVVAGGGVALVRVASK	
1982.92	14336	1982.957	59	75	EIELTDKFENMGAQMVK	
2145.004	16729					
2185.006	15928					
2220.029	23531					
2225.091	17806					
2241.051	15818					
2251.011	14786					
2386.147	19394	2386.247	81	105	ANDAAGDGTTTATVLAQAIVVEGLK	
2444.239	20765	2444.3	323	345	VIITKDNTTIIDGSGDQVQISAR	yes
2466.241	10996					
2497.209	22266	2497.232	59	80	EIELTDKFENMGAQMVKEVASK	
2514.277	58304					
2530.248	14511					
2559.3	23870	2559.37	119	142	GIDKAVIAAVAELKLLSQECSDTK	
2569.285	36534					
2600.342	28029	2600.401	322	345	RVIITKDNTTIIDGSGDQVQISAR	
2605.262	20817	2605.303	143	168	AIAQVGTISANSDESIGDIIATAMEK	
		2613.406	287	311	AMLQDVAILTGGTVIAEEIGLELEK	
2660.408	22676					
2705.245	15200					
2752.421	19632	2752.492	243	268	TGKPLLIVAEDVEGEALATLVVNNMR	yes
2767.326	31510					
2824.334	7606					
2889.406	8792	2889.488	143	171	AIAQVGTISANSDESIGDIIATAMEKVGK	
2906.313	9605	2906.373	172	197	EGVITVEEGQALENELDVVEGMQFDR	yes
		2993.293	471	498	NGEGNFGYNAGNDTYGDMLEMGILDPTK	
3097.491	6155					
3153.503	5534					
		3155.656	198	225	GYLSPYFINKPETGSIELDSPFILLVDK	
3163.403	3351					
3211.43	11672					
		3283.751	198	226	GYLSPYFINKPETGSIELDSPFILLVDKK	
3301.616	8831					
3349.546	10346	3349.511	471	501	NGEGNFGYNAGNDTYGDMLEMGILDPTKVTR	
3740.874	3408	3740.932	81	118	ANDAAGDGTTTATVLAQAIVVEGLKAVAAGMNPMDLKR	
3756.782	2240					
		4290.906	502	545	SALQFAASIAGLMITTEAMIAEVPQDSAPDMGGMGGMGGMGGMM	
		4647.123	499	545	VTRSALQFAASIAGLMITTEAMIAEVPQDSAPDMGGMGGMGGMGGMM	
		7621.398	471	545	NGEGNFGYNAGNDTYGDMLEMGILDPTKVTRSALQFAASIAGLMITTEAMIAEVPQDSAPDMGGMGGMGGMGGMM	

Figure 4.9. Comparison of ions from the tryptic digest of ER-Te-48 selenite reductase to those expected from a similar *in silico* digest of the GroEL protein from *Shewanella frigidimarina*.

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belonging to *Shewanella frigidimarina* (Copeland et al., 2006) (Fig. 4.8, Fig. 4.9). This identification was confirmed by tandem mass spectrometry, but, similar to the tellurite/tellurate reductase, there were many unidentified ions. In other organisms, GroEL folds newly processed enzymes (Kim et al., 2013), but in some cases, it can have another, secondary function (Huberts & I, 2010). Because the bands seen on the SDS gel do not fit with what we know about GroEL in other organisms, this is likely not our protein. When the genome sequence of ER-Te-48 becomes available, we will be able to use these fingerprints to allocate the gene(s), clone them into a high expression vector, and purify proteins at a larger scale. Then the complete enzyme characterization can be accomplished. Our mass spectrometry analysis confirms that we did purify protein with the observed enzymatic activity, but identification based on homology to other proteins is difficult as no other proteins in the database fit within the required taxonomy, thereby indicating that the reductases in this study are unique.

In summary, we have isolated periplasmic tellurite/tellurate and selenite reductases from the deep-ocean hydrothermal vent tube worm epibiont, strain ER-Te-48. This is the only example of Te oxyanion and selenite specific periplasmic reductases obtained from a Gram negative organism. While the optimal conditions and kinetics are similar to the enzymes produced by *Bacillus* sp., STG-83, they are different in both their size and origin. Also, mass spectrometry analysis further confirmed their novelty. Future research will lead to better understanding of this interesting new family of enzymes and may open the possibility to use these unique proteins in bioremediation of highly toxic and harmful oxyanions in nature.

4.5. Acknowledgements

4.7. References

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Chapter 5.

Tellurite and tellurate reduction by the aerobic anoxygenic phototroph *Erythromonas ursincola*, strain KR99 is carried out by a novel membrane associated enzyme

Chris Maltman, Lynda J Donald, Vladimir Yurkov

Applied and Environmental Microbiology
(Submitted)

The first author was the major contributor to research presented.

Dr. L. Donald performed the mass spectrometry and preliminary protein fingerprint data

analysis.

5.1. Abstract

Erythromonas ursincola, strain KR99 isolated from a freshwater thermal spring of Kamchatka Island in Russia, resists and reduces very high levels of toxic tellurite under aerobic conditions. Reduction is carried out by a constitutively expressed membrane associated enzyme, which was purified and characterized. The tellurite reductase has a molecular weight of 117 kDa, and is comprised of 2 subunits (62 and 55 kDa) in a 1:1 ratio. Optimal activity occurs at pH 7.0 and 28°C. Tellurite is reduced with a V_{max} of 5.15 µmol/min/mg protein and a K_m of 3.36 mM. The enzyme can also reduce tellurate with a V_{max} and K_m of 1.08 µmol/min/mg protein and 1.44 mM, respectively. This is the first purified membrane associated Te oxyanion reductase.

5.2. Introduction

Oxyanions of tellurium (Te), especially tellurite (TeO_3^{2-}), are poisonous to not only bacteria, but also to humans. This oxyanion is lethal to most microorganisms at concentrations as low as 1 µg/ml (Yurkov, et al., 1996), with toxicity believed to be a consequence of its properties as a strong oxidant (Taylor, 1999; Lloyd, et al., 2001). However, some microbes are able to resist very high levels of tellurite through its reduction to elemental Te. Even though the capacity to carry out this oxyanion transformation has been known for quite some time, there are very few purified examples of responsible enzymes. In some bacteria, non-specific reduction is accomplished by nitrate reductases (Avazeri, et al., 1997; Sabaty, et al., 2001). Thiol:disulfide oxidoreductase of *Rhodobacter capsulatus* (Borsetti, et al., 2007) and GutS of *E. coli* (Guzzo & Dubow, 2000), among others (Chiang, et al., 2008; Chiong, et al., 1988; Kabiri, et al., 2009; Moscoso, et al., 1998), also play a role in tellurite resistance and/or reduction. However, these enzymes are associated with low level resistance and it is not their primary function. Only one tellurite specific reductase, found in *Bacillus* sp., STG-83, has been published (Etezad, et al., 2009). The cytoplasmic enzyme is 197 kDa, comprised of 3 subunits (66, 43, and 20 kDa), functions optimally at 35°C, pH 8.0, and possesses a K_m of 2.6 mM with a V_{max} of 5.2 µmol/min/mg protein (Etezad, et al., 2009). This bacterium does exhibit increased tellurite resistance (~220 µg/ml) and is believed to be capable of dissimilatory anaerobic reduction, suggesting the enzyme may be involved in respiration.

Reduction of metal(loid) oxyanions can help dispose of excess electrons through the re-oxidation of NADH, $FADH_2$, or quinones, therefore maintaining optimal redox poise *in vivo* as seen in cells of *R. capsulatus* (Moore & Kaplan, 1992; Moore & Kaplan, 1994). Another strategy of this microorganism is based on reduced uptake of the tellurite oxyanion. Acetate

permease is responsible for TeO_3^{2-} influx (Borghese, et al., 2008; Borghese & Zannoni, 2010), therefore, competition between the oxyanion and acetate gives rise to increased resistance. A related approach has been identified in *E. coli*, where a mutated phosphate transport system also resulted in enhanced resistance (Thomas & Kay, 1986). Finally, certain microorganisms can neutralize Te oxyanions through production of volatile organic tellurides such as dimethyltelluride (Ollivier, et al., 2008), but this yields negligible removal. With so little information about specific tellurite reducing enzymes, more work with highly resistant and strongly reducing bacteria would be helpful to expand our knowledge.

Microbial metalloid oxyanion reduction is of great importance for biogeochemical Te cycling in nature. In both anaerobic and aerobic environments, many of these transformations are a direct result of bacterial enzymatic activity (DeMoll-Decker & Macy, 1993; Laverman et al., 1995; Csotonyi et al., 2006). Human behaviours have resulted in the release of vast amounts of toxic chemicals into the biosphere, including Te oxyanions, which contribute to serious pollution problems (Wood, 1974). A potentially attractive and ecologically sound removal method is microbial bioremediation, which has been explored, but only to a very limited extent (Macy et al., 1993; Cantafio et al., 1996; Rajwade & Paknikar, 2003). Therefore, the purification of specific enzymes able to reduce harmful Te oxyanions could aid in the development of environmentally friendly remediation strategies.

Aerobic anoxygenic phototrophs (AAP) are a group of bacteria which have an inherent high level resistance to tellurite (Yurkov & Csotonyi, 2009). Of all taxonomically described AAP, over half originate from extreme habitats, and of those tested, all are resistant to K_2TeO_3 (Yurkov & Csotonyi, 2009). Their MICs are significantly higher than in other studied tellurite reducers. The highest MICs for the purple non-sulfur bacteria *R. capsulatus* and *R. sphaeroides*

are 800 and 900 µg/ml, respectively (Moore & Kaplan, 1992; Moore & Kaplan, 1994), whereas AAP possess MICs up to 2700 µg/ml (Yurkov, et al., 1996). Te crystals can be accumulated inside the cells of AAP (Yurkov, et al., 1996), possibly indicating reduction by a cytoplasmic enzyme. However, many crystals are in close contact with the cell membrane, suggesting a likely membrane associated reductase. Also, it was recently confirmed that some AAP do possess tellurite reducing activity associated with their membranes (Chapter 3).

In this study, tellurite reduction in several AAP (*Erythromicrobium ezovicum*, strain E1; *Erythromicrobium ramosum*, E5; *Erythromonas ursincola*, KR99; and *Sandaracinobacter sibiricus*, RB 16-17) possessing high level resistance to tellurite and the ability to reduce it to elemental tellurium (Te^0) (Yurkov, et al., 1996) were investigated. All species were originally isolated from the hot temperature springs in Russia (Yurkov & Gorlenko, 1990; Yurkov, et al., 1991; Yurkov & Gorlenko, 1992; Yurkov, et al., 1992; Yurkov, et al., 1994).

5.3. Materials and Methods

5.3.1. Tellurite reductase purification and characterization

All species were grown under their optimal conditions, membranes collected, and TeO_3^{2-} reductase activity confirmed by visual blackening, as described (Chapter 3). Membranes were homogenized in Tris HCl buffer, pH 7.8, treated with 2% Triton X-100 and incubated for 30 min at room temperature with gentle shaking, then filtered to remove debris using a 0.2 µm syringe filter. Solubilized membranes were tested for reductase activity by addition of 100 µg/ml tellurite and 25 µl of 10 mM NADH or 1 g/l of their respective carbon sources (Chapter 3) to act as an electron donor(s). Only membrane fractions of strain KR99 retained activity and, therefore, it was used for all other experiments. The solubilized membranes were loaded onto a Superdex S-200 gel column previously equilibrated with 20 mM Tris HCl, pH 8.0, and eluted with a flow

rate of 1.0 ml/min. The 2 ml fractions exhibiting tellurite reduction (Fig. S12) were pooled and concentrated with a 100 kDa membrane cut-off centrifugal concentrator and loaded onto a Source 15 Q anion exchange column equilibrated with 20 mM Tris HCl, pH 8.0. A 0-1 M NaCl gradient was used with a flow rate of 3.0 ml/min. The 2 ml fractions with reductase activity were pooled and concentrated as above (Fig. S13). Native PAGE (5%) confirmed the presence of only a single protein. Molecular weight was estimated with a Superdex S-200 gel filtration column equilibrated with 20 mM Tris HCl, pH 8.0. The protein standards used were: aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa), and aprotinin (6.5 kDa). The column void volume was established with ferritin (440 kDa). Number and size of subunits was determined by SDS PAGE as published (Laemmli, 1970) with 5% stacking and a 12% running gels following denaturation by boiling for 4 min in 1% (w/v) SDS in the presence of 2-mercaptoethanol. Gels were stained with Coomassie brilliant blue (Wilson, 1983).

5.3.2. Enzyme properties and kinetics

Glutamate, aspartate, pyruvate, succinate, malate, lactate, acetate, and NADH were assayed for their capacity as electron donors for oxyanion reduction. Additionally, multiple metal(loid) oxyanions (tellurite, tellurate, selenite, selenate, meta- and orthovanadate (250 µg/ml)) were tested as well as nitrate and nitrite (100 µg/ml) as possible substrates. Reduction of the metal(loid) compounds was monitored visually and nitrate/nitrite reduction spectrophotometrically (Garcia-Robledo et al., 2014). To study the tellurite reductase functional kinetics, the reaction mixture contained: 500 µl Tris HCl pH 7.0, 25 µl NADH (10 mM stock), 50 µl purified enzyme (150 µg/ml), and tellurite (0 - 13 mM) or tellurate (0 – 5 mM). Activity was detected spectrophotometrically at 500 nm based off standard curves (Fig. S14) (Molina et

al., 2010). The K_m of NADH was determined in the same reaction mixture as above by varying the concentration of NADH with 13 mM tellurite. Optimal pH was determined in PBS or Tris HCl buffer adjusted with 0.5 N NaOH or HCl to pH 6.0, 7.0, 8.0, or 9.0. The reaction mixture was incubated at various temperatures (20, 25, 28, 32, 35, 38, and 42°C) to identify the optimum. Enzymatic activity was defined as the amount of enzyme that catalyzes conversion of 1 µmol of substrate to product per minute per mg protein. Protein was measured by the Bradford method (Bradford, 1976).

5.3.3. Mass spectrometry

Protein samples from native gel slices were reduced, alkylated, and digested with trypsin as described (Shevchenko, et al., 2006). Peptides eluted from the gel were mixed with an equal volume of DHB matrix (saturated 2,5-dihydroxybenzoic acid in 50% ACN, 2% formic acid) on a metal target. MALDI spectra were obtained using a prototype quadrupole time-of-flight mass spectrometer built in the Physics and Astronomy department, University of Manitoba (Loboda, et al., 2000). Spectral analysis was done with TOFMA and pTOOL, non-commercial software developed with the instruments. Ions with a signal/noise of 2 were selected, and the list was sent for identification by MASCOT (Matrix Science Limited, 2014). All data were checked against the Swissprot and NCBI databases, limited to bacterial taxonomy and a 30 ppm error level. An open search was also done for contaminants.

5.4. Results and discussion

5.4.1. Physical characteristics of reductase.

After solubilisation, membranes of strains E1, E5, and RB 16-17 could no longer reduce tellurite. If the reduction in these strains is carried out by true membrane proteins, then it is likely that their liberation from the membrane caused changes in either conformation or aggregation,
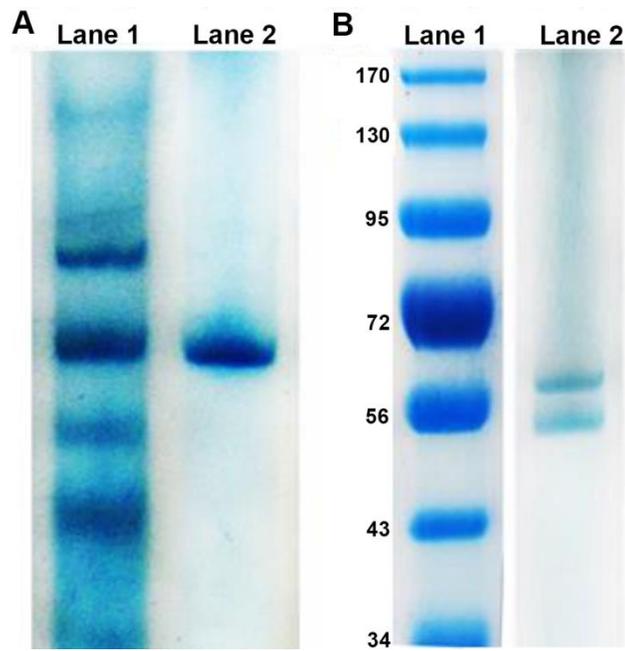
Figure 5.1. Protein purification from membranes of strain KR99. A) Native PAGE: Lane 1, Solubilized membranes; Lane 2, Purified tellurite reductase. B) SDS-PAGE: Lane 1, Molecular mass standards (in kDa); Lane 2, Tellurite reductase.

and led to the loss of activity. In contrast, the KR99 fraction retained the ability to reduce tellurite after treatment, with only NADH capable of serving as an electron donor. Hence, all further enzyme purification was done with KR99 samples alone. Native gel analysis (Fig. 5.1) of the filtrate from the solubilized membranes revealed the presence of several protein bands (Fig. 5.1 A, Lane 1). Identification of the specific reductase was accomplished through size exclusion chromatography followed by anion exchange chromatography, resulting in the 151-fold purification (Table 5.1) of a single protein, confirmed by native PAGE (Fig. 5.1 A, Lane 2). The molecular weight was 117 kDa and it is comprised of two subunits (62 and 55 kDa) in a 1:1 ratio (Fig. 5.1 B).

5.4.2. Biochemistry of reductase.

The tellurite reduction occurred best at 28°C and pH 7.0 (Fig. 5.2). Upon testing alternate Te, Se, and V oxyanions as substrates, the only other that could be reduced was tellurate. Nitrate and nitrite were not reduced. NADH, the only electron donor which could be used, had a K_m of 81.5 μM (Fig. S15). From Lineweaver–Burk plots the K_m and V_{max} values were calculated for each substrate. For tellurite reduction, values of 5.15 μmol/min/mg protein and 3.36 mM were estimated for V_{max} and K_m, respectively (Fig. 5.3 A). The V_{max} for tellurate reduction was 1.08 μmol/min/mg protein with a K_m of 1.44 mM (Fig. 5.3 B). The slower rate for tellurate reduction could be attributed to steric hindrance due to the extra oxygen present or possibly the need for more electrons to transform it to Te^0 as compared to conversion of tellurite. Either may result in the need for more time to carry out reduction. Comparison of the tellurite/tellurate reductase from strain KR99 with other known Te oxyanion reducing enzymes is challenging as this protein is unique. It is from a Gram negative bacterium, membrane associated, and specific to Te oxyanions. The previously published tellurite reductase from *Bacillus* sp., STG-83 appears to

Table 5.1. Isolation of tellurite reductase from the membrane of *E. ursincola*, strain KR99

