IDENTIFICATION OF ORGANO-TELLURIUM AND ORGANO-SELENIUM COMPOUNDS IN THE HEADSPACE GASES ABOVE GENETICALLY MODIFIED *ESCHERICHIA COLI* AMENDED WITH TELLURIUM AND SELENIUM SALTS

A Thesis

Presented to

The Faculty of the Department of Chemistry

Sam Houston State University

In Partial Fulfillment

of the Requirements for the Degree of

Masters of Science

by

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August, 2005
IDENTIFICATION OF ORGANO-TELLURIUM AND ORGANO-SELENIUM COMPOUNDS IN THE HEADSPACE GASES ABOVE GENETICALLY MODIFIED *ESCHERICHIA COLI* AMENDED WITH TELLURIUM AND SELENIUM SALTS

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Abstract

Swearingen Jr., Jerry W., Identification of Organo-tellurium and Organo-selenium Compounds in the Headspace Gases Above Genetically Modified \textit{Escherichia coli} Amended with Tellurium and Selenium Salts. Master of Science (Chemistry), August, 2005, Sam Houston State University, Huntsville, Texas.

Purpose

The purpose of this work was to identify organo-selenium and organo-tellurium compounds produced by genetically modified \textit{Escherichia coli} cells that were amended with selenium or tellurium salts. The goals of this research were to see which genetic modifications were responsible for the production of the organo-chalcogen compounds. Experiments were designed to detect new, never previously reported compounds above the modified \textit{E. coli} cultures. Experiments were also performed to see if the change from aerobic to anaerobic growth conditions affected the bioremediation potential of \textit{Pseudomonas fluorescens} K27.

Recombinant \textit{E. coli} cultures were amended with either tellurate, tellurite, selenate, or selenite or some combination of these salts. Experiments show that ORF 600 and 1VH (which contains ORF 600) produce organo-tellurium and organo-selenium compounds. Expression of ORF 600, which contains the gene encoding for a UbiE methyltransferase, resulted in production of organo-tellurium compounds when the clone was amended with tellurate but did not produce any organo-tellurium compounds when the clone was amended with tellurite. Organo-tellurium compounds that were detected above tellurium amended cultures were dimethyl telluride, methanetellurol, dimethyl tellurenyl sulfide, and dimethyl ditelluride. The last three have never been reported before in the literature as bacterial products. Organo-selenium compounds that were detected were dimethyl selenide, dimethyl seleneny1 sulfide, dimethyl diselenide, and
dimethyl selenodisulfide. The oxidation state of selenium in the amendments did not affect the production of organo-selenium compounds.

Dimethyl selenodisulfide has never been detected in the headspace gases above bacterial cultures but was determined here; its structure was confirmed by GC/MS and compared to a similar mass spectrum found in the literature. Detection of these volatile compounds was performed by using either gas chromatography-sulfur chemiluminescence detection or gas chromatography-mass spectrometry.

Sequentially switching from aerobic to anaerobic growth did not show any improvement or decline of the bioremediation potential of Pseudomonas fluorescens K27. Measurement of dissolved oxygen (D.O.) content of the culture media was done by a D.O. probe that measured the percent of oxygen saturation in the solution. A D.O. probe that measured the solution phase concentration of dissolved oxygen (mg/L) was also used.

KEY WORDS: Genetically modified E. coli, organo-selenium, organo-tellurium, aerobic/anaerobic growth, methyl transferase, dimethyl tellurenyl sulfide.

Approved:

____________________
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Acknowledgments

First of all I would like to thank my girlfriend Carrie Kesler for staying with me, being patient, and helping me during the two years that I have been working towards this degree. I also would like to thank my parents Jerry and Jane Swearingen for financing my college career of seven years. Without them I would not have been able to be here.

For Dr. Chasteen, I would like to thank him for accepting me into his laboratory 4 years ago and giving me the opportunity to conduct research right after my sophomore year of chemistry classes. Without him I would not have made it as far as I have in the field of chemistry.

I would like to thank the people that have worked in this lab with me over the years. There help was appreciated and I hope they appreciated my help in their projects.

Lastly I would like to thank the research group of Dr. Claudio C. Vásquez of the Laboratorio de Microbiologia Molecular of the Facultad de Química y Biología, Universidad de Santiago de Chile. They are responsible for the genetic modifications and for help to the Chasteen laboratory to understand the genetic modifications and the microbiology involved.
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Chapter 1

Introduction

Selenium and tellurium both have an affinity for copper and sulfur. Historically this became apparent when elevated concentrations of selenium and tellurium were found around accumulated copper ores. As a result, the production of these chalcogens is a byproduct of copper ore refining (Solozhenkin et al., 1993). Collectively Se, Te, and S are termed chalcogens.