Fraction	Activity (µM K$_2$TeO$_3$/min)	Total Protein (mg/l)	Specific Activity (µM K$_2$TeO$_3$/min/mg protein)	Yield (%)	Fold Purification
Cell Lysate	8.14	261	0.031	100	1
Membranes	2.25	31	0.073	27.64	2.33
S200 with activity	1.87	0.64	2.94	22.97	94.13
Ion Exchange with activity	1.31	0.28	4.71	16.09	151.09
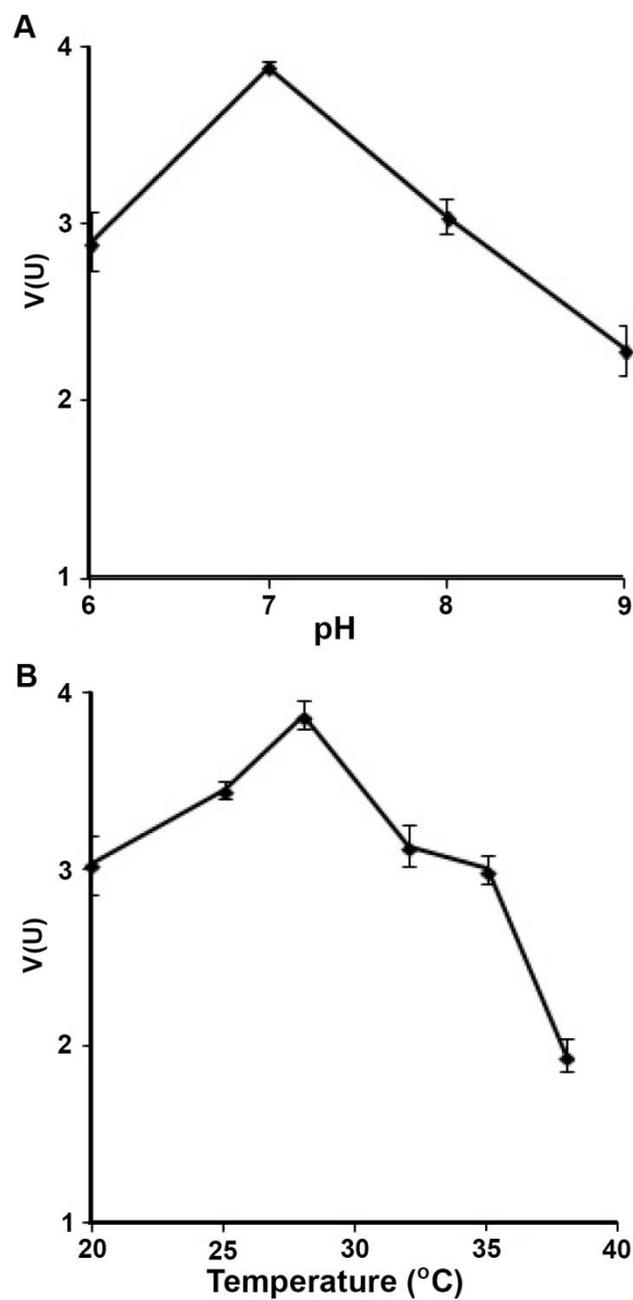
Figure 5.2. Effect of temperature (A) and pH (B) on tellurite reductase activity. Similar results for tellurate (Fig. S16). Error bars represent one standard deviation.
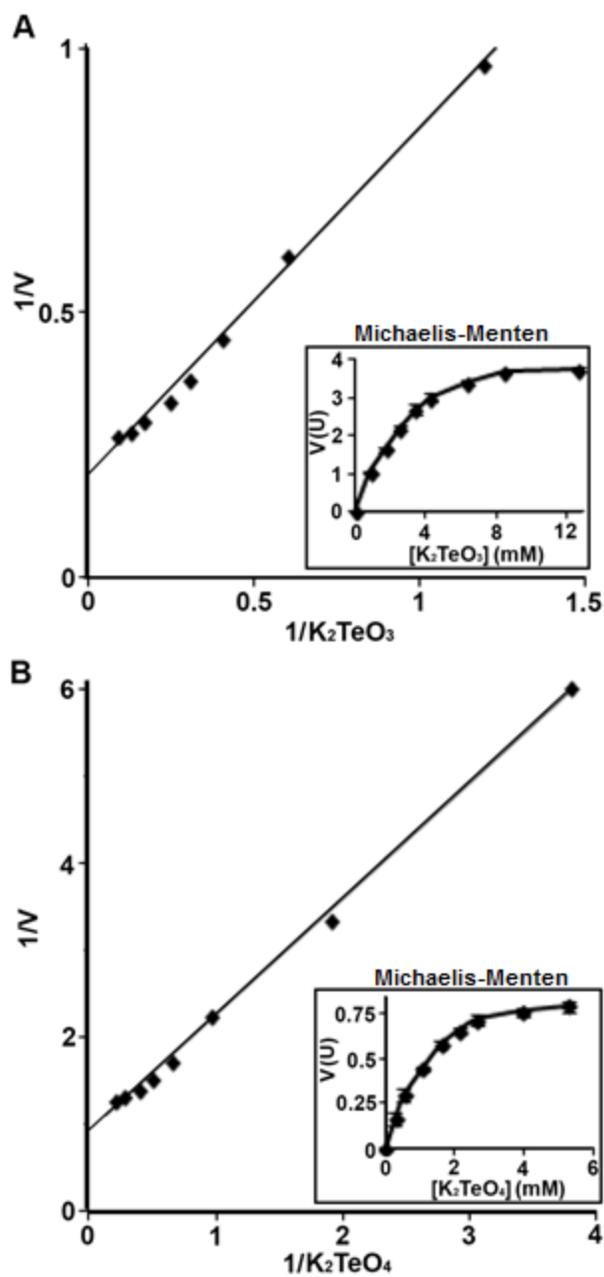
Figure 5.3. Lineweaver–Burk plots. Reduction kinetics of: A) Tellurite. B) Tellurate. The inset shows the Michaelis–Menten plot. Error bars represent one standard deviation.

have a similar V_{max} and K_m, however, it is much larger (197 kDa), comprised of three subunits, cytoplasmic in origin, and purified from a Gram positive organism (Etezad, et al., 2009).

5.4.3. Mass spectrometry analysis

Our first goal was to confirm the isolation of proteins, which was accomplished. The ions generated from the mixture of the two bands observed on the SDS gel were identified by MASCOT (Matrix Science Limited, 2014) as matching to those from the molecular chaperone GroEL of *Blastomonas* sp., CACIA14H2 (Figs. 5.4, 5.5) (Lima, et al., 2014). However, this GroEL has yet to be characterized properly. Its identity was proposed based solely on genomic DNA sequencing and homology to other similar proteins. In some bacteria, GroEL folds newly synthesized enzymes (Kim, et al., 2013), however, in some cases it may have multiple roles (Huberts & van der Klei, 2010). The GroEL monomer from other organisms is very close in mass to the 62 kDa band detected on the SDS gel for our protein, and there may be some homology. When the genome sequence of KR99 becomes available, the mass spectrometry fingerprint can be used to search for the gene(s) encoding the reductase, allowing cloning and protein purification on a larger scale. A proper characterization can then be done, as the identification based on homology to other similar proteins is not the most accurate approach. Nothing else in the database fits within the required taxonomy, indicating that the reductase in this study is unique.

In summary, we have isolated a membrane associated tellurite reductase, which is also capable of reducing tellurate, from the AAP *E. ursincola*, strain KR99. This is the first example of its kind, as well as the first from an AAP. Unlike the reductase of *Bacillus* sp., STG-83, this enzyme could not be used for anaerobic respiration as KR99 does not grow anaerobically. In some cases, nitrate reductases are implicated in tellurite reduction (Avazeri et al., 1997; Sabaty et

>gi|563284320|gb|ESZ88862.1| molecular chaperone GroEL [*Blastomonas* sp.

CACIA14H2]

MAAKDVKFGRDARERILRGVDILADAVKVTLGPKGRNVVIDKSFGAPRITKDGV

SVAKEIELKDKFENMGAQMVKEVASKTNDIAGDGTTTATVLAQAIVREGMKSV

AAGMNPMDLKRGIDLAVTKVVENLKSRSKDVAGSNEIAQVGIISANGDREVGEK

IAEAMERVGKEGVITVEEAKGLEFELDVVEGMQFDRGYLSPYFITNPDKMTVEL

DNPYILIHEKKLSNLQAMLPILEAVVQTGRPLLIIAEDIEGEALATLVVNKLRGGL

KVAAVKAPGFGDRRKAMLEDIAILSKGEMISEDLGIKLENVTLGMLGQAKRVSI

DKDNTTIVDGAGEADAIKARVEAIRTQIDNTTSDYDREKLQERLAKLAGGVAVI

KVGGASEVEVKEKKDRVDDALHATRAAVEEGIVPGGGTALLYATSALEGLTGE

NDDQTRGIDIIRKALFAPVRQIAQNAGHDGAVVSGKLLDGNDPTLGFNAATDTY

ENLVAAGVIDPTKVVRAALQDAASVAGLLITTEAAICDAPEDKAAAGGMGGMP

GGMGGMGGMDF

Figure 5.4. Mass spectrometry sequence analysis for the membrane associated tellurite/tellurate reductase from strain KR99 compared to its nearest match GroEL from *Blastomonas* sp., CACIA14H2. Sequence identified by data matching is underlined. All sequences are from the NCBI database.

KR99	*Blastomonas*				
Observed ions	Expected ions	Peptide		MSMS[1]	Sequence of *Blastomonas* GroEL
1302.687	1302.717	37	48	okay	NVVIDKSFGAPR
1498.822	1498.884	119	132		GIDLAVTKVVENLK
1514.722	1514.753	198	210	poor	GYLSPYFITNPDK
1595.906	1595.937	19	34	poor	GVDILADAVKVTLGPK
1741.917	1742.018	119	134		GIDLAVTKVVENLKSR
1814.905	1814.936	211	225		MTVELDNPYILIHEK
1883.854	1883.885	182	197		GLEFELDVVEGMQFDR
1978.157	1978.206	16	34	no match	ILRGVDILADAVKVTLGPK
1985.871	1985.989	137	156		DVAGSNEIAQVGIISANGDR
2088.049	2088.094	81	101	poor	TNDIAGDGTTTATVLAQAIVR
2132.028	2132.072	323	343		VSIDKDNTTIVDGAGEADAIK
2306.167	2306.237	446	468	poor	ALFAPVRQIAQNAGHDGAVVSGK
2714.309	2714.356	503	529		AALQDAASVAGLLITTEAAICDAPEDK
2743.333	2743.386	135	161		SKDVAGSNEIAQVGIISANGDREVGEK
3311.612	3310.671	198	225		GYLSPYFITNPDKMTVELDNPYILIHEK
3375.608	3375.656	405	438	excellent	AAVEEGIVPGGGTALLYATSALEGLTGENDDQTR
3559.763	3559.828	469	502	good	LLDGNDPTLGFNAATDTYENLVAAGVIDPTKVVR
4172.104	4171.152	405	445		AAVEEGIVPGGGTALLYATSALEGLTGENDDQTRGIDIIRK

[1]Tandem mass spectrometry to test whether the sequence of the peptides matched the expected.

Figure 5.5. Comparison of ions from the tryptic digest of KR99 tellurite reductase to those expected from a similar *in silico* digest of the GroEL protein from *Blastomonas* sp., CACIA14H2 (NCBI gi|563284320).

al., 2001), therefore it could be argued the protein isolated here belongs to this family. However, KR99 is not capable of nitrate reduction, and neither is the isolated Te oxyanion reductase, ruling out such an option (Yurkov, et al., 1991). Furthermore, mass spectrometry analysis confirmed its uniqueness. The exact role in cells remains unclear, though strain KR99 grown in the presence of tellurite demonstrates an increase of biomass (Chapter 3), attributing to some important physiological influence.

The fact that the discovered reductase is NADH dependant could indicate the reduction of Te oxyanions is involved in disposal of electron excess through the re-oxidation of NADH, maintaining optimal redox poise in cells (Moore & Kaplan, 1992 and 1994), which permits optimal growth and explains the observed increase in biomass. AAP are known to have an inherent ability to resist and reduce extremely high levels of different toxic metal(loid) oxyanions, especially tellurite. Therefore, continued research is leading to 'greener' ecologically friendly methods of bioremediation of these highly toxic substances (Turner et al., 2012; Bonificio & Clarke, 2014; Luek et al., 2014).

5.5. Acknowledgements

5.6. References

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Chapter 6.

Tellurite-, tellurate-, and selenite-based anaerobic respiration by strain CM-3 isolated from gold mine tailings

Chris Maltman, Michele D. Piercey-Normore, and Vladimir Yurkov

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Dr. Piercey-Normore assisted with the 16S rRNA gene sequencing.

6.1. Abstract

The newly discovered strain CM-3, a Gram negative, rod shaped bacterium from gold mine tailings of the Central Mine in Nopiming Provincial Park, Canada, is capable of dissimilatory anaerobic reduction of tellurite, tellurate, and selenite. CM-3 possesses very high level resistance to these oxyanions, both aerobically and anaerobically. During aerobic growth, tellurite and tellurate resistance was up to 1500 and 1000 µg/ml, respectively. In the presence of selenite, growth occurred at the highest concentration tested, 7000 µg/ml. Under anaerobic conditions, resistance was decreased to 800 µg/ml for the Te oxyanions; however, much like under aerobic conditions, growth with selenite still took place at 7000 µg/ml. In the absence of oxygen, CM-3 couples oxyanion reduction to an increase in biomass. Following an initial drop in viable cells, due to switching from aerobic to anaerobic conditions, there was an increase in CFU/ml greater than one order of magnitude in the presence of tellurite (6.6×10^3 to 8.6×10^4 CFU/ml), tellurate (4.6×10^3 to 1.4×10^5 CFU/ml), and selenite (2.7×10^5 to 5.6×10^6 CFU/ml). A control culture without metalloid oxyanions showed a steady decrease in CFU/ml with no recovery. ATP production was also increased in the presence of each oxyanion, further indicating anaerobic respiration. Partial 16S rRNA gene sequencing revealed a 99.0% similarity of CM-3 to *Pseudomonas reactans*.

Keywords: tellurite, anaerobic respiration, metalloid oxyanions, selenite, tellurate, Pseudomonas

6.2. Introduction

Extreme environments have provided insight into how microorganisms can adapt to survive and thrive under the harshest of conditions. Microorganisms have been found in many environments where they are least expected such as arid deserts and hydrothermal vents (Csotonyi et al., 2006; Csotonyi et al., 2010). Bacteria have evolved unique abilities that allow them to inhabit ecological niches that would be considered to be extreme for most forms of life. Some of the localities have elevated concentrations of highly toxic oxyanions of tellurium (Te) and selenium (Se) (Cooper, 1971; Knott et al., 1995), leading to the evolution of microbes capable of tolerating the increased presence of toxins (Yurkov & Csotonyi, 2003).

Te is a metalloid element related to oxygen and sulfur in group 16 of the Periodic Table. It possesses stable oxidation states of VI (tellurate), IV (tellurite), 0 (elemental tellurium), and II (telluride). Tellurate is most common in the hydrosphere, while in the lithosphere it is found as tellurides of gold and silver (Cooper, 1971). Overall, Te has a very low global abundance (10^{-2} to 10^{-8} ppm) and its distribution is not homogenous (Yurkov & Csotonyi, 2003). For example, in gold mines it can be significantly concentrated (14.8 ppm) (Wray, 1998). Oxyanions of Te are highly toxic to microorganisms at levels as low as 1 µg/ml (Yurkov et al., 1996), but through aerobic reduction, certain species can resist levels as high as 2,500 to 4,000 µg/ml (Pearion & Jablonski, 1999; Yurkov et al., 1999). The majority of tellurite and tellurate resistance is found under aerobic conditions, although there are some exceptions. *Rhodobacter sphaeroides* can reduce up to 600 µg/ml tellurite during anaerobic photosynthesis to dispose of excess reducing equivalents (Moore & Kaplan, 1994). In the case of *Desulfovibrio desulfuricans*, resting cells can couple the reduction of tellurite to the oxidation of formate, but no energy is conserved (Lloyd et al., 2001).

Se parallels Te in many instances. It is also a group 16 element related to sulfur and oxygen with the same oxidation states of VI (selenate), IV (selenite), 0 (elemental selenium), and II (selenide). However, the toxicity of Se oxyanions is lower than that of Te, resulting in microbial resistance in excess of 7000 µg/ml (Rathgeber et al., 2006). Global abundance of Se, while very low, is still much higher than that of Te, around 0.05 ppm (Krauskopf, 1982) and like Te, it can be locally concentrated. Sulfide ores of iron, nickel, copper, zinc and lead result in increased Se levels in mine tailings, and natural Se abundance in bedrock can lead to increased levels in soils (Haug et al., 2007). Selenite and selenate are the most prevalent forms found, sometimes at levels so high that their toxic effect can be observed in animals inhabiting those areas in the form of selenosis (Tinggi, 2005). Much like Te, aerobic microbial resistance and reduction is more commonly reported than anaerobic resistance and reduction.

Dissimilatory electron transport (anaerobic respiration) to tellurite, tellurate, and selenite is a rare ability. To date, there are only seven examples of bacteria that have been confirmed to utilize these oxyanions as terminal electron acceptors during anaerobic growth. The first report of dissimilatory anaerobic use of tellurate was first published for the taxonomically unclassified strain ER-Te-48 (Csotonyi et al., 2006). Both *Bacillus selenitireducens* and *B. beveridgei* are the only known bacteria able to respire anaerobically using all three oxyanions (Baesman et al., 2007; Baesman et al., 2009). *Sulfurospirillum barnesii* can undergo respiration while growing on tellurite and tellurate (Baesman et al., 2007). Lastly, there are three isolates (*Desulfurispirillium indicum*, strain HGMK1, and HGMK3), which can use selenite for respiration (Takai et al., 2002; Rauschenbach et al., 2011). Some other bacteria are suspected to have this capability (Klonowska et al., 2005; Etezad et al., 2009), but none have been confirmed. The use of metalloid oxyanions as terminal electron acceptors in respiratory pathways is consistent with our

current knowledge of energetics. The redox couple of TeO_3^{2-}/Te (0.827 V), TeO_4^{2-}/Te (1.719 V), and SeO_3^{2-}/Se (0.875 V) are more favorable for anaerobic respiration than the SO^{2-}/HS^- redox couple (-0.217 V) utilized by sulfate reducers (Bouroushian, 2010). Further supporting this line of logic, reduction of selenite to Se (ΔG_f^o = -132.4 kJ (mol electrons)$^{-1}$) (Blum et al., 1998) and tellurite to Te (ΔG_f^o = -71.3 kJ (mol electrons)$^{-1}$) coupled to the oxidation of lactate is highly exergonic (Baesman et al., 2009), providing more than enough energy for growth. Likely factors contributing to the infrequent use for respiration is the low global abundance and high toxicity. Nevertheless, respiration on other toxic oxyanions is known (Lovley, 1993), indicating toxicity does not always prevent inclusion in metabolism. Until recently, Te has been considered a biologically unimportant element. However, the discovery of respiration based on tellurite and tellurate revealed newly discovered biological relevance (Csotonyi et al., 2006). Much like Se, which was considered biologically insignificant for many years, it is now understood that Se is essential for some forms of life (Haug et al., 2007), and Te may follow a path similar to Se as future research focuses on the metalloid.

The goal of this work was to identify bacteria possessing very high levels of aerobic and anaerobic resistance to metal(loid) oxyanions, as well as the ability to perform anaerobic respiration, isolated from tailings of the Central Mine in Nopiming Provincial Park, Manitoba, Canada. This gold mine opened in 1927 and closed in 1937 (Sherriff et al., 2007) and is known to have increased levels of Se (up to 121 ppm) (Renault et al., 2000). Since then, the site has been virtually untouched, providing ideal conditions for resident bacteria to establish a stable community. We describe a novel bacterium, strain CM-3, capable of very high level aerobic and anaerobic resistance to tellurite, tellurate, and selenite and of anaerobic respiration.

6.3. Materials and Methods

6.3.1. Isolation and metalloid oxyanion resistance

Samples from the tailings of the Central Mine in Nopiming Provincial Park, Manitoba, Canada were obtained in August of 2011. A 1 cm^3 sample was resuspended in sterile water (10 ml) and used to directly inoculate crimp sealed, vacuum degassed Balch tubes under a headspace of N_2 containing Minimal Salts (MS) medium (Yurkov et al., 1996) supplemented with 1 g/l lactate and 25 µg/ml K_2TeO_3 for enrichment of anaerobic tellurite resistant bacteria. Tubes were incubated at 28°C in the dark and observed for visual blackening due to reduction of tellurite to elemental Te (Yurkov et al., 1996) as well as monitoring of tellurite concentration in the medium by the $NaBH_4$ method (Molina et al., 2010). A sample was taken from tubes showing reduction and used to inoculate rich organic (RO) (Yurkov et al., 1994) plates containing 25 µg/ml K_2TeO_3, which were incubated at 28°C in the dark in a Forma Scientific anaerobic chamber. Resulting colonies were selected and replated on RO plates and incubated the same as previously for both aerobic and anaerobic conditions. Phase contrast microscopy was performed using a Ziess Axioskope 2 microscope on the selected strains and a representative was chosen for all further testing.

A 5% inoculum of an overnight culture of aerobically grown cells was used to determine resistance to tellurite, tellurate, selenite, and selenate. Aerobic resistance was tested in RO liquid medium containing 100 µg/ml of one of these four oxyanions. Cultures were incubated at 28°C in the dark on an incubator shaker at 200 rpm. Resistance and reduction were monitored visually in terms of blackening (tellurite and tellurate) or reddening (selenite, selenate) of the culture indicating the appearance of elemental Te and Se, respectively (Rathgeber et al., 2006). The level of resistance was examined using 250, 500, 750, 1000, 1500, 2000 µg/ml for tellurite and tellurate and 1000, 2000, 3000, 4000, 5000, 6000, and 7000 µg/ml for selenite. Balch tubes,

prepared as above, containing liquid anaerobic metalloid respiration (AMR) medium (in g/l: KH_2PO_4, NH_4Cl, (0.5); $CaCl_2$, (0.1); yeast extract, lactate (1); $MgSO_4$, (0.01); vitamins and trace metals solution (2 ml/l) (Drews 1983)) and 100 µg/ml of one of K_2TeO_3, K_2TeO_4, Na_2SeO_3 or Na_2SeO_4 were used to investigate anaerobic resistance. Tubes were incubated at $28^{o}C$ in the dark. Level of resistance anaerobically was monitored using (in µg/ml) 200, 400, 600, 800, and 1000 for the Te oxyanions and 500, 1000, 1500, 2000, 3000, 4000, 5000, 6000 and 7000 for selenite. All experiments were performed in triplicate.