No biological role for tellurium has been assigned but the concentration of this element in human blood is approximately 6 ppb and 15 ppb in tissue. Tellurium is the 72nd most abundant element on the earth with concentrations in soil ranging from 0.05 ppm to 30 ppm and 0.15 parts per trillion in sea water. Common minerals that contain tellurium are calaverite (AuTe₂), sylvanite (AgAuTe₄), and tellurite (TeO₂) (Emsley, 2002). Uses of this element include its being an additive to steel to improve machinability, an additive to lead to improve hardness and acid-resistance, and a semi-conducting material used for the production of thermoelements and photoelements (Craig, 1986).

Selenium has been established as a component in a number of enzymes. The first such report in human tissue came from Awasthi et al. (1975) while analyzing an antioxidant enzyme, glutathione peroxidase in erythrocytes. Another discovery of selenium’s biological importance was when Se was found as a component in deiodinase, an enzyme that promotes hormone production in the thyroid gland. Seleno-amino acids selenomethionine and selenocystine have been identified in soil-sediment extracts (Zhang et al., 1999) and in Bertholletia excelsa, the Brazil Nut (Chunhieng et al., 2004).
Selenium is the 67th most abundant element with an earth’s crustal concentration of 50 ppb, soil concentration of 5 ppm, and sea water concentration of 0.2 ppb. Atmospheric Se concentration is 1 nanogram per cubic meter of air usually in the form of methylated derivatives that mimic those of sulfur, for instance, CH$_3$SeSeCH$_3$ or CH$_3$SeCH$_3$.

Economically the conductive properties of selenium contribute to the design and function of photoelectric cells, light meters, solar cells and photocopiers. Selenium is also used to decolorize glass in that industry, in the form of cadmium selenide, larger amounts of this compound resulting in a ruby color in glass to which it is added. Even though selenium is toxic at low levels, the presence of this element in glutathione peroxidase can act as an antagonist of metals such as mercury, arsenic, and thallium in biological systems and thereby lowering these elements already significant toxicity (Emsley, 2002).

Methods of Detecting Organo-Chalcogen Compounds

Organic sulfur compounds of interest produced by biological organisms are methanethiol (MeSH, CH$_3$SH), dimethyl sulfide (DMS, CH$_3$SCH$_3$), dimethyl disulfide (DMDS, CH$_3$SSCH$_3$), and dimethyl trisulfide (DMTS, CH$_3$SSSCH$_3$). A review by Bentley and Chasteen describes a sulfur biospheric cycle. This involves solution-phase sulfate and sulfite being biologically reduced to H$_2$S. Bioproduction of methionine, dimethylsulfiniopropionate, MeSH, and 3-methylthiopropionic acid has been reported once hydrogen sulfide is produced. These thiol-containing compounds are all produced by enzymatic action on H$_2$S. Further bioproduction will result in the release of DMS in the atmosphere. DMS is then photochemically oxidized into compounds such as C-O-S, DMSO [CH$_3$S(=O)CH$_3$], DMSO$_2$ [CH$_3$S(=O$_2$)CH$_3$], and eventually to the sulfate and sulfite ions that return to the marine or terrestrial ecosystems (Bentley and Chasteen,
Because of the similar chemistry of the other chalcogens, selenium and tellurium, they can possibly replace sulfur in these cyclic processes to establish a selenium or tellurium biospheric cycle (Thayer, 1995). In biological systems, replacing sulfur- or selenium-containing proteins with tellurium can have disastrous consequences (Garberg et al., 1999); however, Bentley and Chasteen still hypothesize that tellurium, like Se before it, will ultimately be found to be a required trace element for some organisms even though both Se and Te are on the same order of toxicity as arsenic for their common oxyanions.