6.3.2. Kinetics experiments

Aerobically grown cells of CM-3 were injected into vacuum degassed 120 ml crimp sealed bottles containing 75 ml of AMR medium amended with tellurate, tellurite, or selenite at 50 µg/ml under a headspace of N_2. Samples were taken every 24 h for CFU/ml and ATP counts. CFU/ml was monitored as an assay for growth by serial dilution to 10^{-5} followed by plating on RO plates incubated at $28^{o}C$. Colonies were counted after 7 days. ATP was measured using an ATP Bioluminescence Kit from Sigma-Aldrich as outlined in section 2.3.2.

6.3.3. Phylogenetic analysis

Isolation of genomic DNA was performed as described (Chen & Kuo, 1993). Partial sequencing of the 16S rRNA gene was performed by PCR utilizing bacterial primers U1 and U1R (James 2010). Amplifications were performed in 50 µl reaction volumes containing 25 µl DreamTaq PCR Master Mix, 0.25 µM of each primer, and between 10 and 50 ng of DNA in a thermal cycler with the following conditions: initial denaturing at 95°C for 5 min, then denaturing at 95°C for 30 sec, annealing at 46°C for 30 sec, extension at 72°C for 1.5 min for 35 cycles with a final extension at 72°C for 10 min ending with a $7^{o}C$ hold. Preparation and sequencing of the PCR product, nucleotide sequence editing was carried out as published

(Fontaine et al., 2012). A NCBI Blast search was performed to determine the nearest relative and a Maximum Likelihood phylogenetic tree created using MEGA 6.0 (Tamura et al., 2013). Bootstrap analyses were performed with 1000 replicates.

6.3.4. Nucleotide sequence accession number

The 16S rRNA gene sequence determined in this study for CM-3 was deposited in GenBank under the accession number KR150623.

6.4. Results and Discussion

6.4.1. Strain isolation and metalloid oxyanion resistance analysis

Extreme environments have provided us with numerous isolates capable of resistance to and reduction of metalloid oxyanions (Yurkov et al., 1999; Yurkov & Csotonyi, 2003; Csotonyi et al., 2006; Rathgeber et al., 2006; Baesman et al., 2007; Baesman et al., 2009). The same is true for the microbial community inhabiting the tailings of the Central Mine, which are spread out over a wide area (Fig 6.1 a). Sites chosen for sampling were representative of the environmental conditions found throughout the location. Site 1 (Fig 6.1 b), referred to as the Blue Pond, was a transient pond, with water levels dependent on precipitation. This location experienced fluctuating humidity, temperature, and pH. Oxyanion concentrations would also vary through dilution (when water is present) and concentration (when dry). At the time of sampling, the Blue Pond was virtually dry due to lack of rain, with a pH of 4.1 at the moist bottom. Site 2 (Fig 6.1 c) was at the former mine drainage system and today where precipitation runs off from the mine site proper. The site was arid above and below the surface, with a pH of 3.9. Site 3 (Fig. 6.1 d) was similar to Site 2 except while dry on the surface, there was still moisture below, resulting in a pH of 7.0. Lastly, Site 4 (Fig. 6.1 e) was at a location called the Green Pond, which unlike the Blue Pond, was permanent, however, as it had been an extremely hot and dry summer, there was
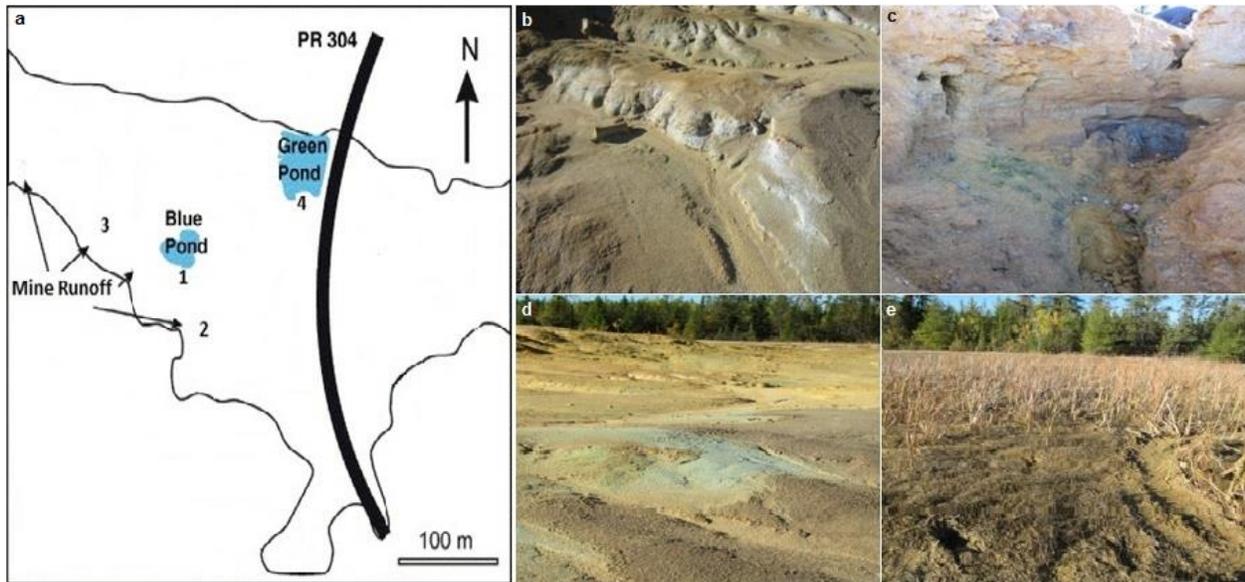
Figure 6.1. a) Schematic map of mine tailings from the Central Mine in Nopiming Provincial Park, Manitoba, Canada showing location of sampling; Photographs of b) Site 1, c) Site 2, d) Site 3, and e) Site 4.

little water remaining, but was still wet at the time of sampling with a pH of 6.9.

Upon observation of Balch tubes with tellurite containing medium and inoculated with an environmental sample from each of the four sites, we noted blackening of the culture only at Site 3. To confirm formation of the elemental Te as a result of anaerobic tellurite reduction, levels of TeO_3^{2-} in the growth medium were monitored over time (Fig. 6.2). Tellurite was decreasing in conjunction with visible darkening, confirming anaerobic reduction. To identify the microorganism(s) responsible, samples taken from an actively reducing enriched Balch tube were transferred onto plates. After 7 days, several small black colonies appeared and they were selected for further analysis. All isolates were facultative anaerobes growing under both aerobic and anaerobic conditions, forming small, circular colonies 1-2 mm in diameter and microscopically cells were similar (Fig. 6.3). One strain, CM-3, was further investigated for its resistance to Te and Se oxyanions in both aerobic and anaerobic conditions. This rod shaped bacterium is 1.8-2.6 µm in length, Gram negative, and motile. During aerobic growth, CM-3 possesses very high level resistance to tellurite (1500 µg/ml), tellurate (1000 µg/ml), and selenite (>7000 µg/ml). No resistance to or reduction of selenate was observed. The range of oxyanion resistance was comparable under anaerobic conditions (Fig. 6.4), with level of resistance decreased, but still remaining very high (800 µg/ml for tellurite and tellurate and 7000 µg/ml for selenite). Hence, strain CM-3 can reduce and resist extreme levels of tellurite, tellurate, and selenite under both aerobic and anaerobic conditions.

6.4.2. Kinetics of growth and ATP production

Reduction of an oxyanion under anaerobic conditions does not automatically confirm respiration. For evidence, we monitored growth (CFU/ml) in AMR medium containing one of tellurite, tellurate, or selenite as the only possible terminal electron acceptor. Growth and
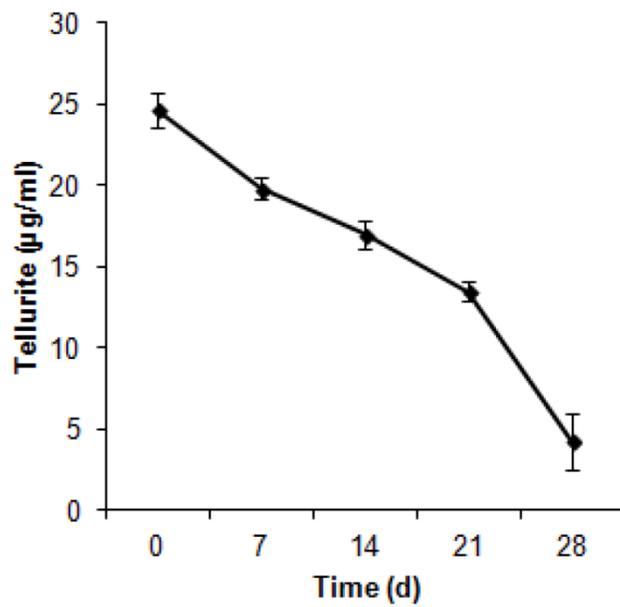
Figure 6.2. Change in concentration of tellurite in the growth medium over time by CM-3 samples taken from Site 3 grown under anaerobic conditions.
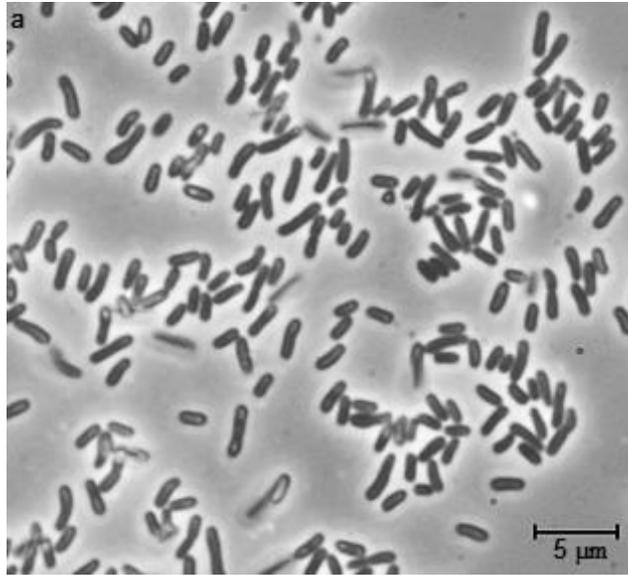
Figure 6.3. Phase contrast micrograph of strain CM-3 isolated from Site 3 showing rod-shaped bacteria.
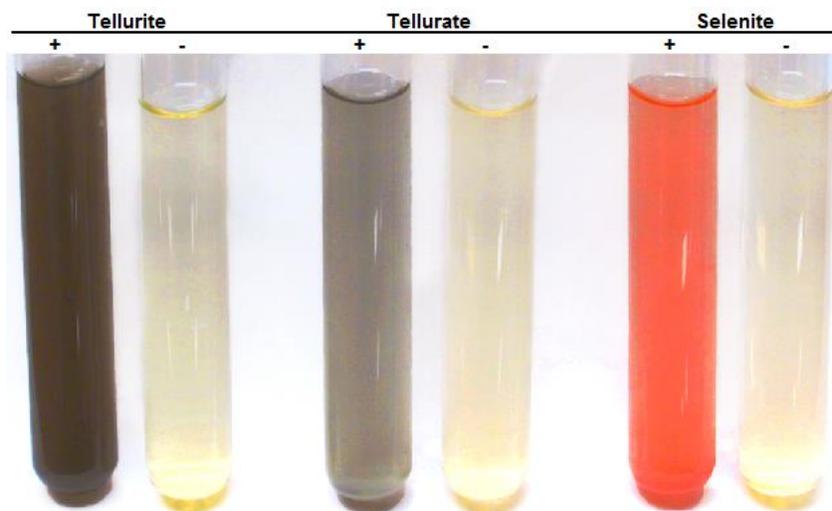
Figure 6.4. Results of the test for anaerobic growth in the presence of 100 µg/ml tellurite, tellurate and selenite as the sole terminal electron acceptor by CM-3. The color change to black is due to reduction of tellurite/tellurate to elemental Te and reddening is due to reduction of selenite to elemental Se. - ; no oxyanion present, + ; oxyanion present.

reduction in such conditions would indicate anaerobic respiration. An increase in CFU/ml of greater than an order of magnitude was detected for all three oxyanions. Initially there was a significant drop in CFU/ml for the Te oxyanions, which can be attributed to CM-3 adapting to anaerobic growth as well as to the initial toxic shock from newly introduced tellurite or tellurate. However, shortly after, CFU/ml increased from 6.6×10^3 to 8.6×10^4 in the presence of tellurite over 5 days (Fig. 6.5 a) and 4.6×10^3 to 1.4×10^5 with tellurate over 7 days (Fig. 6.5 b). In the case of the anaerobic selenite experiment, the initial drop in CFU/ml was not as significant as it might be less toxic to cells than the Te oxyanions and recovery was faster. The CFU/ml increased from 2.7×10^5 to 5.6×10^6 over three days (Fig. 6.5 c). During the same time, the number of cells in control tubes without an oxyanion immediately began to decline without recovery.

The previous data showed anaerobic growth, with oxyanions as terminal electron acceptors. Next, ATP levels in the presence and absence of metalloid oxyanions were monitored to further confirm anaerobic respiration. As expected, ATP production per CFU/ml was much higher during log phase (Fig. 6.5 d, e, f). Growth coupled to increased ATP production, along with cell death in the absence of an oxyanion, strongly confirms that CM-3 is performing dissimilatory reduction of tellurite, tellurate, and selenite under anaerobic conditions.

6.4.3. Phylogenetic analysis

Partial sequencing of the 16S rRNA gene (726 bp) revealed CM-3 is a member of the genus *Pseudomonas* and most closely related to *Pseudomonas reactans* (99.0%) (Fig 6.6). Some members of this genus are capable of aerobic Te and Se oxyanion resistance and reduction (Lortie et al., 1992; Malik & Jaiswal, 2000; Rajwade & Paknikar, 2003; Hunter & Manter, 2009), however, the reported resistance (eg. tellurite MIC of 150 µg/ml (Zanaroli et al., 2002)) is
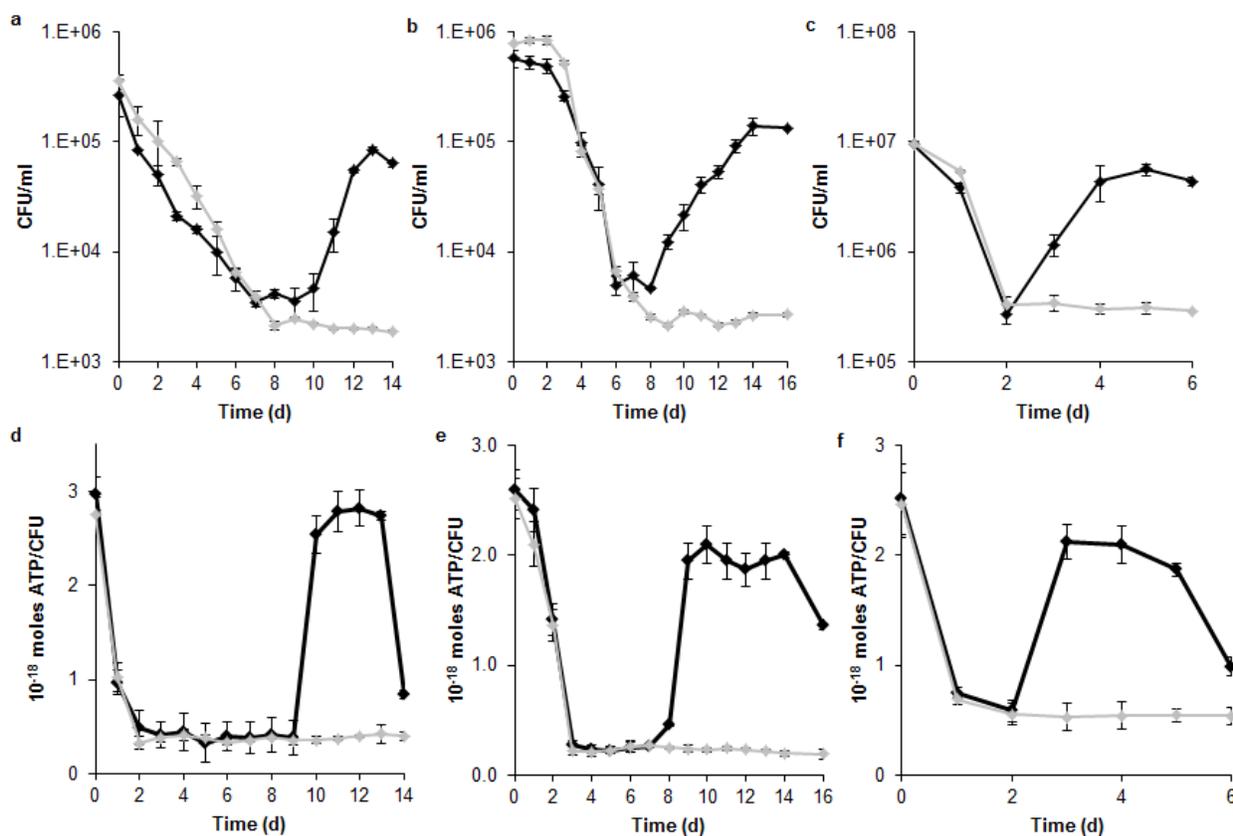
Figure 6.5. Changes in growth of CM-3 (CFU/ml) over time (days) with added oxyanions as the sole terminal electron acceptor. a) K_2TeO_3, b) K_2TeO_4, and c) Na_2SeO_3. Change in ATP production (ATP/CFU/ml) over time (days) by cells of CM-3 during anaerobic growth in the presence of metalloid oxyanions. d) K_2TeO_3, e) K_2TeO_4, and f) Na_2SeO_3. ♦ – With oxyanion; ♦ - Without oxyanion Error bars represent one standard deviation.
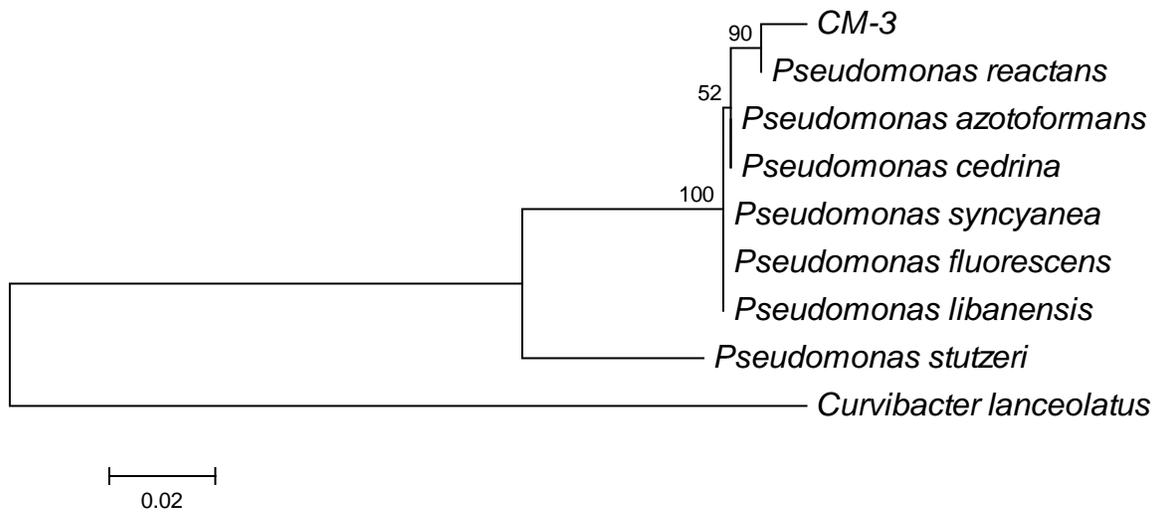
Figure 6.6. Maximum likelihood phylogenetic tree of strain CM-3 showing its position among species of the genus *Pseudomonas*. (Bar represents number of base pair substitutions per site).

extremely low when compared to CM-3. *Pseudomonas stutzeri* pn1 is able to anaerobically respire on selenate (Narasingarao & Haggblom, 2007), while *P. isachenkovii* and *P. vanadiumreductans* can use metavanadate (Yurkova & Lyalikova, 1990). However, there are no reports of either selenite or Te oxyanion respiration, and strain CM-3 makes the first example.