Other organic chalcogens of interest are dimethyl selenide (DMSe, CH$_3$SeCH$_3$), dimethyl selenenyl sulfide (DMSSe, CH$_3$SeSCH$_3$), dimethyl diselenide (DMSdSe, CH$_3$SeSeCH$_3$), dimethyl telluride (DMTe, CH$_3$TeCH$_3$), and dimethyl ditelluride (DMDTe, CH$_3$TeTeCH$_3$). Analysis of these volatile organo-chalcogen compounds can be achieved by using gas chromatography (GC) coupled with detectors such as flame ionization detection (FID) for carbon-containing compounds (Van Fleet-Stalder and Chasteen, 1998), flame photometric detection (FPD) for sulfides (Kataoka et al., 2000), and fluorine-induced chemiluminescence for volatile sulfides, selenides, and tellurides (Van Fleet-Stalder and Chasteen, 1998; Basnayake et al., 2001). Mass spectrometry (MS) has also been a useful tool as a general detector used to determine unknown compound identities. FID offers an overall view of the volatiles (hydrocarbons and organometalloids) in headspace, but the concentration of any organometalloid has to be relatively high for FID determination of these heteroatom-containing compounds (Holm, 1997). FPD detection also does not offer selectivity or linearity of organometalloid detection comparable to those of fluorine-induced chemiluminescence (Van Fleet-Stalder
and Chasteen, 1998; Aue and Singh, 2001). The fluorine-induced chemiluminescence
detector offers a selective and sensitive means of headspace analysis of organosulfur, -
selenium, and -tellurium compounds with a linear range of three orders of magnitude and
picogram detection limits for the organometalloids under study here. GC/MS can be used
to identify unknown headspace components but this method requires relatively high
concentrations of the analytes and cryogenic trapping if the boiling points of some
components are below ~20 °C. An alternative approach is to use a thick-film capillary
column (5 μm film) to more successfully separate low boiling point species; however,
thick-film columns also have higher amounts of column bleed at moderate temperatures
and this increases background and detection limits for GC/MS which responds to the
components of the column bleed.

Extraction methods for these compounds in complex samples range from
derivatization and manual headspace extraction to solid phase micro extraction (SPME).
An example of using derivatization reagents to detect organo-chalcogens would be the
use of 1-fluoro-2,4-dinitrobenzene (FDNB) to determine concentrations of
dialkyldiselenides in water. The diselenide is reduced to a selenol (RSeH) by the
addition of zinc and hydrochloric acid. The selenol then reacts with FDNB to form HF
and RSe-DNP. After derivatization of organo-diselenides, analysis of the extracted
selenide derivatives is performed by GC/MS. This method offers a detection limit on the
order of nanograms (Gómez-Ariza et al., 1999).

Manual headspace extraction is another simple yet reproducible extraction
technique for the collection of volatile compounds. The equipment used consists of a
syringe of known volume with a needle and a locking valve to prevent sample loss.
Detection of DMTe was accomplished by using this method when searching for organo-tellurium compounds above tellurium amended cultures of *Pseudomonas fluorescens* K27 (Basnayake et al., 2001). Using culture flasks with specially-designed, reusable, enclosure caps allowed for multiple extractions over a period of time without risk of the sample escaping the flask (Stalder et al., 1995).

Solid phase micro extraction (SPME) is a relatively new technique in sample collection for chemical analysis. Unlike manual headspace extraction, this method can be used in both liquid and gas phases. SPME’s improvement over manual extraction is that it offers a method that pre-concentrates the sample in order for analysis to be performed on a less sensitive instrument (Kataoka et al., 2000; Ábalos et al., 2002). SPME was used to identify volatile organic compounds that were being emitted from naturally-aged books. GC/MS was used in the sample analysis. The SPME fiber was used in two ways, headspace analysis and contact extraction. Headspace extraction collected volatiles that were in the gas phase while contact extraction was used to collect less volatile compounds on the surface of the paper (Lattuati-Derieux, 2004). SPME extraction methods have also been used in the collection and concentration of forensic specimens. In the analysis of explosive and ignitable residues, an SPME fiber was used in three different types of extraction methods, headspace, partial headspace, and direct immersion. Headspace analysis using SPME collects volatile compounds in the gas phase while partial headspace involves the partial immersion of the SMPE fiber into the solution phase of the sample. Half of the fiber is immersed in the solution phase while the other half of the fiber is exposed to the gas phase. This type of extraction can decrease the detection limits of insoluble, less volatile compounds that occupy a thin
layer right above the solution phase. Direct immersion is accomplished when all of the
SPME fiber is exposed to the solution phase with none of the fiber exposed to headspace
gas. This extraction technique allows for the collection of non-volatile compounds that
remain in solution. It also can collect volatiles that are not in the gas phase which
partition between the gas and solution phase, based on Henry’s Law (Robbins et al.,
1993). Adjustable parameters using SPME include fiber absorption time and
temperature, solution ionic strength, agitation, and SPME fiber solid-phase selection
(Furton et al., 2000).

The solid phase of the SPME fiber is very similar to the stationary phase found on
the inside of a capillary column used for gas chromatography. Table 1 lists the different
types of stationary phase components available and the type of analysis for which the
stationary phase is designed. A SMPE fiber is selected based on the type of analysis that
needs to be performed. Typical SPME applications include industrial applications,
headspace analysis, environmental analysis, flavors, odors, toxicology, and forensic
analysis.
Table 1. Description of SPME extraction phases.

<table>
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<th>Solid Phase</th>
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<td>75 μm – 85 μm</td>
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<tr>
<td>Polydimethylsiloxane</td>
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</tr>
<tr>
<td>Polydimethylsiloxane/divinylbenzene</td>
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<td>65 μm</td>
</tr>
<tr>
<td>Polyacrylate</td>
<td>Polar Semivolatiles</td>
<td>85 μm</td>
</tr>
<tr>
<td>Polydimethylsiloxane</td>
<td>Nonpolar High Molecular Weight</td>
<td>7 μm</td>
</tr>
<tr>
<td>Polydimethylsiloxane</td>
<td>Nonpolar Semivolatiles</td>
<td>30 μm</td>
</tr>
<tr>
<td>Carbowax/divinylbenzene</td>
<td>Alcohols and Polar Compounds</td>
<td>65 μm - 70 μm</td>
</tr>
<tr>
<td>Carbowax/templated resin</td>
<td>Surfactant and Other Polar Analytes</td>
<td>50 μm</td>
</tr>
<tr>
<td>Divinylbenzene/Carboxen</td>
<td>Trace Level Analysis</td>
<td>50 μm / 30 μm</td>
</tr>
</tbody>
</table>

This extraction method can be used in both gas chromatography and liquid chromatographic separations. The technology that has been developed allows for the automation of the SPME sampling process and injection into a chromatographic device (Supleco, 2005).

**Bacterial Production of Organo-Chalcogen Compounds**

*Volatile Organo-Sulfur or Organo-Metalloidal Compounds*

Biomethylation of the sulfur, selenium, and tellurium (the chalcogens) has been reported previously (Van Fleet-Stalder and Chasteen, 1998). Fungi and bacteria have been shown to either reduce toxic metalloidal anions to the relatively nontoxic elemental form or to reduce and methylate the metalloid to even more negative oxidation states to produce volatile forms. The earliest systematic reports of this were Challenger's in the 1930s, collected in his seminal 1945 review (Challenger, 1945).
Organosulfur compounds detected in bacterial headspace include MeSH, DMS, DMDS, and DMTS (Ishihara et al., 1995; Stalder et al., 1995). Biogenic, volatile selenium compounds detected include DMSe, DMDSe (Doran, 1982), and DMSeS (Chasteen, 1993; Chasteen, 1998). The only volatile tellurium derivative that has been detected in bacterial headspace is dimethyl telluride (Basnayake et al., 2001); however, analysis of *Acremonium falciforme* headspace has also yielded one report of fungal production of dimethyl ditelluride (Chasteen et al., 1990).

Organisms most sensitive to tellurium and selenium oxyanions are Gram-negative bacteria. The relative toxicity of the common tellurium oxyanions are $\text{TeO}_3^{2-} > \text{TeO}_4^{2-}$ (Scala and Williams, 1963). Selenium oxyanions are less toxic than tellurium oxyanions; however the same toxicity trend still applies; Se(IV) toxicity is greater than Se(VI) (Yu et al., 1997). Since very low concentrations of these anions inhibit the growth of a wide variety of bacterial species, these salts are routinely added to selective culture media for the purpose of isolating certain Se or Te resistant species from natural sources (Shimada, et al., 1990; Zadik et al., 1993).

Naturally occurring organisms (harvested from the environment) that have shown resistance to selenium and tellurium salts include *Pseudomonas fluorescens* K27 (Burton et al., 1987), *Thermus thermophilus* HB8 and *Thermus flavus* AT-62 (Chiong et al., 1988a; 1988b), a group of phototrophic bacteria including *Rhodobacter sphaeroides* 2.4.1 (Van Fleet-Stalder et al., 1997), and *Geobacillus stearothermophilus* V (Vásquez, 1985; Moscoso et al., 1998). These metalloid-resistant bacteria have varying abilities to reduce selenium and tellurium oxyanions [oxidation states: Se(IV), Se(VI), Te(IV), Te(VI)] to the elemental form Se$^0$ and Te$^0$ (Basnayake et al., 2001; Pearion and Jablonski, 1999;
Further reduction of added metalloids to the -2 oxidation state also occurs (Bentley et al., 2002). This has been shown by the detection in the headspace above bacterial cultures of methylated metalloids (Se and Te) having a significant vapor pressure (relatively low boiling points). Organo-tellurides contribute to the garlic odor found around microorganisms which had lengthy exposure to inorganic tellurium compounds (Hansen, 1853), in rodents (Larner, 1995), and the breath of humans (Xu et al., 1997).