6.5. Summary

This work identified CM-3, a novel bacterium related to *Pseudomonas reactans* (99.0%), which possesses very high levels of aerobic and anaerobic resistance to tellurite, tellurate, and selenite, as well as the ability to use oxyanions for dissimilatory anaerobic respiration. CM-3 is only the fourth reported case of a tellurite respiring microbe and fifth for tellurate and selenite. Also, other than *B. selenitireducens* and *B. beveridgei*, this is the only other isolate ever found capable of utilizing all three oxyanions and is the only Gram negative representative. Our discovery provides evidence that *Pseudomonas* species possess the rare ability to anaerobically respire on tellurite, tellurate, and selenite as well as a wider range and greater level of metalloid resistance than previously thought.

With the search for more environmentally friendly methods for cleanup of locations contaminated by oxyanions, microbes, such as CM-3, show great potential. The use of bacteria for bioremediation might provide a more ecologically responsible method for removal of contaminants. As well, the ability to transform oxyanions to pure elemental forms may be useful in bacterial recycling and recovery of metal(loid)s, such as Te, for industrial purposes with the least amount of negative impact on the environment.

6.6. Acknowledgements

6.7. References

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Chapter 7.

A diverse community of metal(loid) oxyanion respiring bacteria is associated with tube worms in the vicinity of the Juan de Fuca Ridge black smoker field

Chris Maltman, Graham Walter, and Vladimir Yurkov

The first author was the major contributor the research presented.

Graham Walter assisted with prepping genomic DNA for 16S rRNA gene sequencing.

7.1. Abstract

Epibiotic isolates from the vent tube worms *Paralvinella sulfincola* and *Ridgeia piscesae* inhabiting the Axial Volcano (AV) caldera and Explorer Ridge (ER) vent field of the Juan de Fuca Ridge in the Pacific Ocean were investigated for the ability to respire anaerobically on tellurite, tellurate, selenite, selenate, metavanadate and orthovanadate as terminal electron acceptors. Out of 107 isolates tested, 106 were capable of respiration on one or more of these oxyanions, indicating that metal(loid) oxyanion based respiration is not only much more prevalent in nature than is generally believed, but also is an important mode of energy generation in the habitat. Partial 16S rRNA gene sequencing revealed the bacterial community to be rich and highly diverse, containing many potentially new species. Furthermore, it appears that the worms not only possess a close symbiotic relationship with chemolithotrophic sulfide-oxidizing bacteria, but perhaps also with the metal(loid) oxyanion transformers. Possibly they protect the worms through reduction of the toxic compounds that would otherwise be harmful to the host.

7.2. Introduction

Bacterial respiration on oxyanions of metal(loid)s is known (Lovley, 1993), however, it was not believed to be widespread. Due to the high toxicity, especially of tellurium oxyanions, it has long been believed they have no significant (if any, in the case of Te) role in biological processes. However, microbes have adapted and evolved to incorporate oxyanions of Te, Se, and V into metabolic processes, especially in metal(loid) rich environments (Holden & Adams, 2003). In regards to dissimilatory electron transport to metal(loid)s, strong support comes from the physical/chemical features of the redox couples for Te, Se, and V oxyanions (TeO_3^{2-}/Te = 0.827 V; TeO_4^{2-}/TeO_3^{2-} = 0.885 V; SeO_3^{2-}/Se = 0.885 V; VO_2^+/VO^{2+} = 1.000 V). Although highly toxic, they are more favorable for anaerobic respiration than that of SO_4^{2-}/HS^- (-0.217 V) couple widely used by sulfate reducers (Bouroushian, 2010). No dissimilatory anaerobic reduction of Te oxyanions was known until 2006, when strain ER-Te-48 from a deep sea hydrothermal vent tube worm was found to be capable of anaerobic tellurate based respiration (Csotonyi et al., 2006). Since then, four other bacteria have been shown to respire on Te oxyanions (Baesman et al., 2007; Baesman et al., 2009; Chapter 6). The dissimilatory use of Se and V oxyanions has been known for some time, however, it is limited to only a select few species (Yurkova & Lyalikova, 1990; Macy et al., 1993; Blum et al., 1998; Stolz & Oremland, 1999; Blum et al., 2001; Takai et al., 2002; Ortiz-Bernard et al., 2004; Carpentier et al., 2005; Csotonyi et al., 2006; Narasingarao & Haggblom, 2006; Baesman et al., 2007; Narasingarao & Haggblom, 2007; Narasingarao & Haggblom, 2007a; Baesman et al., 2009; Rauschenbach et al., 2011; Chapter 6). The majority are halophiles from locales lacking any detectable metal(loid)s, suggesting the ability to respire on oxyanions was not directly evolved for survival.

Deep sea hydrothermal vents, so-called Black Smokers, are geological formations, which release subterranean seawater that has been superheated to more than 400°C by magma pockets beneath the sea floor. Through this process, metal(loid)s are mobilized from the crustal basalts, highly enriching the vent plumes and chimneys (Knott et al., 1995; Yurkov & Csotonyi, 2003). Levels of Te can be in excess of 200 ppm (Bogdanov et al., 2008) and Se can exceed 4000 ppm (Keith et al., 2015). The harsh environment suggests life there should be scarce, but numerous unique organisms call this ecological niche home. The sulfide and tube worms surrounding vents are of particular interest with regards to bacterial-metal(loid) interactions. Due to the proximity to the plume waters, they and their associated microbes are in close contact with elevated levels of metal(loid)s (Van Dover, 2000). These animals harbor a community of metal resistant bacteria (Jeanthon & Prieur, 1990), indicating that the microbial population does experience, and has adapted to metal(loid) exposure. Such conditions offer the perfect environment for the evolution of biological processes dependant on metal(loid)s. Another feature of these worms providing selective pressure in favor of bacteria capable of metal(loid) resistance/respiration is their vanadium enriched blood (Michibata et al., 2002). Since these creatures possess conditions ideal for dissimilatory metal(loid) reduction, it is not surprising their epibionts gave us not only the first example of anaerobic respiration on Te oxyanions (Csotonyi et al., 2006), but also on metavanadate (Yurkova & Lyalikova, 1990), and orthovanadate (Csotonyi et al., 2006).

As mentioned prior, there are very few known examples of microbes utilizing Te, Se, or V oxyanions as terminal electron acceptors during anaerobic growth. They are spread out among different genera (Yurkova & Lyalikova, 1990; Macy et al., 1993; Blum et al., 1998; Stolz & Oremland, 1999; Blum et al., 2001; Takai et al., 2002; Ortiz-Bernard et al., 2004; Carpentier et al., 2005; Csotonyi et al., 2006; Narasingarao & Haggblom, 2006; Baesman et al., 2007;

Narasingarao & Haggblom, 2007; Narasingarao & Haggblom, 2007a; Baesman et al., 2009; Rauschenbach et al., 2011; Chapter 6), suggesting metal(loid) oxyanion respiring microbes are phylogenetically diverse and not limited to a single taxonomic group. In this study, we investigated 107 epibiotic isolates from the vent tube worms *Paralvinella sulfincola* and *Ridgeia piscesae* of the Axial Volcano (AV) caldera and Explorer Ridge (ER) vent field of the Juan de Fuca Ridge (Csotonyi et al., 2006) for the ability to respire anaerobically on tellurite, tellurate, selenite, selenate, metavanadate, and orthovanadate. Partial sequencing of the 16S rRNA gene was then carried out to determine their phylogenetic diversity.

7.3. Materials and Methods

7.3.1. Growth and respiration with metal(loid) oxyanions

Sampling and collection of both the sulfide tube worm (*R. piscesae*) and tube worm (*P. sulfincola*) from Axial Volcano (Hell Vent: $45^o56'00''$N, $130^o00'51''$W; 1,543 m) and Explorer Ridge (Lucky Find: $49^o45'38''$N, $130^o15'23''$W; 1,791 m) of the Juan de Fuca Ridge in the Pacific Ocean in 2003 was as previously published (Csotonyi et al., 2006). Tissue from the worms was rinsed, homogenized, and used for inoculation of enrichment cultures. 107 metal(loid) reducing epibiotic bacterial strains were isolated as described (Csotonyi et al., 2006). Each was grown aerobically at 28^oC in the dark on rich organic (RO) (Yurkov et al., 1994) plates containing 2% NaCl and used to inoculate crimp sealed, vacuum degassed Balch tubes, under a headspace of N_2, filled with anaerobic metal(loid) respiration (AMR) liquid medium, containing (g/l): KH_2PO_4, 0.5; NH_4Cl, 0.5; $CaCl_2$, 0.1; yeast extract, 1.0; lactate, 1.0; and $MgSO_4$, 0.01. Vitamin and trace microelements solutions (Yurkov et al., 1994) were added at 2 ml/l. Medium was amended with one of tellurite, tellurate, selenite, selenate (100 µg/ml), or metavanadate, orthovanadate (500 µg/ml). Tubes were incubated at 28^oC in the dark and monitored for

respiration over two weeks. A representative strain was chosen for each oxyanion reducing group. Aerobically grown cells of ER-Te-40B, ER-Te-57, AV-Te-18, ER-V-8, AV-V-4, and ER-Te-41 were used to inoculate degassed 120 ml crimp-sealed bottles containing 100 ml of AMR medium with one of tellurite, tellurate, selenite, selenate, metavanadate or orthovanadate at the concentrations listed above, under a headspace of N_2. Control tubes were not supplemented with any of these oxyanions. Protein yield was measured by Bradford assay (Bradford, 1976). ATP was measured using an ATP Bioluminescence Kit from Sigma-Aldrich (see section 2.3.2). All experiments were performed in triplicate.

7.3.2. Phylogenetic analysis

Genomic DNA was extracted from pure cultures of each isolate as published (Chen & Kuo, 1993). Partial 16S rRNA gene amplification by PCR was carried out using universal bacterial primers (James, 2010), in 50 µl reaction volumes containing: 25 µl DreamTaq PCR Master Mix, 0.25 µM of each primer, and between 10 and 50 ng of DNA. The amplification cycle was as follows: Initial denaturing at 95°C for 5 min, denaturing at 95°C for 30 sec, annealing at 46°C for 30 sec, extension at 72°C for 1.5 min for 35 cycles with a final extension at 72°C for 10 min, ending with a hold at 7°C. Preparation of the PCR products was as described (Fontaine et al., 2012). Samples were sequenced by the Manitoba Institute of Cell Biology. The nucleotide sequences were edited and phylogenetic relatedness determined as reported (Fontaine et al., 2012). All sequences were deposited in GenBank under the accession numbers provided in Table 7.1. Maximum likelihood phylogenetic trees were created using Phylogeny.fr (Dereeper et al., 2008).

7.4. Results and Discussion

7.4.1. Growth and reduction with metal(loid) oxyanions

Table 7.1. GenBank accession numbers for 16S rRNA gene sequences obtained in this study

Strain	Sequence Accession Number	Strain	Sequence Accession Number
AV-Se-12	KT277103	ER-Se-21-light	KT277155
AV-Se-13	KT277104	ER-Se-22-dark	KT277156
AV-Se-15-dark	KT277105	ER-Se-22-light	KT277157
AV-Se-16	KT277106	ER-Se-3	KT277158
AV-Se-17	KT277107	ER-Te-2-brown	KT277159
AV-Se-18	KT277108	ER-Te-2-grey	KT277160
AV-Se-19	KT277109	ER-Te-40	KT277161
AV-Se-2-dark	KT277110	ER-Te-40B	KT277162
AV-Se-3	KT277111	ER-Te-41	KT277163
AV-Te-17	KT277112	ER-Te-41B	KT277164
AV-Te-18	KT277113	ER-Te-42	KT277165
AV-Te-19	KT277114	ER-Te-42B-dark	KT277166
AV-Te-20	KT277115	ER-Te-42B-light	KT277167
AV-Te-21-dark	KT277116	ER-Te-43	KT277168
AV-Te-21-light	KT277117	ER-Te-44	KT277169
AV-Te-22	KT277118	ER-Te-45	KT277170
AV-Te-23-dark	KT277119	ER-Te-46	KT277171
AV-Te-23-light	KT277120	ER-Te-47	KT277172
AV-Te-24	KT277121	ER-Te-49	KT277173
AV-Te-25	KT277122	ER-Te-50	KT277174
AV-Te-26	KT277123	ER-Te-50-white	KT277175
AV-Te-27	KT277124	ER-Te-51	KT277176
AV-V-1	KT277125	ER-Te-52	KT277177
AV-V-10-1	KT277126	ER-Te-53	KT277178
AV-V-10-2	KT277127	ER-Te-54-dark	KT277179
AV-V-11	KT277128	ER-Te-54-light	KT277180
AV-V-12	KT277129	ER-Te-55	KT277181
AV-V-13	KT277130	ER-Te-56	KT277182
AV-V-14	KT277131	ER-Te-57	KT277183
AV-V-15	KT277132	ER-Te-58	KT277184
AV-V-17	KT277133	ER-Te-59	KT277185
AV-V-19	KT277134	ER-Te-60	KT277186
AV-V-2	KT277135	ER-Te-61	KT277187
AV-V-20	KT277136	ER-Te-63	KT277188
AV-V-21	KT277137	ER-Te-64-fast	KT277189
AV-V-22	KT277138	ER-Te-64-slow	KT277190
AV-V-23	KT277139	ER-Te-65	KT277191
AV-V-3	KT277140	ER-Te-66	KT277192
AV-V-4	KT277141	ER-V-10	KT277193
AV-V-5	KT277142	ER-V-11	KT277194
AV-V-6	KT277143	ER-V-12	KT277195
AV-V-7	KT277144	ER-V-13	KT277196
ER-Se-1	KT277145	ER-V-14	KT277197
ER-Se-13	KT277146	ER-V-15	KT277198
ER-Se-14	KT277147	ER-V-2	KT277199
ER-Se-15	KT277148	ER-V-3	KT277200
ER-Se-16	KT277149	ER-V-4	KT277201
ER-Se-18	KT277150	ER-V-5	KT277202
ER-Se-19-dark	KT277151	ER-V-7	KT277203
ER-Se-2	KT277152	ER-V-8	KT277204
ER-Se-20	KT277153	ER-V-9	KT277205
ER-Se-21-dark	KT277154		

Upon visual investigation for change of colorless water soluble oxyanions to colored elemental forms (black for Te, red for Se, and grey/black/brownish for V (Csotonyi et al., 2006)) due to microbial activity, we found that under anaerobic conditions, all isolates but ER-V-1 were capable of reducing at least one (Fig. 7.1), and many could use more than one of the oxyanions tested (Tables 7.2 and 7.3). While the color transformation indicated the possibility of anaerobic respiration, experimental proof was required. For each oxyanion reduction group, one representative strain was chosen and protein levels with and without the oxyanion added to the growth medium were analyzed. Protein increased significantly in the presence of tellurite (5.6 fold), tellurate (10 fold), selenite (4.6 fold), selenate (4.3 fold), metavanadate (6.2 fold), and orthovanadate (4.8 fold), while no increase, only decrease, was observed in their absence (control tubes), due to no growth (Fig. 7.2). There was no other electron acceptor in the medium, therefore, growth was clearly supported by metal(loid) oxyanions, confirming all strains were obtaining energy from anaerobic respiration. When ATP levels were monitored, they also increased during growth in the presence of the tested oxyanions and declined in their absence, further confirming respiration (Fig. 7.3, Fig. S17). As discussed above, currently there are only 5, 17, and 6 strains confirmed to respire on Te, Se, and V oxyanions, respectively (Yurkova & Lyalikova, 1990; Macy, et al., 1993; Blum, et al., 1998; Stolz & Oremland, 1999; Blum, et al., 2001; Takai, et al., 2002; Ortiz-Bernard, et al., 2004; Carpentier, et al., 2005; Csotonyi, et al., 2006; Narasingarao & Haggblom, 2006; Baesman, et al., 2007; Narasingarao & Haggblom, 2007; Narasingarao & Haggblom, 2007a; Baesman, et al., 2009; Rauschenbach, et al., 2011; Chapter 6). Our work adds significant numbers to the list (105, 85, 101, 1, 47, and 17 for tellurite, tellurate, selenite, selenate, metavanadate, and orthovanadate, respectively) and proves that anaerobic metal(loid) oxyanion based respiration is a quite common, well established mode
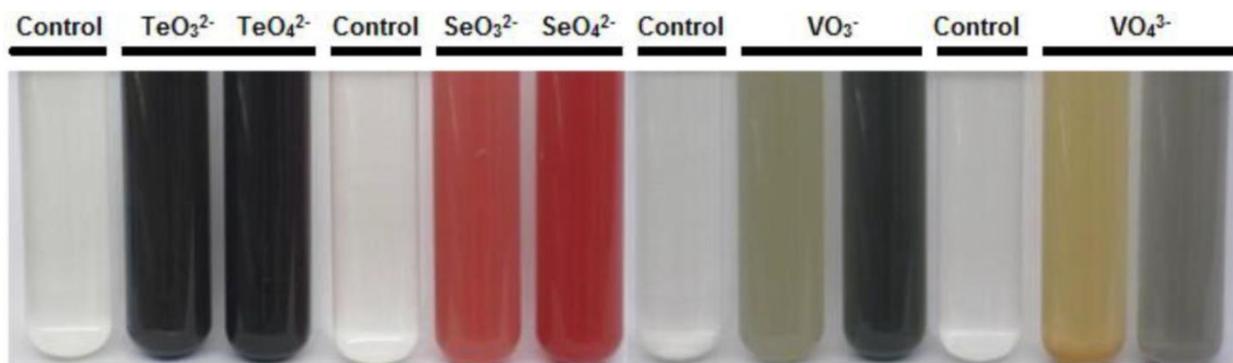
Figure 7.1. Anaerobic respiration resulting in visible reduction of tellurite, (strain ER-Te-40B), tellurate (ER-Te-57), selenite (AV-Te-18), selenate (ER-V-8), metavanadate (AV-V-4 - brown, AV-V-5 - black), and orthovanadate (ER-Te-41 - brown, AV-V-19 - grey/black) by isolates from deep sea hydrothermal vent worms. For Te oxyanion containing cultures, black coloration indicates reduction of oxyanion to elemental Te. Dissolved Se oxyanion color change from clear to red due to reduction to elemental Se. Change in color for V oxyanions is a result of reduction to lower oxidation state.

Table 7.2. Range of metal(loid) oxyanions use for anaerobic respiration by strains epibiotically associated with vent worms at Axial Volcano.

Strain	Metal(loid) Oxyanion						Strain	Metal(loid) Oxyanion					
	TeO_3^{2-}	TeO_4^{2-}	SeO_3^{2-}	SeO_4^{2-}	VO_3^-	VO_4^{3-}		TeO_3^{2-}	TeO_4^{2-}	SeO_3^{2-}	SeO_4^{2-}	VO_3^-	VO_4^{3-}
AV-Se-12	+	+	+	-	-	-	AV-V-1	+	+	+	-	+	-
AV-Se-13	+	+	+	-	-	-	AV-V-10-1	+	+	+	-	-	-
AV-Se-15-dark	+	+	+	-	-	-	AV-V-10-2	+	+	+	-	+	-
AV-Se-16	+	+	+	-	-	-	AV-V-11	+	+	+	-	+	-
AV-Se-17	+	+	+	-	-	-	AV-V-12	+	+	+	-	+	-
AV-Se-18	+	+	+	-	-	-	AV-V-13	+	+	+	-	+	-
AV-Se-19	-	+	+	-	-	-	AV-V-14	+	+	+	-	+	+
AV-Se-2-dark	+	+	+	-	+	-	AV-V-15	+	+	+	-	+	+
AV-Se-3	+	+	+	-	-	-	AV-V-17	+	+	+	-	+	+
AV-Te-17	+	-	+	-	-	-	AV-V-19	+	+	+	-	+	+
AV-Te-18	+	+	+	-	+	+	AV-V-2	+	+	+	-	-	-
AV-Te-19	+	-	+	-	-	-	AV-V-20	+	-	-	-	-	-
AV-Te-20	+	-	+	-	-	-	AV-V-21	+	+	+	-	+	-
AV-Te-21-dark	+	+	+	-	-	-	AV-V-22	-	-	-	-	+	-
AV-Te-21-light	+	+	+	-	-	-	AV-V-23	+	-	+	-	+	-
AV-Te-22	+	-	+	-	-	-	AV-V-25	+	+	+	-	+	-
AV-Te-23-dark	+	+	+	-	-	-	AV-V-3	+	-	+	-	-	-
AV-Te-23-light	+	+	+	-	-	-	AV-V-4	+	+	+	-	+	-
AV-Te-24	+	+	+	-	+	+	AV-V-5	+	+	+	-	+	-
AV-Te-25	+	+	+	-	-	-	AV-V-6	+	+	+	-	-	-
AV-Te-26	+	+	+	-	-	-	AV-V-7	+	+	+	-	+	+
AV-Te-27	+	+	+	-	-	-							

+, Anaerobic respiration taking place; -, No anaerobic respiration occurring.

Table 7.3. Range of metal(loid) oxyanions use for respiration by the Explorer Ridge vent worm epibionts.

Strain	Metal(loid) Oxyanion						Strain	Metal(loid) Oxyanion					
	TeO_3^{2-}	TeO_4^{2-}	SeO_3^{2-}	SeO_4^{2-}	VO_3^-	VO_4^{3-}		TeO_3^{2-}	TeO_4^{2-}	SeO_3^{2-}	SeO_4^{2-}	VO_3^-	VO_4^{3-}
ER-Se-1	+	+	+	-	-	-	ER-Te-51	+	+	+	-	-	-
ER-Se-13	+	-	+	-	-	-	ER-Te-52	+	+	+	-	-	+
ER-Se-14	+	+	+	-	-	-	ER-Te-53	+	+	+	-	-	-
ER-Se-15	+	-	+	-	+	-	ER-Te-54-dark	+	+	+	-	+	-
ER-Se-16	+	+	-	-	-	-	ER-Te-54-light	+	+	+	-	-	+
ER-Se-18	+	+	+	-	+	-	ER-Te-55	+	+	+	-	+	+
ER-Se-19-dark	+	+	-	-	-	-	ER-Te-56	+	+	+	-	+	-
ER-Se-2	+	-	+	-	-	-	ER-Te-57	+	+	+	-	+	+
ER-Se-20	+	+	+	-	-	-	ER-Te-58	+	+	+	-	+	-
ER-Se-21-dark	+	+	+	-	+	-	ER-Te-59	+	+	+	-	+	-
ER-Se-21-light	+	+	+	-	-	-	ER-Te-60	+	+	+	-	+	-
ER-Se-22-dark	+	-	+	-	-	-	ER-Te-61	+	+	+	-	-	-
ER-Se-22-light	+	-	+	-	-	-	ER-Te-63	+	-	+	-	-	-
ER-Se-3	+	-	+	-	-	-	ER-Te-64-fast	+	+	+	-	-	-
ER-Te-2-brown	+	-	+	-	-	-	ER-Te-64-slow	+	+	+	-	-	-
ER-Te-2-grey	+	-	+	-	-	-	ER-Te-65	+	+	+	-	-	-
ER-Te-40	+	+	+	-	+	-	ER-Te-66	+	+	+	-	-	-
ER-Te-40B	+	+	+	-	+	-	ER-V-10	+	+	+	-	+	-
ER-Te-41	+	+	+	-	-	+	ER-V-11	+	+	+	-	-	-
ER-Te-41B	+	+	+	-	+	-	ER-V-12	+	+	+	-	+	+
ER-Te-42	+	+	+	-	+	+	ER-V-13	+	+	+	-	-	-
ER-Te-42B-dark	+	+	+	-	-	-	ER-V-14	+	+	+	-	+	-
ER-Te-42B-light	+	+	+	-	-	+	ER-V-15	+	+	+	-	+	-
ER-Te-43	+	+	+	-	-	-	ER-V-2	+	-	-	-	+	-
ER-Te-44	+	+	+	-	+	-	ER-V-3	+	-	+	-	+	-
ER-Te-45	+	+	+	-	-	-	ER-V-4	+	+	-	-	+	-
ER-Te-46	+	+	+	-	+	-	ER-V-5	+	-	+	-	-	-
ER-Te-47	+	+	+	-	+	-	ER-V-6	+	+	+	-	+	+
ER-Te-48	+	+	+	-	+	+	ER-V-7	+	+	+	-	+	-
ER-Te-49	+	+	+	-	+	+	ER-V-8	+	+	+	+	+	-
ER-Te-50	+	+	+	-	-	-	ER-V-9	+	+	+	-	-	-
ER-Te-50-white	+	-	+	-	-	-	ER-V-1	-	-	-	-	-	-

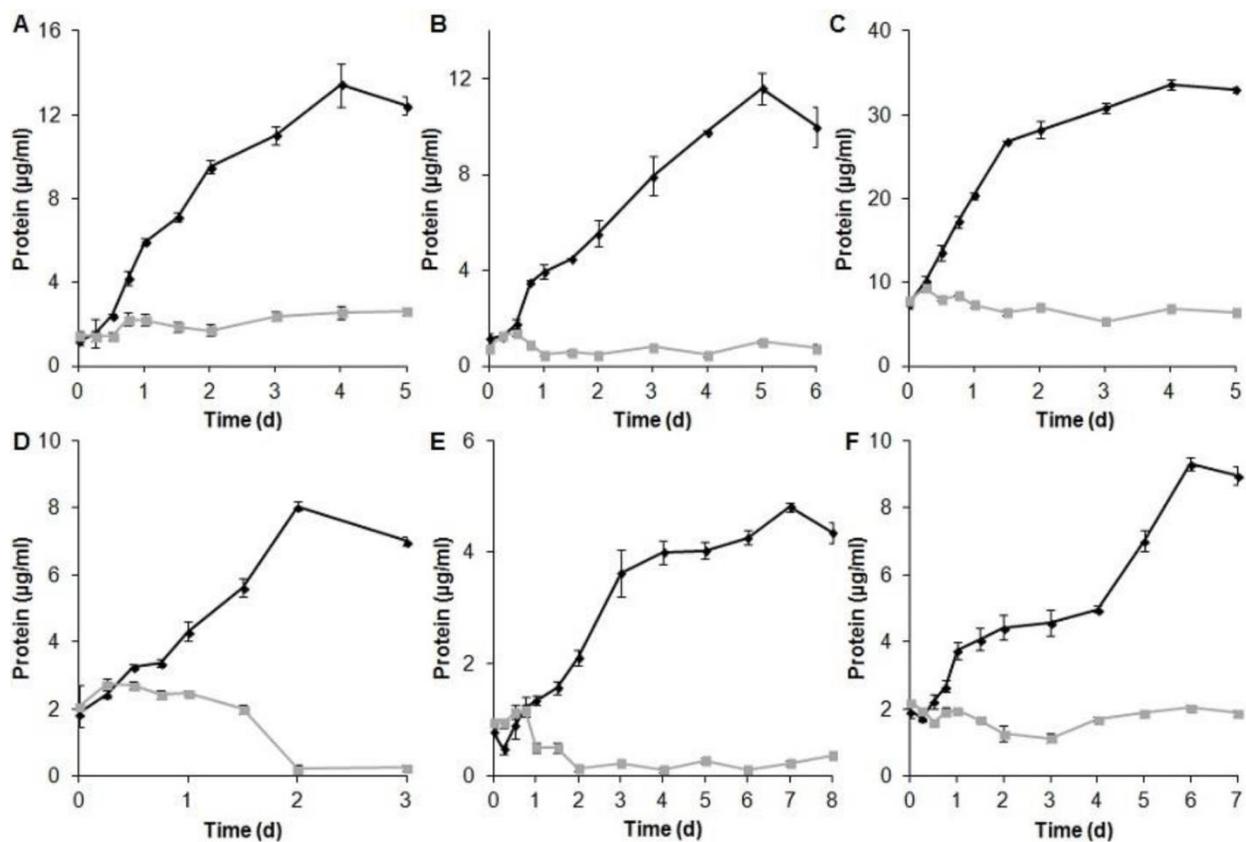+, Anaerobic respiration taking place; -, No anaerobic respiration occurring.

Figure 7.2. Growth as determined by protein production in the presence versus absence of metal(loid) oxyanions. A) Strain ER-Te-40B with tellurite; B) ER-Te-57 with tellurate; C) AV-Te-18 with selenite; D) ER-V-8 with selenate; E) AV-V-4 with metavanadate and F) ER-Te-41 with orthovanadate. ♦ – With metal(loid) oxyanion; ■ - Without oxyanion. Error bars represent on standard deviation.
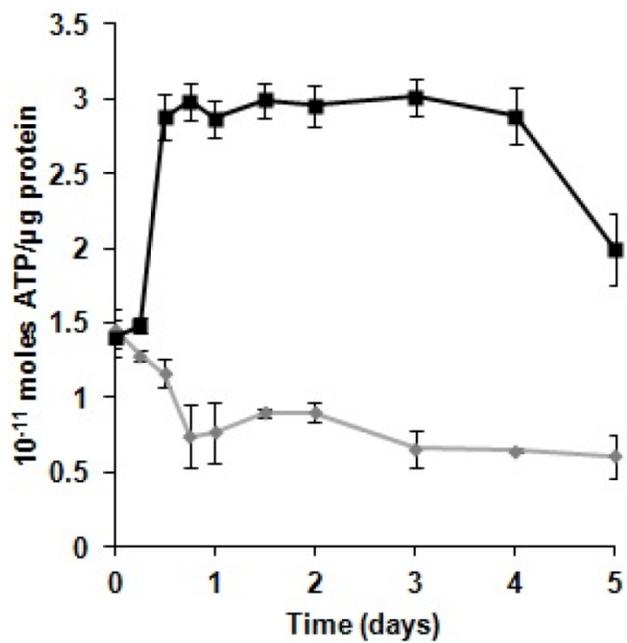
Figure 7.3. ATP production by cells of ER-Te-40B during anaerobic growth in presence of K_2TeO_3. A similar trend was seen for all remaining strains. ■ – With metal(loid) oxyanion; ♦- Without oxyanion. Error bars represent one standard deviation.

of energy generation supporting life of epibiotic microorganisms associated with worms at deep sea hydrothermal vents. Interestingly, only one strain (ER-V-8) used selenate, which is possibly not a common form of Se in the habitat. Also, of the 107 isolates tested, 105 utilized more than one oxyanion. Hence, their metabolic capabilities are not narrowed on usage of a single element. Te oxyanions and selenite are preferred for respiration among those tested. Possibly these are the most prevalent forms vent worms are exposed to, resulting in their widespread use. Another possibility might be that the pathway (enzyme(s)) expression needed for Te and Se oxyanion respiration is simpler than what is required for the V oxyanion based reactions. This idea is supported by the fact that Te and Se oxyanion reduction can be accomplished by the activity of a specific single enzyme (Schroder et al., 1997; Guzzo & Dubow, 2000; Etezad et al., 2009; Chapters 4 & 5), whereas V oxyanion based respiration involves multiple proteins (Myers et al., 2004). As protein synthesis is energy intensive (Jewett et al., 2009), for a cell to produce a single protein only in the presence of the inducing compound, instead of several, is less taxing.

7.4.2. Phylogenetic analysis

Research into the microbial species makeup of specific locales in or around vents has been previously undertaken, focusing on low-temperature diffuse flow deep sea vents (Huber et al., 2007), vent plume waters (Huber et al., 2010), hydrothermal sediments (Teske et al., 2002), and microbial mats covering vent chimneys (Moyer et al., 1995; Brazelton & Baross, 2009; Xie et al., 2011). These studies have shown extremely diverse bacterial populations that can differ significantly between neighbouring vents. The metabolic diversity has also received some attention (Wang et al., 2009) as well as the epibionts of vent inhabitants such as sulfide tube and tube worms (Jeanthon & Prieur, 1990; Jeanthon, 2000). *Riftia pachyptila* possesses chemolithoautotrophic, sulphur-oxidizing endosymbionts, which autotrophically fix carbon

dioxide, using reduced sulphur compounds from vent fluids as electron donors, thereby cleaning the blood from toxic sulfide, and synthesizing organic compounds for their host (Van Dover, 2000). However, the presence, and especially diversity, of metal(loid) oxyanion respiring bacteria, which can obviously help to remove toxic metal(loid)s dissolved in surrounding water and, therefore, detoxify the blood of their hosts, has not been considered yet. Our collection of strains is shedding some light on this important component of epibiotic populations.

Three strains (ER-Te-48, ER-V-6, and AV-V-25) previously had their 16S rRNA gene sequenced (Csotonyi et al., 2006). Partial 16S rRNA gene sequencing of the 103 remaining isolates revealed a highly diverse group (Fig. 7.4). Worms from the two sampling locations had a distinct phylogenetic suite of microbes (Fig. 7.5). Epibionts originating from the vent worms living at Axial Volcano were dominated by *Vibrio* (41.9%) and *Pseudoalteromonas* (39.5%) relatives, with *Curvibacter* (9.3%) and *Shewanella* (9.3%) relatives making up the remainder. The Explorer Ridge worms had different distribution and composition. The isolates had greater variety, dominated by *Curvibacter* (36.5%) and *Shewanella* (30.2%) relatives. The remaining organisms were comprised of *Pseudomonas* (12.7%), *Pseudoalteromonas* (7.9%), *Marinobacter* (3.2%), *Thalassospira* (3.2%), *Vibrio* (3.2%), *Aquabacterium* (1.6%), and *Okibacterium* (1.6%) relatives. Some of these genera are already known for their metal(loid) oxyanion respiring capabilities (Yurkova & Lyalikova, 1990; Macy et al., 1993; Blum et al., 1998; Stolz & Oremland, 1999; Blum et al., 2001; Takai et al., 2002; Ortiz-Bernard et al., 2004; Carpentier et al., 2005; Csotonyi et al., 2006; Narasingarao & Haggblom, 2006; Baesman et al., 2007; Narasingarao & Haggblom, 2007; Narasingarao & Haggblom, 2007a; Baesman et al., 2009; Rauschenbach et al., 2011; Chapter 6). *Shewanella* species are metabolically diverse and versatile in regards to metal resistance and respiration (Myers et al., 2004), the best characterized
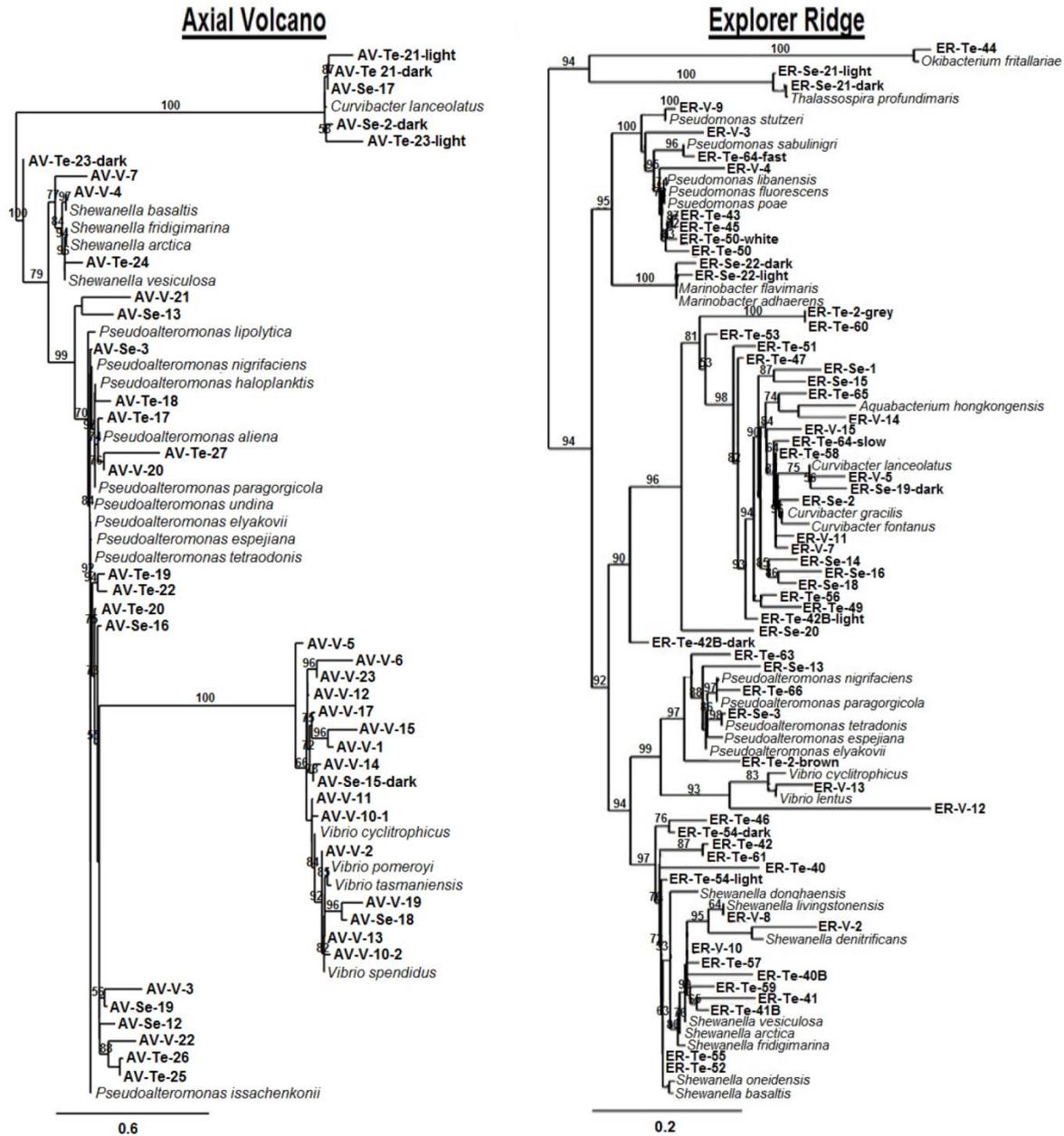
Figure 7.4. Maximum likelihood phylogenetic tree of strains based on partial 16S rRNA gene sequences (avg. 400 bp) showing position of new metal(loid) respiring isolates from vent worms of Axial Volcano and Explorer Ridge vent worms and their closest neighbors. (Scale bars represent number of base pair substitutions per site)
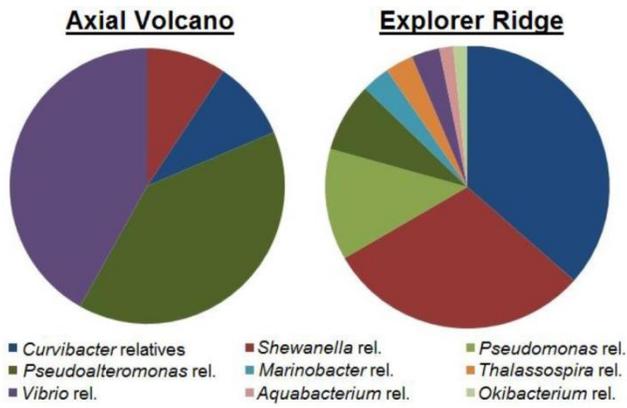
Figure 7.5. Distribution of metal(loid) oxyanion respiring epibionts of vent worms at Axial Volcano compared to those of Explorer Ridge.

being *S. oneidensis,* MR-1. Therefore, it is not surprising that they comprise a significant proportion of the isolates (39.5%). The same can be said about the *Pseudomonas* relatives. While they were not as abundant (12.7% of total isolates in samples), the genus is known to respire on selenate (*Pseudomonas stutzeri,* pn1) (Narasingarao & Haggblom, 2007) and metavanadate (*P. isachenkovii* and *P. vanadiumreductans*) (Yurkova & Lyalikova, 1990). Our work indicates both genera contain many members capable of metal(loid) oxyanion respiration.

In concurrence with previous publication (Huber et al., 2007), our data demonstrate that neighbouring deep sea habitats, including epibiotic bacterial populations of worms, may vary significantly in terms of composition. Comparing the two samples in our hands, we see the majority of isolates are ɤ-proteobacteria, however, the dominant genera associated with Axial Volcano tube worms comprise a minor fraction in the Explorer Ridge hosts. Such unequal distribution is likely due to the nature of this extraordinary environment. Deep ocean vent habitats are unstable, with features such as temperature, flow rate, water composition, and overall activity being highly variable and very often changing (Hessler et al., 1988). Radical fluctuations in parameters as a result of hydrothermal activity can lead to abolishment of life in the vent vicinity (Tunnicliffe & Juniper, 1990). However, following these drastic changes, life is quick to recover. While it is unknown if there was a recent change in activity at either of the two sampling sites, lower bacterial diversity could be a result of this phenomenon, causing re-establishment of life in the surrounding area. The subsequent primary microbial succession would lead to a more narrow range of microorganisms. Faster growing bacteria, such as members of the genus *Vibrio* (Wang et al., 2013), would dominate during initial colonization, followed by a progression of slower growing microbes, ultimately resulting in a climax population with greater variety. The more mature diverse population was seen in worms at ER,

which had an increased number of genera represented (9 total), while the population of AV worms was comprised of only 4, the majority of which are *Vibrio* relatives. A second possibility may be related to the age of the worms. Similar to above, if a worm is young, it will not have a mature climax population of epibionts. However, an older worm is more likely to have a much greater diversity of bacteria residing in/on it. Therefore, while the true age of the worms sampled was not known, it may be the cause of the difference observed between ER and AV symbionts, not necessarily a drastic geological event, causing a major disturbance to the ecosystem.