Mixed Sulfur/Metalloid Compounds

Challenger (1945), Reamer and Zoller (1980), and Doran (1982) have proposed widely-discussed mechanisms for the reduction and methylation of metalloids. A mechanism for the headspace production of DMSeS has also been proposed (Chasteen, 1993). The formation of this mixed organo-chalcogen involves the bacterial production of DMDSe and then disproportionation with DMDS to form two molecules of DMSeS. Another possible pathway is an exchange reaction of MeSH with DMDSe to form DMSeS and MeSeH (methaneselenol). Previously unreported mixed species (sulfur-metalloid) metabolites and compounds have recently been detected in or above biological sources. Possibly the most unusual occurrence was the detection of the first arsinothioyl metabolite from a biological source. High performance liquid chromatography/mass spectrometry was used to identify 2-methylarsinothioyl acetic acid ((CH$_3$)$_2$As(=S)CH$_2$COOH) in the urine of sheep which had consumed large amounts of arenosugars from seaweed in daily diets (Hansen et al., 2004). To date, methanetellurol (MeTeH), DMDTe, and dimethyl tellurenyl sulfide (DMTeS, CH$_3$-Te-S-CH$_3$) (Klayman and Günther, 1973) have not been detected in bacterial headspace. This last compound,
DMTeS, is analogous to DMSeS which has been detected in bacterial headspace. We have named it based on the nomenclature of Klayman and Günther (1973).

**Trisulfide and Selenodisulfide**

Dimethyl trisulfide plays a key role in the taste of food products at very low concentrations (0.1 ppb in beer, for instance) (Peppard, 1978). One study indicated that a precursor of DMTS production in aged beer was 3-methylthiopropionaldehyde and its reduced form 3-methylthiopropanol. There was also a pH dependence on taste of the aged beer: high pH resulted in an onion-like off-flavor and a low pH enhanced the cardboard flavor (Gijs et al., 2000). DMTS has also been detected as an aroma component of cooked brassicaceous (cabbage family) vegetables. A proposed mechanism (illustrated in Figure 1) of trisulfide formation involves the production of DMDS and its subsequent reaction with pyruvic acid and ammonia. Finally, in this sequence of events, an unstable sulfenic acid is produced and this reacts with hydrogen sulfide to produce the trisulfide. The proposed initial precursor of DMDS production was S-methyl-L-cysteine sulfoxide (Maruyama, 1970).

\[
\text{H}_3\text{CSSCH}_3 + \text{O} \quad \rightarrow \quad 2 \text{H}_3\text{CSOH} + \text{H}_2\text{S} \quad \rightarrow \quad \text{H}_3\text{CSSSCH}_3
\]

**Figure 1.** Proposed mechanism for the formation of dimethyl trisulfide.

DMTS can also be produced by the decomposition of larger dimethyl polysulfides. Dimethyl tetrasulfide decomposes over time into DMTS, dimethyl disulfide, - pentasulfide, and - hexasulfide at 80 °C. In work examining this decomposition, DMTS could be detected right after the start of the experiment (the last
data point collected was at 1828.8 hours). Banfield’s free radical \((N-[1,1\text{-dimethyl }3-(N-oxido \text{phenylimine)}\text{butyl}]-N\text{-phenylaminyloxy})\) was added to see if the decomposition of dimethyl tetrasulfide increased, however, this addition yielded inconclusive results (Pickering et al., 1967). These workers proposed that the possible biological formation of DMTS in cabbage tissues may involve reactions with methyl methanethiosulfinate with hydrogen sulfide or methyl methanethiosulfinate with hydrogen sulfide along with the involvement of C-S lyase on S-methyl-L-cysteine sulfoxide. Chin and Lindsay (1994) also suggested that DMDS and MeSH are also produced using variations of this mechanism in biological systems containing methyl methanethiosulfinate or methyl methanethiosulfonate.