The diversity of epibionts in our study is similar to those found in other worms living in related habitats. Bacterial populations associated with *Lamellibrachia* sp. and *Escarpia southwardae*, from cold seeps in the eastern Mediterranean, are also dominated by ɤ-proteobacteria (Duperron et al., 2009; Duperron et al., 2014). However, despite this similarity in sharing this ɤ subclass affiliation, the genera and species composition does vary. When looking at the microbial populations of the tubes, there is little similarity to our results obtained from tissue samples. The biofilms on tubes of *R. pachyptila* are comprised of primarily ε-proteobacteria, as are those of *P. sulfincola* (Lopez-Garcia et al., 2002; Page et al., 2004). It is interesting that *P. sulfincola* tissue has a much different group of epibiotic bacteria in comparison to its tube, suggesting microbes inhabiting tubes do not necessarily colonize the body of the worm, creating significantly different communities even in such close proximity.

Lastly, the greatly varying sequence similarities to known species (from as low as 90.6 to as high as 100%) indicate diverse microbial populations (Table 7.4). Surprisingly, two strains (ER-Se-20 and ER-Te-44) are close relatives of non-marine bacteria (*Aquabacterium* and *Okibacterium*, respectively). The first genus is freshwater (Kalmbach et al., 1999) and the latter is comprised of a sole aerobic species associated with plant seeds (Evtushenko et al., 2002).

Table 7.4. Nearest phylogenetic relative for each isolate as determined by partial 16S rRNA gene sequencing.

Strain	Nearest Relative (% 16S rRNA Similarity)	Strain	Nearest Relative (% 16S rRNA Similarity)
AV-Se-12	*Pseudoalteromonas espejiana* (96.2)	ER-Se-21-light	*Thalassospira profundimaris* (99.7)
AV-Se-13	*Pseudoalteromonas issachenkonii* (91.3)	ER-Se-22-dark	*Marinobacter adhaerens* (98.2)
AV-Se-15 dark	*Vibrio splendidus* (98.3)	ER-Se-22-light	*Marinobacter flavimaris* (98.6)
AV-Se-16	*Pseudoalteromonas elyakovii* (99.1)	ER-Se-3	*Pseudoalteromonas nigrifaciens* (98.5)
AV-Se-17	*Curvibacter lanceolatus* (98.3)	ER-Te-2-brown	*Pseudoalteromonas paragorgicola* (93.5)
AV-Se-18	*Vibrio cyclitrophicus* (97.1)	ER-Te-2-grey	*Curvibacter gracilis* (95.9)
AV-Se-19	*Pseudoalteromonas elyakovii* (97.3)	ER-Te-40	*Shewanella frigidimarina* (92.5)
AV-Se-2-dark	*Curvibacter lanceolatus* (97.9)	ER-Te-40B	*Shewanella vesiculosa* (92.4)
AV-Se-3	*Pseudoalteromonas paragorgicola* (99.5)	ER-Te-41	*Shewanella vesiculosa* (95.6)
AV-Te-17	*Pseudoalteromonas elyakovii* (98.0)	ER-Te-41B	*Shewanella vesiculosa* (98.5)
AV-Te-18	*Pseudoalteromonas nigrifaciens* (96.3)	ER-Te-42	*Shewanella arctica* (94.2)
AV-Te-19	*Pseudoalteromonas haloplanktis* (98.0)	ER-Te-42B-dark	*Shewanella oneidensis* (93.4)
AV-Te-20	*Pseudoalteromonas tetradonis* (99.0)	ER-Te-42B-light	*Curvibacter lanceolatus* (95.8)
AV-Te-21-dark	*Curvibacter lanceolatus* (98.6)	ER-Te-43	*Pseudomonas libanensis* (98.3)
AV-Te-21-light	*Curvibacter lanceolatus* (96.8)	ER-Te-44	*Okibacterium fritillariae* (98.2)
AV-Te-22	*Pseudoalteromonas undina* (96.8)	ER-Te-45	*Pseudomonas poae* (98.6)
AV-Te-23-dark	*Shewanella arctica* (95.5)	ER-Te-46	*Shewanella oneidensis* (96.3)
AV-Te-23-light	*Curvibacter lanceolatus* (92.1)	ER-Te-47	*Curvibacter lanceolatus* (96.1)
AV-Te-24	*Shewanella vesiculosa* (95.2)	ER-Te-48	*Shewanella frigidimarina*[a]
AV-Te-25	*Pseudoalteromonas haloplanktis* (93.4)	ER-Te-49	*Curvibacter lanceolatus* (94.6)
AV-Te-26	*Pseudoalteromonas espejiana* (94.4)	ER-Te-50	*Pseudomonas fluorescens* (96.5)
AV-Te-27	*Pseudoalteromonas tetradonis* (90.6)	ER-Te-50-white	*Pseudomonas libanensis* (98.5)
AV-V-1	*Vibrio tasmaniensis* (95.1)	ER-Te-51	*Curvibacter gracilis* (93.9)
AV-V-10-1	*Vibrio splendidus* (98.4)	ER-Te-52	*Shewanella basaltis* (99.7)
AV-V-10-2	*Vibrio cyclitrophicus* (98.7)	ER-Te-53	*Curvibacter lanceolatus* (92.8)
AV-V-11	*Vibrio cyclitrophicus* (99.6)	ER-Te-54-dark	*Shewanella basaltis* (97.7)
AV-V-12	*Vibrio splendidus* (99.1)	ER-Te-54-light	*Shewanella basaltis* (99.0)
AV-V-13	*Vibrio splendidus* (99.9)	ER-Te-55	*Shewanella oneidensis* (99.0)
AV-V-14	*Vibrio splendidus* (96.7)	ER-Te-56	*Curvibacter lanceolatus* (97.3)
AV-V-15	*Vibrio pomeroyi* (91.4)	ER-Te-57	*Shewanella vesiculosa* (98.3)
AV-V-17	*Vibrio cyclitrophicus* (98.6)	ER-Te-58	*Curvibacter lanceolatus* (100)
AV-V-19	*Vibrio cyclitrophicus* (94.3)	ER-Te-59	*Shewanella vesiculosa* (97.4)
AV-V-2	*Vibrio splendidus* (100)	ER-Te-60	*Curvibacter fontanus* (95.4)
AV-V-20	*Pseudoalteromonas aliena* (98.1)	ER-Te-61	*Shewanella donghaensis* (97.3)
AV-V-21	*Pseudoalteromonas elyakovii* (91.7)	ER-Te-63	*Pseudoalteromonas elyakovii* (96.3)
AV-V-22	*Pseudoalteromonas haloplanktis* (91.3)	ER-Te-64-fast	*Pseudomonas sabulinigri* (98.5)
AV-V-23	*Vibrio splendidus* (98.6)	ER-Te-64-slow	*Curvibacter lanceolatus* (98.2)
AV-V-25	*Vibrio pomeroyi*[a]	ER-Te-65	*Curvibacter lanceolatus* (95.4)
AV-V-3	*Pseudoalteromonas lipolytica* (90.8)	ER-Te-66	*Pseudoalteromonas tetradonis* (99.6)
AV-V-4	*Shewanella basaltis* (99.7)	ER-V-10	*Shewanella vesiculosa* (99.7)
AV-V-5	*Vibrio tasmaniensis* (96.7)	ER-V-11	*Curvibacter lanceolatus* (97.5)
AV-V-6	*Vibrio cyclitrophicus* (92.6)	ER-V-12	*Vibrio cyclitrophicus* (92.7)
AV-V-7	*Shewanella frigidimarina* (94.7)	ER-V-13	*Vibrio lentus* (95.8)
ER-Se-1	*Curvibacter lanceolatus* (94.2)	ER-V-14	*Curvibacter lanceolatus* (95.6)
ER-Se-13	*Pseudoalteromonas espejiana* (95.4)	ER-V-15	*Curvibacter lanceolatus* (95.3)
ER-Se-14	*Curvibacter lanceolatus* (95.9)	ER-V-2	*Shewanella denitrificans* (97.5)
ER-Se-15	*Curvibacter lanceolatus* (94.6)	ER-V-3	*Pseudomonas marincola* (97.3)
ER-Se-16	*Curvibacter lanceolatus* (94.5)	ER-V-4	*Pseudomonas fluorescens* (95)
ER-Se-18	*Curvibacter lanceolatus* (96.7)	ER-V-5	*Curvibacter lanceolatus* (97.0)
ER-Se-19-dark	*Curvibacter lanceolatus* (96.4)	ER-V-6	*Shewanella frigidimarina*[a]
ER-Se-2	*Curvibacter lanceolatus* (97.8)	ER-V-7	*Curvibacter lanceolatus* (97.9)
ER-Se-20	*Aquabacterium hongkongensis* (92.9)	ER-V-8	*Shewanella livingstonensis* (100)
ER-Se-21-dark	*Thalassospira profundimaris* (99.9)	ER-V-9	*Pseudomonas stutzeri* (99.7)

[a]Csotonyi et al, 2006.

Also, 28 *Curvibacter* relatives were identified (Table 7.4), even though all published members have been isolated from freshwater wells (Ding & Yokota, 2004; Ding & Yokota, 2010). Clearly, sequencing hints on several potentially new taxonomic genera and species inviting further study for definitive identification.

In summary, it has been a long held belief that Te is a biologically insignificant element and its oxyanions were only considered as strong toxins to life. However, the recent discovery of bacteria capable of incorporating its oxyanions into metabolic pathways indicates otherwise (Csotonyi et al., 2006; Chapter 6). The discoveries of our previous work and this study show that Te in some habitats definitely supports life. Around black smokers in particular, metal(loid) oxyanion respiration is not simply an ability possessed by a few select bacteria, but is an established method of energy generation for a vast diversity of microbes. Very importantly, such epibiotic microbial suites may provide protection from toxic Te, Se, and/or V compounds present in vent fluids and, therefore, diffused into the host blood, by their removal via anaerobic respiration. It has long been known that these worms have an important symbiotic relationship with sulfur bacteria (Van Dover, 2000), which remove toxic sulfides from the blood by its conversion. Our data suggests a similar positive relationship may exist between worms and metal(loid) oxyanion converting bacteria described in this paper.

7.5. Acknowledgments

7.6. References

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Chapter 8.

Conclusions and Future Perspectives

8.1. Major Thesis Discoveries

This study has focused on microorganisms originating from extreme environments capable of transforming very toxic Te Se, and V oxyanions from higher oxidation states to less toxic elemental forms. The two main areas of the project were: (1) The impact of tellurite on cells of highly resistant microorganisms and the strategies employed for resistance/reduction and (2) Bacteria with the ability to carry out anaerobic respiration on metal(loid) oxyanions as terminal electron acceptors. The limited investigation and lack of strong knowledge in the field highlights the importance of this work. Our study also emphasizes the role of extreme environments, specifically mine tailings, thermal springs, and deep sea hydrothermal vents, in serving as repositories for bacteria with new interesting and important abilities. By virtue of their unique features, we have been able to show the significance of such ecosystems in providing us with microorganisms having the potential for biotechnological use in bioremediation of Te, Se, and possibly V compounds.

The first objective was to elucidate the physiological impact of tellurite on bacteria from extreme environments possessing very high level resistance to the oxyanion. The discovery that its influence can be positive was contrary to the generally held belief that exposure can only have a negative impact. Following exposure, strains KR99 and E5 both had an increase in biomass and ATP levels (Fig. 3.1), suggesting a benefit from TeO_3^{2-} presence. Once the effect on cells had been recorded, the question arose as to how reduction is carried out. Much still remains unclear (Turner, 2013), therefore, any additional information is highly beneficial. We identified four distinct cellular strategies for dealing with tellurite. Firstly, a periplasmic based inductive reductase was found in ER-Te-48. The second was a constitutive membrane associated reductase, possessed by strains E1, E4, KR99, RB3, and RB 16-17. Cells of JF1, T4, and Te-2-2

required *de novo* protein synthesis and an intact cytoplasmic membrane. Lastly, E4(1) and Se-1-2-red needed an intact cell and induced protein synthesis (Chapters 2 and 3). The final goal of this part of the thesis was to identify the enzymes responsible for reduction. Based on our experiments, the isolation, purification, and characterization of two tellurite/tellurate specific reductases (one periplasmic and the other membrane associated) and one selenite specific periplasmic reductase, was accomplished (Chapters 4 and 5). These enzymes are the first of their kind ever reported. Since the only other known tellurite reductase originated from the cytoplasm of a Gram positive bacterium (Etezad et al., 2009), those found here are clearly new to science and they greatly expand information on the enzymology of bacterial metalloid interactions.

Anaerobic respiration with Te, Se, and/or V oxyanions as terminal electron acceptors was believed to be an extremely rare phenomenon. Only a handful of confirmed cases existed prior to our work (Table 1.1). However, in spite of high toxicity, use of these compounds in this pathway is logical (Chapter 1). As extreme environments have provided examples of not only high level resistance (Yurkov & Csotonyi, 2003; Csotonyi et al., 2006; Plaza et al., 2016), but the first examples of their use for dissimilatory anaerobic reduction (Macy et al., 1989; Yurkova & Lyalikova, 1990; Csotonyi et al., 2006), it was reasonable to expect to find more cases by studying the inhabitants of such locales. The result was isolation of strain CM-3. This *Pseudomonas reactans* phylogenetic relative was isolated from gold mine tailings and possessed not only the ability to resist and reduce very high levels of tellurite, tellurate, and selenite both aerobically and anaerobically, but also anaerobically respire using these same compounds (Chapter 6). Upon discovery of CM-3, we decided to check other bacteria from metal(loid) rich habitats. Therefore, a laboratory collection of 107 isolates from deep sea hydrothermal vent tube worms, known to resist and reduce Te, Se, and/or V compounds (Csotonyi et al., 2006) was

tested. Our results confirmed anaerobic respiration using these oxyanions is a valid means of energy generation for the members of this deep ocean microbial community (Chapter 7). Out of 107 strains, 106 could use at least one of the oxyanions, proving toxicity does not prevent their inclusion in biological process.

Overall, our study produced two major discoveries. The first was an understanding of the physiological response of bacteria to a challenge by tellurite and strategies involved in resistance and reduction. It showed that approaches are not shared, even by isolates from the same or similar environments. Secondly, we found that despite their toxicity, Te, Se, and V oxyanions do appear to have a role in the life of some vent worm bacterial epibionts in the vicinity of deep sea hydrothermal vents.

8.2. Future perspectives

Our results have significantly enhanced the knowledge of bacterial metal(loid) interactions. Nevertheless, many questions still remain. While we were able to identify four distinct strategies for reduction, only a small group of bacteria (11 strains) was reviewed. In order to determine if the approaches found in the selected microbes are in fact common in those possessing very high level resistance, a broader range of isolates from various environments must be considered. In doing this, a better picture of the concept could be obtained.

This research isolated the first ever reported periplasmic Te and selenite oxyanion reductases as well as the first membrane associated Te oxyanion reductases. While some basic characterization has been achieved, much more remains to study. Genome sequencing of strains ER-Te-48 and KR99 would be the first step. Together with the mass spectrometry fingerprints, it could provide the information needed to ascertain the gene(s) encoding these enzymes.

Following identification, the gene(s) could be cloned into a high expression plasmid, allowing addition of a polyhistadine-tag and large scale protein production. One could then easily purify sizable quantities via a Ni-column, providing the volume of pure protein needed for more in-depth analysis such as metal content, detailed rate kinetics, and crystallography. The DNA sequencing could also be used to develop a molecular approach/marker to isolate metal(loid) reducers from environmental samples. Due to the lack of a genetic marker for screening strains for the presence of metal(loid) reductase genes, current metagenomic analysis of natural samples cannot tell us if the bacteria present are capable of high level resistance to Te, Se, and/or V, or anaerobic respiration. The possibility to recognize the genetic elements encoding the key participating component(s) of reduction also has a second potential application, providing a non-culture based molecular technique to screen environmental samples. Currently, the only method to test if an isolate is resistant to, or can anaerobically respire on Te, Se, and/or V compounds is to work with pure cultures, which can be tedious. Obviously, the development of such a molecular biological method would be highly beneficial. With the required operon(s) identified, PCR amplification of reductase gene(s) could give a genetically based method, which may be faster and easier to perform than employing classical culturing techniques. The shortcoming of a method based solely on sequence is while it can identify the presence of a homologous gene and indicate the possibility of function, one cannot confirm activity without culturing. Therefore, it is best to use both approaches in tandem.

With 'green' technologies becoming main stream, bacteria, such as in our study, are of great importance. Today, there is more attention on Te, Se, and V contamination and bioremediation has been gaining popularity (Gadd, 2010). The main issue with using microorganisms for this purpose is the insignificant amount of oxyanion removed and the length

of time taken to achieve the goal (Cantafio et al., 1996; Bonificio & Clarke, 2014). In order to improve the process, strains with higher levels of resistance and faster removal rates are sought after. Many microbes in our work show great potential. Preliminary results with some of the isolates has revealed that they can remove 10-100 fold more tellurite and/or selenite within a drastically shorter time compared to those species currently published (Cantafio et al., 1996; Bonificio & Clarke, 2014). All this is quite promising, but requires research to fine tune and develop a practical and economically viable process.

Much like bioremediation, potential biological recovery of rare and valuable metals from ores has been gaining ground (Marwede & Reller, 2012; Ilyas & Lee, 2014). Many sought after elements are omnipresent in nature, but at concentrations so low that conventional mining techniques are not feasible. Bacteria capable of bioaccumulation of such elements internally may provide a way of overcoming their disperse distribution. All of the strains studied here accumulated elemental Te and/or Se intracellularly in relatively large amounts. In terms of capturing pure elements this is ideal. Developing a method to recover cells containing the desired metal(loid) may potentially eliminate the need for massive mining operations and the use of harsh chemicals. The microorganisms investigated here are very promising in this regard.

Obviously, heavy metal(loid) reducing bacteria are of great interest for many different fields of applied science (Turner et al., 2012; Nancharaiah & Lens, 2015). Future research, following the paths laid out above, has the chance to yield some important discoveries in bioremediation, bioreclamation, biometallurgy, enzymology, microbial ecology, microbial physiology, and taxonomy. One can only imagine the new prospectives that lie ahead.

8.3. References

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Appendix I.

Supplementary Materials

Table S1. Level of tellurite resistance for selected strains in RO liquid medium.

Strain	Tellurite Concentration (µg/ml)					
	500	750	1000	1500	2000	2500
ER-Te-48	100 ± 4.8	95 ± 4.7	92 ± 6.1	86 ± 4.8	77 ± 5.0	64 ± 5.2
JF1	100 ± 3.9	93 ± 5.5	88 ± 4.4	76 ± 3.8	52 ± 3.2	9 ± 2.1
Se-1-2-red	100 ± 4.1	97 ± 7.1	85 ± 5.1	74 ± 6.3	61 ± 4.2	11 ± 3.1
T4	100 ± 5.3	94 ± 6.2	81 ± 5.2	58 ± 3.7	5 ± 2.2	NG
Te-2-2	100 ± 6.1	92 ± 8.3	87 ± 6.9	73 ± 7.4	54 ± 6.2	12 ± 7.3

Growth determined by A_{950} and represented as percent maximal absorbance. NG – No growth

Table S2. Level of tellurite resistance for selected aerobic anoxygenic phototroph strains in RO liquid medium.