\[
\text{CH}_3\text{SSeSCH}_3
\]

has been variously named as dimethyl 2-selena-1,3-disulfide or dimethyl selenotrisulfide (Ganther, 1971). Chemical abstract service (CAS) naming would be dimethyl bis(thio)selenide (Loening, 1972); however, to be consistent with nomenclature from previous published work with mixed species sulfur/tellurium or selenium compounds (Klayman and Günther, 1973), dimethyl selenodisulfide (DMSeDS) will be used in this work. Selenodisulfides have been used in \textit{in vitro} experiments involving selenium derivatives of glutathione that were reduced to a persulfide analog by glutathione reductase. Those experiments were undertaken to mimic and investigate biochemical processes. Excesses of the reduced form of glutathione (GSH) were reacted with selenious acid [Se(IV)] to form GSSeSG. Reaction of GSSeSG with glutathione reductase forms GSeSH and after this point many pathways for the fate of selenium have been proposed. One pathway, illustrated in Figure 2, involves the formation of reduced GSH and elemental selenium. Another involves the production of hydrogen selenide
which reacts with S-adenosyl-methionine to form dimethyl selenide (Figure 2) (Ganther, 1971). Methanethiol can be substituted in the reaction pathway to form CH$_3$S-SeSCH$_3$ (Figure 2, bottom reaction) (Painter, 1941).

Figure 2. Illustrations of mechanisms proposed in Ganther, 1971 and Painter, 1941.

Another study of selenodisulfide involved the addition of selenious acid to a ribonuclease (RNase) which contained eight thiol (-SH) groups. The oxidized form of this enzyme has four sulfur-sulfur linkages; the reduced form has free thiol groups and is inactive. The reaction of selenious acid with RNase involves the formation of a selenodisulfide (R-S-SeS-R) linkage within the enzyme. The enzymatic activity of both the oxidized native form (R-SS-R) and the selenodisulfide form have been compared at various pH values and the affect of the selenodisulfide linkage reduced the enzymatic activity by a factor of ~100 (Ganther and Corcoran, 1969).
Sequential Anaerobic/Aerobic Conditions for a Facultative Anaerobe

A facultative anaerobe is an organism that can switch its metabolism from anaerobic grown in the absence of oxygen to aerobic growth when oxygen is present (Chapelle, 2000). Very little work has been published about the oxic/anoxic conditions that affect facultative anaerobes which have become resistant to selenium. Until recently (Hapuarachchi et al., 2004), no work had been published that determined the differences in metalloid conversion by facultative anaerobes under differing gas purge conditions; however, alternating purge cycles or aerobic conditions have been used to optimize color removal of synthetic dyestuffs (Kapdan et al., 2003; Isik and Sponza, 2003; Wong and Yuen, 1996) and to degrade hydrocarbons (Grishchenkov et al., 2000; Stephenson et al., 1999; Zitomer and Speece, 1993). The headspace purging experiments undertaken here were meant to fill in that gap.

Genetic Modification of Organisms for Mechanistic Studies

Efforts have been made to understand the detoxification mechanisms of toxic selenium and tellurium salts by metalloid-resistant cells along with the biochemical and genetic basis of that resistance. These efforts have resulted in finding tellurium-resistance (Te\(^R\)) determinants on plasmids and bacterial chromosomes and have revealed a diversity of structure and organization of the involved genes (Taylor, 1999; Walter and Taylor, 1992). In order to identify any genetic material that contributes to the tellurium resistance of the organism, the genome of the resistant bacteria is cleaved with an endonuclease, one of the enzymes that hydrolyzes the interior phosphodiester bonds of a nucleic acid. These enzymes recognize specific DNA sequences and cleave at a particular point within the nucleotide sequence for which the enzyme recognizes (Nelson
and Cox, 2000). An example is the *Hind*III restriction endonuclease that recognizes the DNA sequence of AAGCTT (5’ to 3’) and then cleaves the DNA at the A-A nucleic acid residues. Once the restriction enzyme cleaves the DNA at all of the sites at which the proper sequence is present, PCR (polymerase chain reaction) can be used to amplify specific DNA fragments. Once PCR is completed these fragments are then ligated to a plasmid vector. A plasmid is a circular, extrachromosomal, independently replicating DNA molecule that is often used in genetic engineering (Nelson and Cox, 2000). An illustration of this process is found in Figure 3.
Figure 3. Illustration of the restriction enzyme activity on the HindIII plasmid and the ligation of foreign DNA into the plasmid. Electroporation is used to insert the plasmid into the host cell.

The plasmid vector is then transferred by electroporated into cells that lack tellurium resistance. Electroporation is the introduction of a macromolecule into a cell by applying a high-voltage electric pulse that renders the cell membrane permeable to large molecules (Nelson and Cox, 2000). These transformed cells are then grown in the presence of tellurite or tellurate. Colonies that show growth are isolated and the proteins are extracted. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is then used to separate the proteins produced by the tellurium-resistant clone. The SDS-
PAGE of a control (no plasmid is present) and cells with the plasmid present (and tellurium amended) are compared to check for production of any new proteins. Detection of the proteins is usually accomplished by using a dye to stain the location of the separated proteins in the gel. If there is overproduction of a protein from the tellurium-amended culture compared to the control, the protein band, on the gel, is extracted and amino acid sequencing takes place. This step allows for the determination of the RNA and DNA genetic sequence from which this protein was coded. The sequence can then be submitted to a genetic library where comparisons can be made between different organisms that may share the same genetic sequence for this protein (Araya et al., 2004; Tantaleán et al., 2003).