Strain	Tellurite Concentration (µg/ml)					
	100	250	500	750	1000	1500
E1	NA	NA	100$\pm$3.3	79$\pm$5.1	64$\pm$5.6	10$\pm$2.8
E4(1)	NA	NA	100$\pm$4.8	71$\pm$2.8	46$\pm$4.3	15$\pm$3.1
E5	NA	NA	100$\pm$4.9	84$\pm$5.6	73$\pm$5.3	54$\pm$6.7
KR99	NA	NA	100$\pm$5.5	95$\pm$5.8	85$\pm$6.1	68$\pm$4.9
RB3	100$\pm$6.1	82$\pm$8.3	47$\pm$6.9	21$\pm$7.4	NG	NG
RB 16-17	100$\pm$7.2	82$\pm$6.1	61$\pm$4.7	49$\pm$5.5	5$\pm$2.2	NG

Growth determined by A_{950} and represented as percent maximal absorbance. NG – No growth; NA – Not applicable, was not tested.
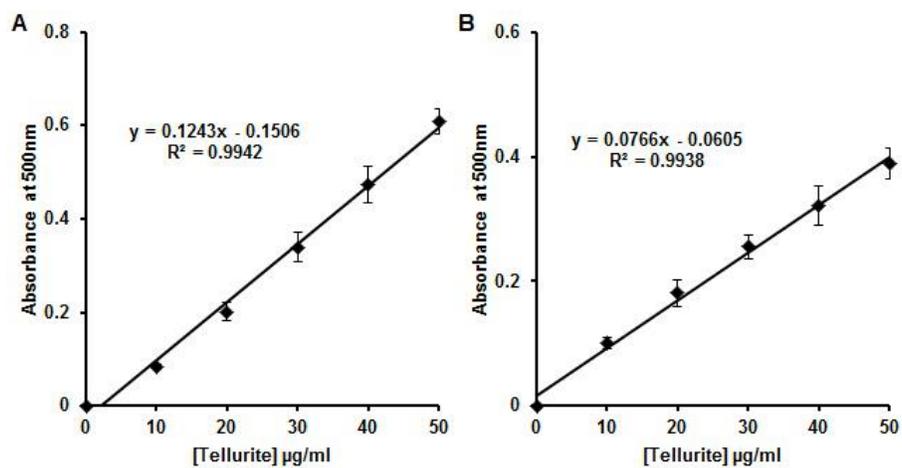
Figure S1. Standard curves for elemental tellurium production from various concentrations of tellurite in A) RO NaCl medium and B) MS NaCl medium. Error bars represent one standard deviation.
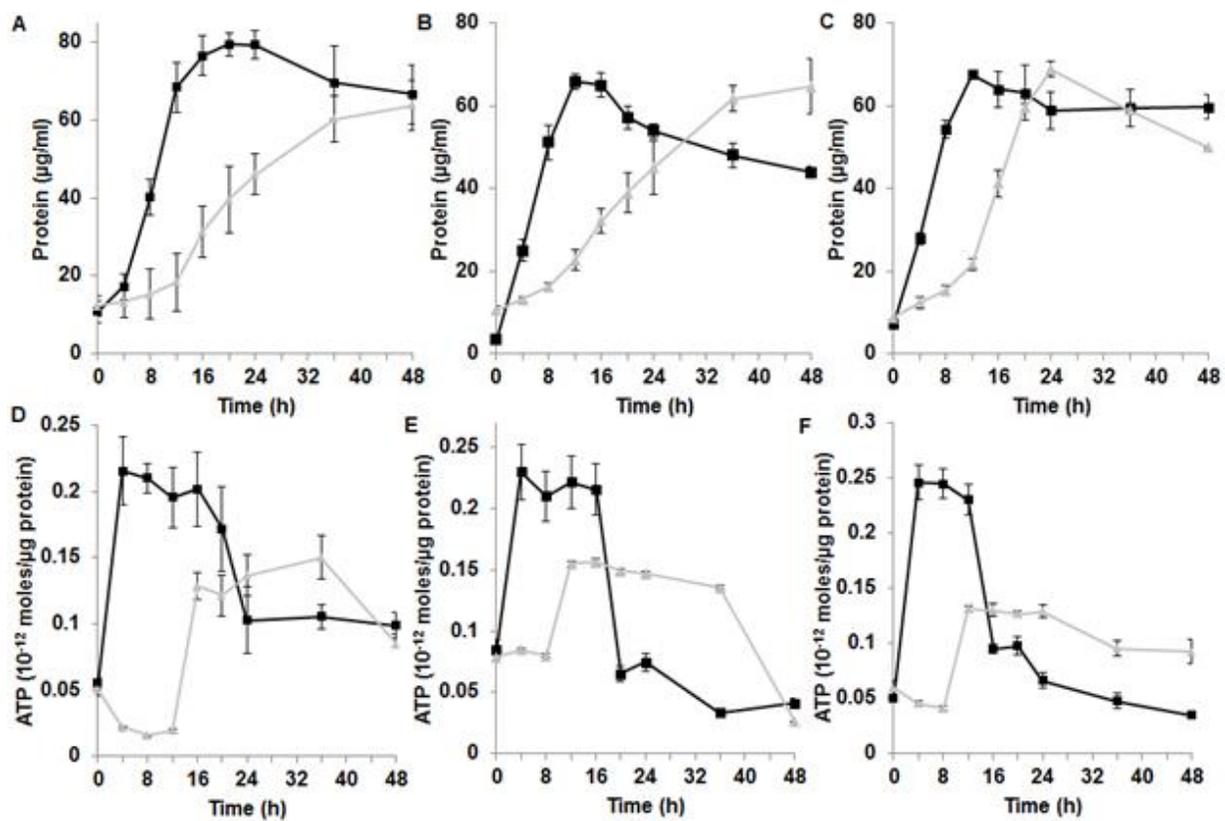
Figure S2. Protein and ATP production in presence versus absence of K_2TeO_3. A) Strain T4. B) Te-2-2. C) JF1. D) T4. E) Te-2-2. F) JF1. ■ – No K_2TeO_3; ▲ – 500 µg/mL K_2TeO_3. Error bars represent one standard deviation.
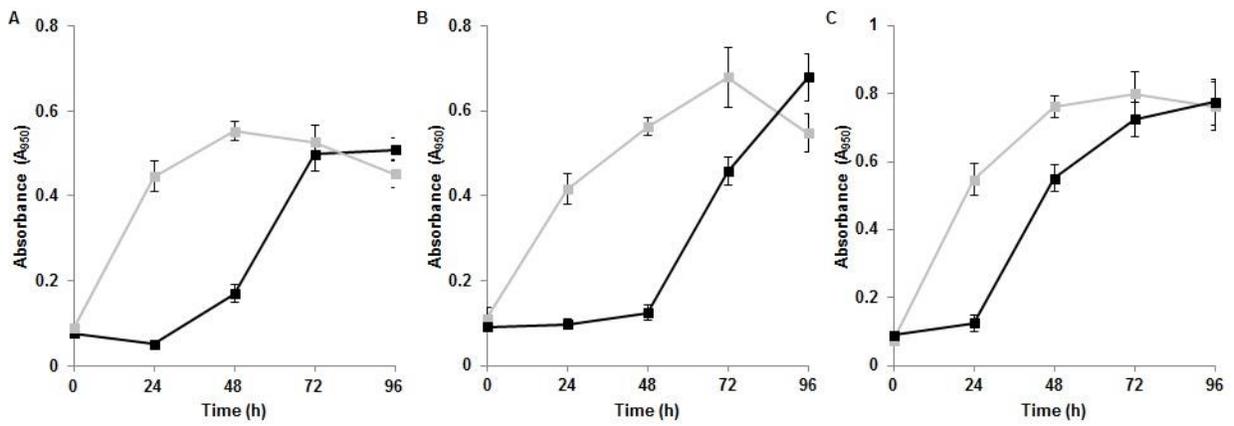
Figure S3. Growth during primary vs. secondary exposure to 500 µg/ml K_2TeO_3. A) Te-2-2, B) JF1, C) T4. ■ - Primary exposure; ■ - Secondary exposure. Error bars represent one standard deviation.
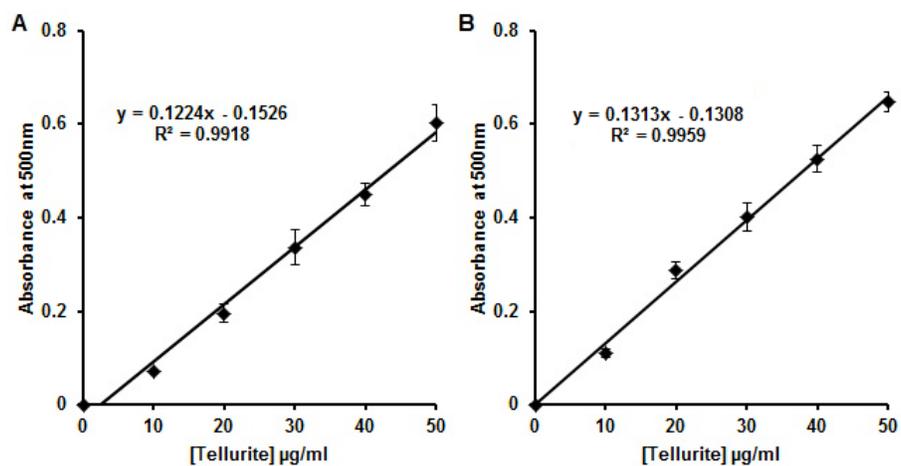
Figure S4. Standard curves for elemental tellurium production from various concentrations of tellurite in A) RO medium and B) MS medium. Error bars represent on standard deviation.
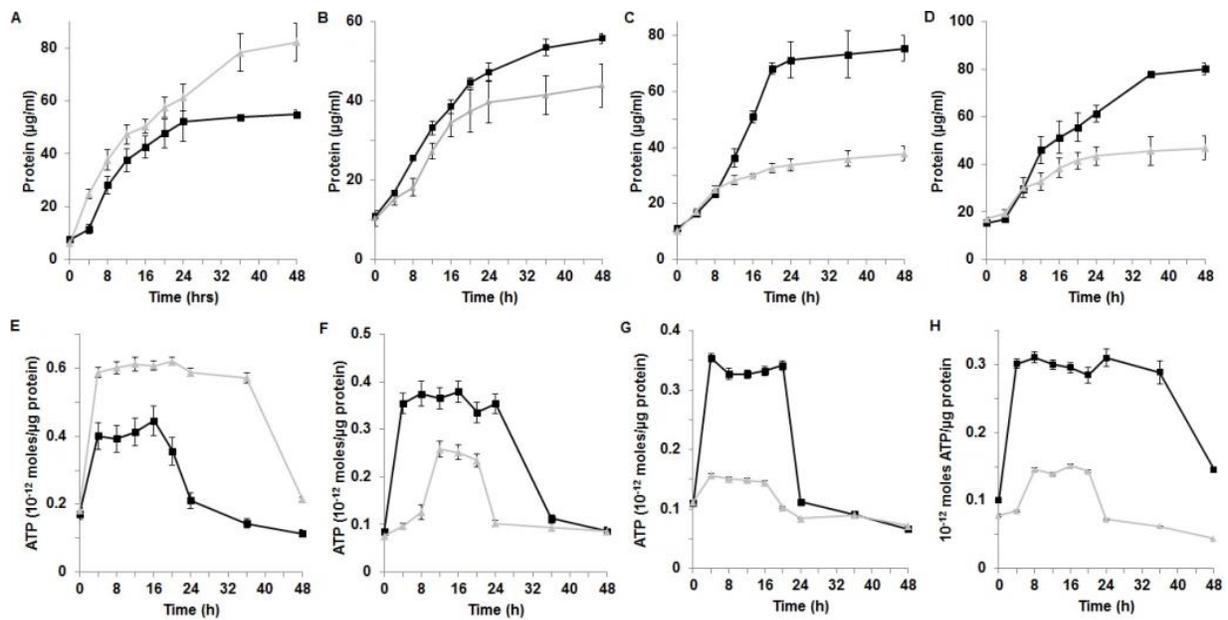
Figure S5. Protein and ATP production in presence versus absence of K_2TeO_3. A) Strain E5. B) E4(1). C) RB3. D) RB 16-17. E) E5. F) E4(1). G) RB3. H) RB 16-17. ■ – No K_2TeO_3; ▲ – 500 µg/mL K_2TeO_3 for E5 and E4(1) or 100 µg/mL K_2TeO_3 for RB3 and RB 16-17. Error bars represent one standard deviation.
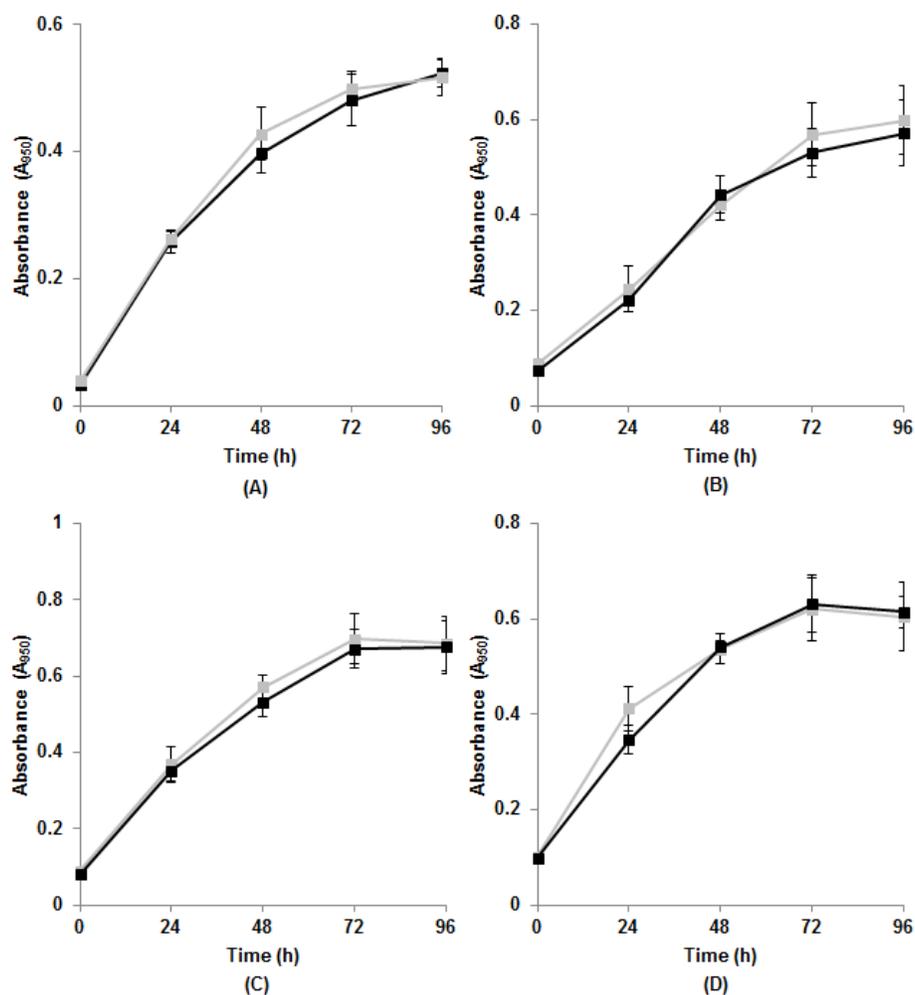
Figure S6. Growth during primary vs. secondary exposure to K_2TeO_3. A) E5, B) RB3, C) RB 16-17, D) E1. ■ - Primary exposure; ■ - Secondary exposure. Error bars represent one standard deviation.
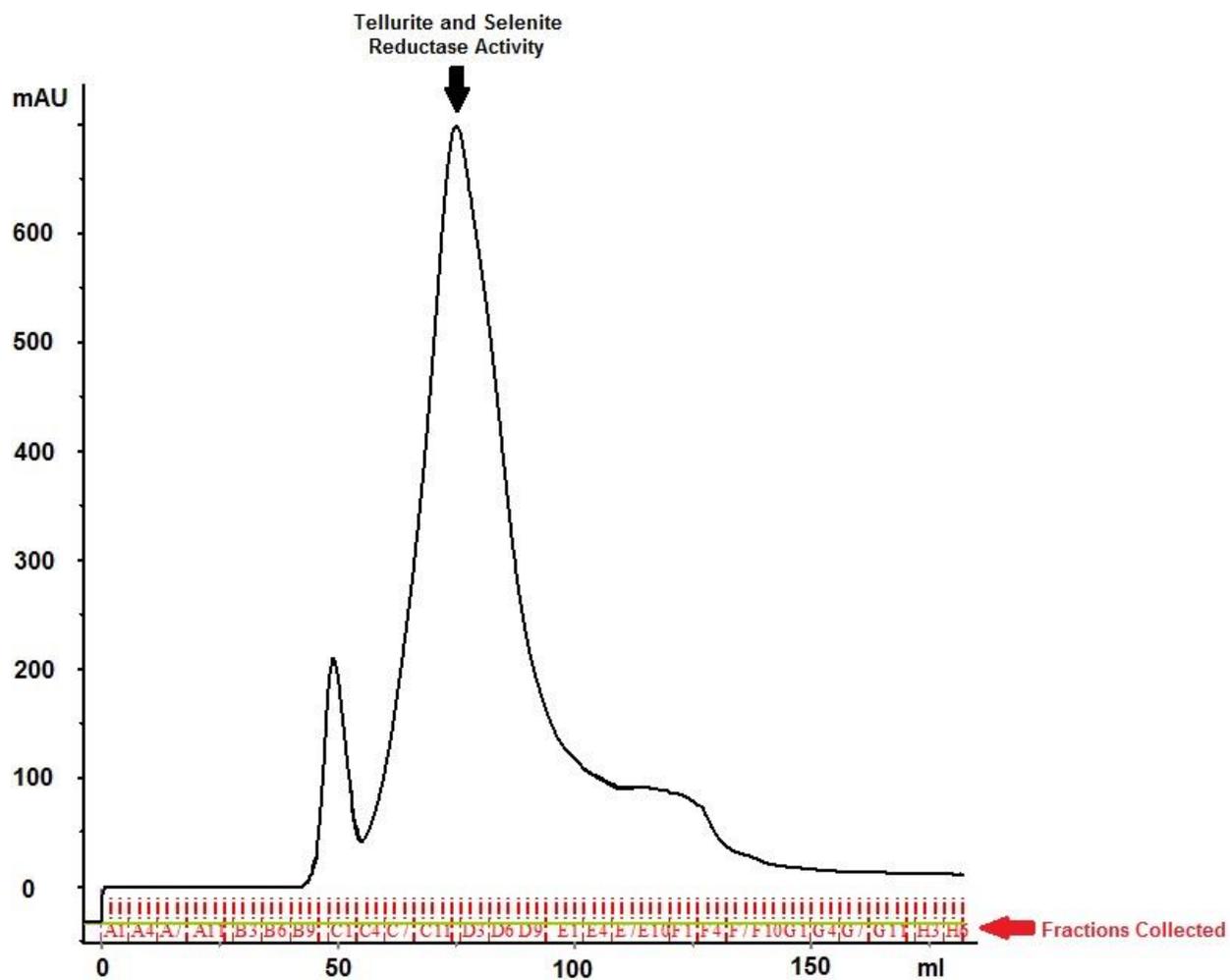
Figure S7. S200 gel filtration column elution profile for >100 kDa periplasmic fraction of ER-