**Relevance of this Research**

This research involves experiments that look into the production of methylated chalcogens from genetically modified *E. coli*. The effect of the oxidation state of both selenium and tellurium on headspace production was also studied. Gas chromatography-sulfur chemiluminescence detection and gas chromatography-mass spectrometry were used to identify organo-sulfur, -selenium, and -tellurium compounds produced by these recombinant bacteria. Recombinant cultures that contain different inserted open reading frames (ORFs) were grown and amended with selenium or tellurium salt in order to determine if the function of the added genetic material promoted tellurium or selenium resistance and the biomethylation of these metalloids as measured by volatile organo-metalloidal production. Mixed aerobic/anaerobic conditions were also studied to see if there were improvements/changes in the bioreduction of selenium. Dissolve oxygen
content was monitored and the switching from aerobic to anaerobic culture conditions was examined.
Chapter 2
Experimental

Part 1: Aerobic/Anaerobic Bioreactor Experiments

Reagents

All reagents were used without further purification. Sterile tryptic soy broth (1 \% w/v; DIFCO Laboratories; Detroit, MI, USA) with 0.3 \% (w/v) nitrate in a 2.7 L glass bioreactor (BioFlow III batch/continuous fermentor; New Brunswick Scientific; Edison, NJ USA) was inoculated with a liquid culture of *Pseudomonas fluorescens* K27 previously grown aerobically for 48 h (10\%/vol. inoculum). Within a few minutes of inoculation, sterile solutions of sodium selenate (Na$_2$SeO$_4$) or sodium selenite (Na$_2$SeO$_3$; Aldrich Chemicals; Milwaukee, WI USA) were added to produce predetermined, initial oxyanion concentrations in the bioreactor. Nitrate, which acts a terminal electron acceptor during anaerobic growth for this microbe, was present at initial 0.3\% concentrations in all experiments.

Dissolved Oxygen Monitoring

Dissolved oxygen (D.O.) was determined using an Ingold polarographic dissolved oxygen probe calibrated using sequential nitrogen and air purging (degassing method) as specified by the bioreactor’s manufacturer (New Brunswick Scientific BioFlo III manual). The range that this process reports (0\% to 100\%), therefore, is percent saturation level based on air (Aguiar Jr. et al., 2002). The absolute concentration of dissolved oxygen was checked using a handheld, dissolved oxygen instrument (Model 55; YSI Inc.; Yellow Spring, OH USA) calibrated in air.
Strictly anaerobic bioreactor runs were purged continuously with $\text{N}_2$: 50 mL technical grade $\text{N}_2$/min, passed through a 1-µm sterile filter (Nalgene; Rochester, NY USA), into a purge tube terminating at the bottom of the reactor vessel. The pressure of the reactor vessel was held constant at ~1 atm. Sequential anaerobic/aerobic runs were alternatively purged with $\text{N}_2$ (always 50 mL/min ±10%) or sterile air, initially at 50 mL min$^{-1}$. In later experiments, 250 mL/min air purges were carried out. The liquid cultures were incubated at 30°C with constant mixing (200 rpm) for 72 h. Though growth rates varied with oxyanion amendment concentrations, even 10 mM amended cultures (the slowest growing) reached stationary phase after approximately 50 hours based on optical density measurements using absorbance/scattering at 526 nm (Van Fleet-Stalder and Chasteen, 1998; Yu et al., 1997).

**Part 2: Analysis of Headspace Gases Produced by Genetically-Modified** *E. coli*

**Reagents**

The following reagents were used without further purification. Dimethyl disulfide ($\text{CH}_3\text{SSCH}_3$), dimethyl diselenide ($\text{CH}_3\text{SeSeCH}_3$), sodium tellurite ($\text{Na}_2\text{TeO}_3$) sodium selenite ($\text{Na}_2\text{SeO}_3$), and sodium selenate ($\text{Na}_2\text{SeO}_4$) from Aldrich Chemical (Milwaukee, WI, USA). Ampicillin, acetonitrile, and DL-dithiothreitol (DTT) was purchased from Sigma (St. Louis, MO, USA). Dimethyl telluride ($\text{CH}_3\text{TeCH}_3$) and dimethyl ditelluride ($\text{CH}_3\text{TeTeCH}_3$) were obtained from Organometallics, Inc. (East Hampstead, NH, USA). Certified A.C. S *Plus* hydrochloric acid, sulfuric acid, and certified zinc metal dust were obtained from Fisher Scientific (Houston, TX, USA). 'Baker Analyzed' sulfuric acid was obtained from J.T. Baker (Phillipsburg, NJ, USA).
Bacterial growth