Te-48 containing tellurite and selenite reductase activity.
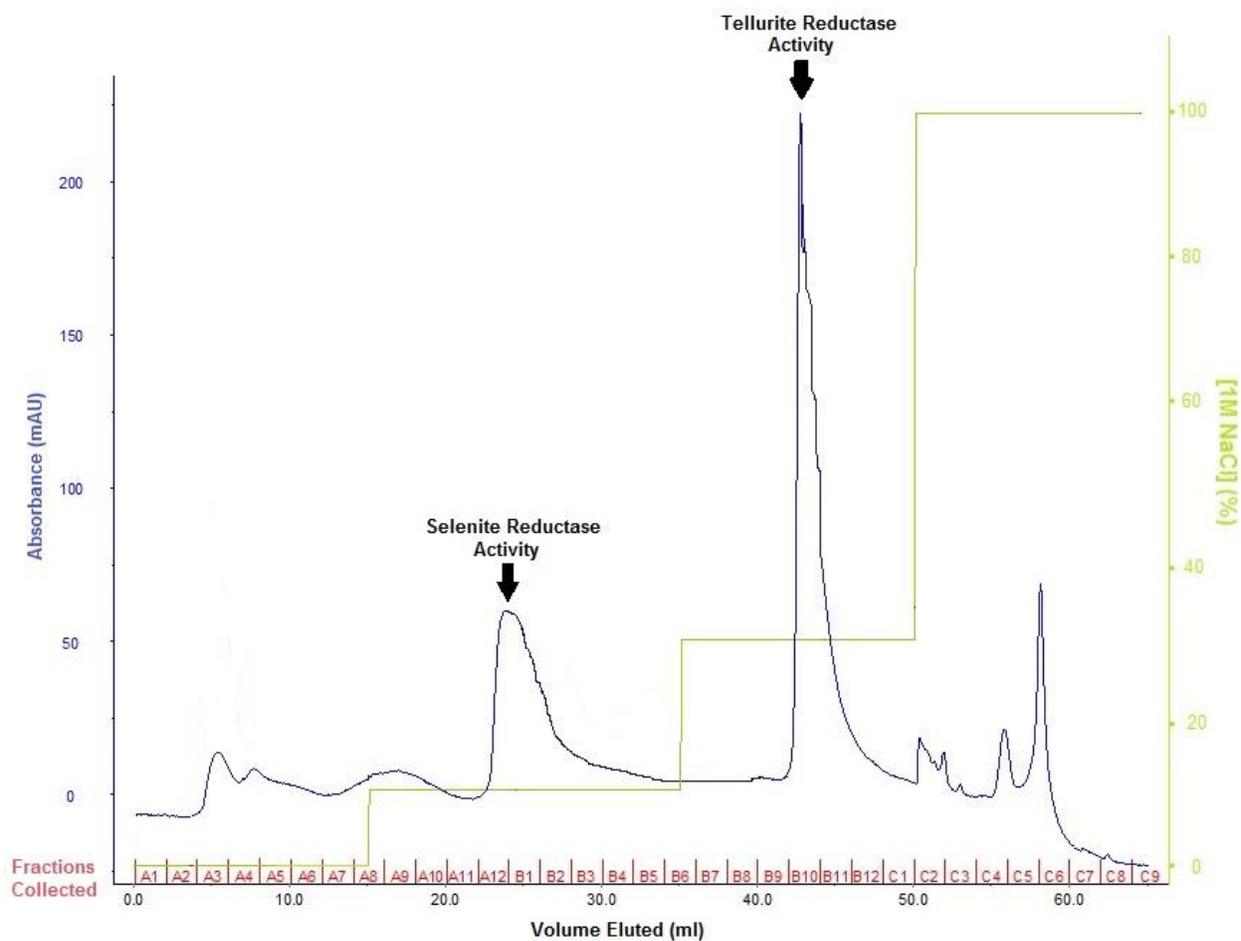
**Figure S8.** Source 15Q anion exchange column elution profile for ER-Te-48 tellurite and

selenite reductase.
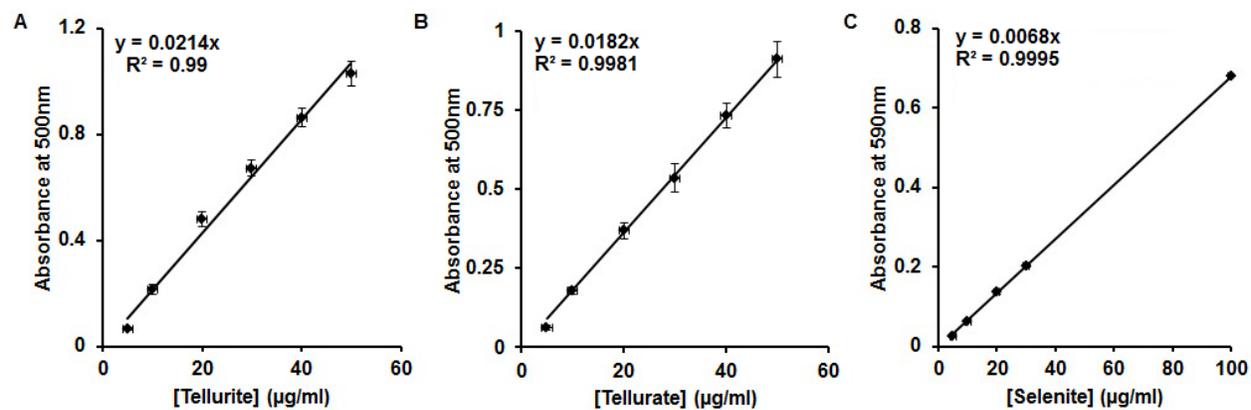
Figure S9. Standard curves for elemental tellurium production from A) tellurite and B) tellurate in 20 mM TRIS HCl pH 8.0 and elemental selenium production from C) selenite in 20 mM PBS pH 6.0. Error bars represent on standard deviation.
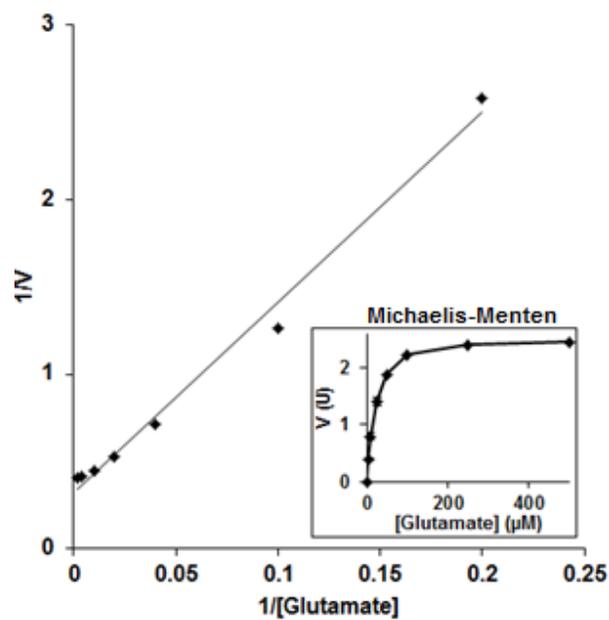
Figure S10. Lineweaver–Burk plot of the electron donor glutamate for selenite reduction. The insert shows the Michaelis–Menten plot. Error bars represent one standard deviation.
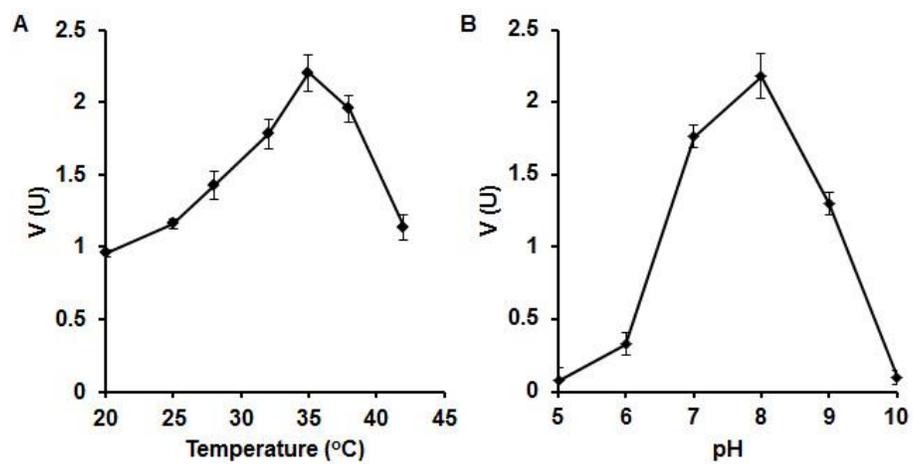
Figure S11. Effect of A) temperature and B) pH on tellurate reduction. Error bars represent one standard deviation.
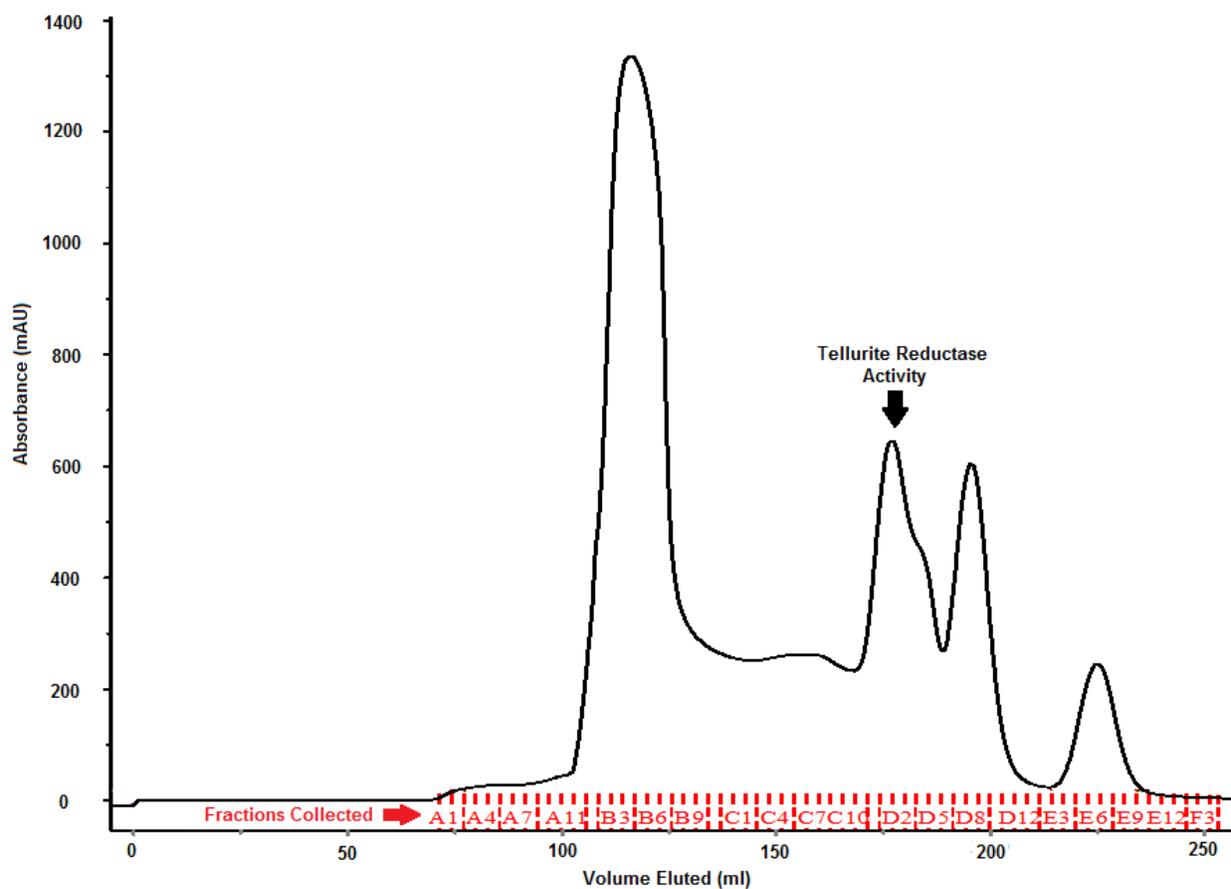
Figure S12. S200 gel filtration column elution profile for solubilized membranes of

Erythoromonas ursincola, strain KR99, containing tellurite reductase activity.
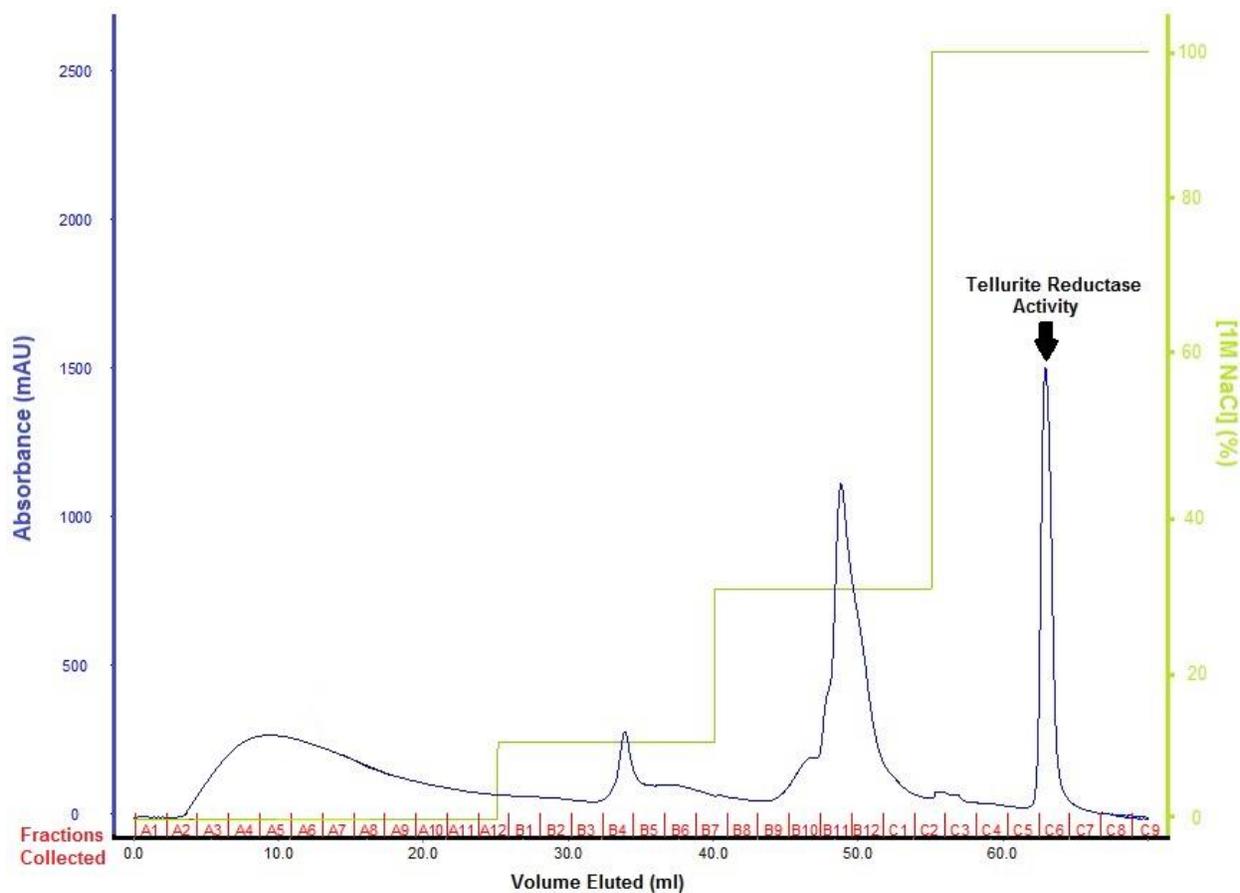
Figure S13. Source 15Q anion exchange column elution profile for tellurite reductase for

Erythromonas ursincola, strain KR99.
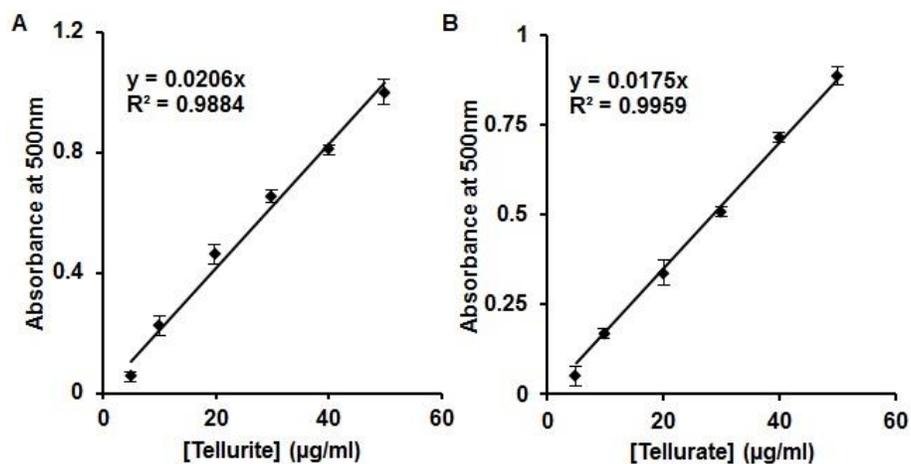
Figure S14. Standard curves for elemental tellurium production from A) tellurite and B) tellurate in 20 mM TRIS HCl pH 7.0.
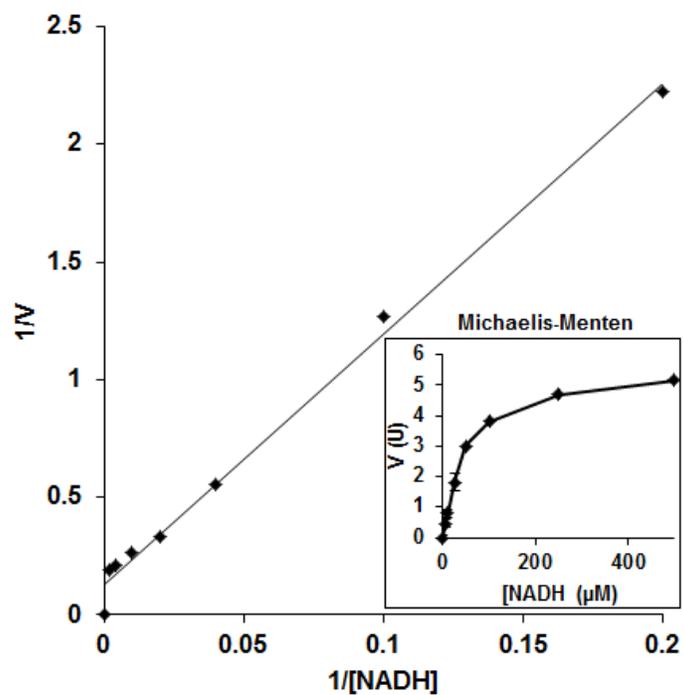
Figure S15. Lineweaver–Burk plot of the electron donor NADH for tellurite reduction. The insert shows the Michaelis–Menten plot. Error bars represent one standard deviation.
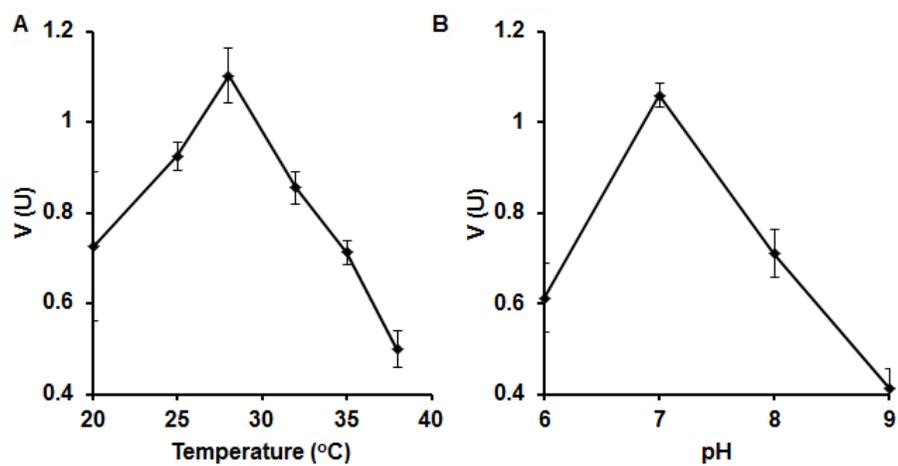
Figure S16. Effect of A) temperature and B) pH on tellurate reduction. Error bars represent one standard deviation.
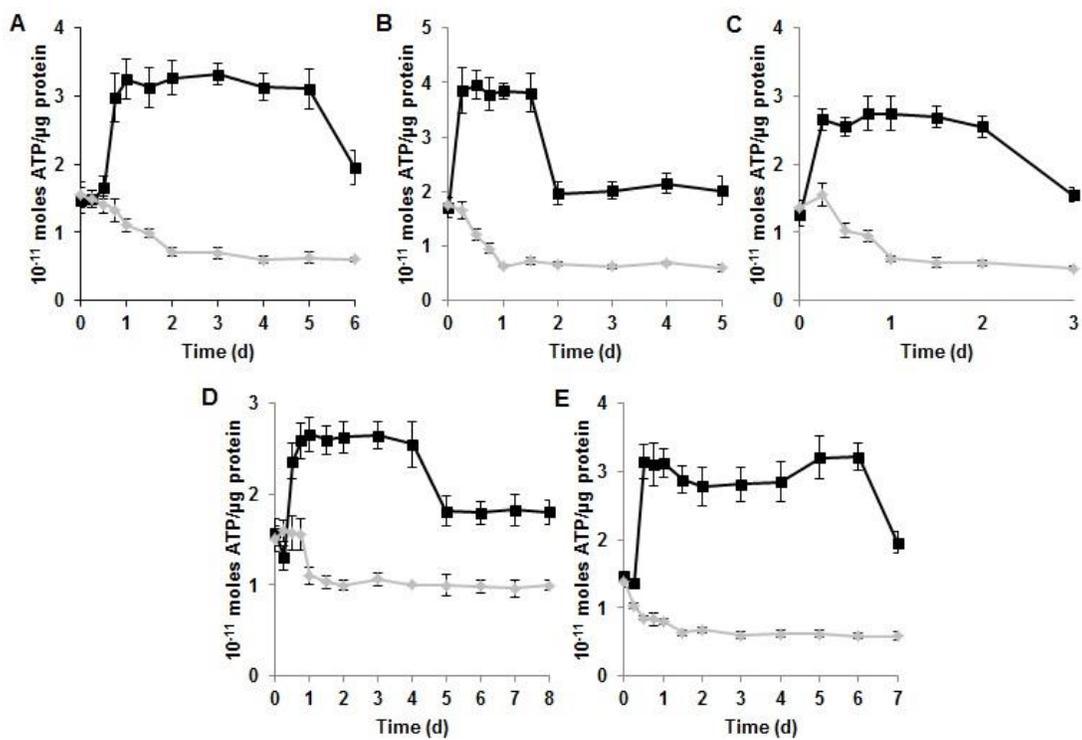
Figure S17. ATP production during anaerobic growth in presence of K_2TeO_3. A) ER-Te-57. B) AV-Te-18. C) ER-V-8. D) AV-V-4. E) ER-Te-41. ■ – With metal(loid) oxyanion; ♦- Without oxyanion. Error bars represent one standard deviation.