*G. stearothermophilus* V was from the collection of Dr. Claudio C. Vásquez of the Universidad de Santiago de Chile and grown as described (Vásquez, 1985). *E. coli* JM109 (*endA1, recA1, gyrA96, thi, hsdR17 (rK-, mK+), relA1, supE44, Δ(lac-proAB), [F', traD36, proA+B+, lacIqZM15]) was from Promega (USA). Cells were grown in LB medium (10 g/L tryptone, 5 g/L yeast extract and NaCl, pH = 7) (Sambrook et al., 1989) at 37° C. When appropriate, ampicillin (100 μg/mL) was added to the medium. The selenium amendment (0.01 mM or 0.2 mM selenate or selenite) or tellurium amendment (0.01 mM or 0.2 mM tellurite or tellurate) was added to a 10% inoculum which had grown for 24 hours. The Se or Te amendment solutions came from sterile filtered (0.2-μm pore size) solutions. Sampling occurred 48 hours after the amendment was added.

Genetic manipulations

*E. coli* 1VH was isolated as one of the recombinant tellurite-resistant clones upon transformation of tellurite-sensitive *E. coli* JM109 cells with a gene library of *G. stearothermophilus* V. The library was constructed using HindIII restriction endonuclease and pSP72 cloning vector (Promega) as described earlier (Vásquez et al., 2001; Tantaleán et al., 2003).

Synthesis of mixed tellurium/sulfur compounds

Chemical reduction of disulfides in the presence of either diselenide or ditelluride was carried out to make mixed tellurium/sulfide or selenium/sulfide compounds (Noller, 1965; Virtue and Lewis, 1934; Singh and Kats, 1995). A 1.0 M solution of HCl or H₂SO₄ was prepared and 1.0 mL was transferred to 16 mm x 125 mm borosilicate glass test tubes. Twenty-five μL of liquid of the appropriate organo-metalloid were added to the
aqueous acid in the tube. After the addition of the organo-metalloid, ~60 mg of zinc metal were added and the tubes were immediately capped with open-top screw caps with Teflon®/silicon liners (Alltech, Deerfield, IL, USA). Headspace sampling, to observe the chemical products of this reaction, took place over a period stretching to as long as 24 hours. A second reduction procedure using dithiothreitol (DTT) involved the addition of 25 µL dimethyl disulfide and 25 µL dimethyl ditelluride to 1.0 mL of 5.0 mM DTT (Singh and Kats, 1995) followed immediately by capping as before. Headspace sampling took place over a 24 hour span.

**Headspace sampling**

*Gas syringe*

The collection and sampling of static headspace above bacterial cultures were accomplished by using a manual, gas syringe with a locking valve used to keep the sample gas enclosed and a 21-gauge needle (2.5 cm length; VICI, Baton Rouge, LA, USA). The volume of the gas syringe was 1 mL. Gas syringes used in this study were cleaned at 50°C in a 2-L enclosed, glass vessel purged with nitrogen (Van Fleet-Stalder and Chasteen, 1998; Zhang and Chasteen, 1994). Contamination/carry over by the syringe was checked by injecting 1 mL of laboratory air into the gas chromatograph; all lab air blanks showed no chromatographic peaks above detection limits. Syringes were cleaned in this manner before use in GC/MS also. The sampling of small gas volumes of headspaces above reaction mixtures carried out in test tubes was accomplished using a 10-µL liquid GC syringe (Alltech).

Using the 1.0-mL gas syringe, the Teflon/silicon liners of the caps were pierced with the syringe needle and 1 mL of bacterial headspace was collected. The locking valve
was closed, allowing for a gas tight seal. The syringe was transferred to the hot injector of
the GC, the locking valve was opened, and the 1 mL injection made. The 10-μL syringe
was used in a similar manner; however, gas volumes of 2, 5, and 10 μL were collected
from the reaction headspace of the test tubes. The smaller volume syringes had no
locking valve system.

**Solid phase micro extraction (SPME)**

SPME fibers of 75 μm (thickness) carboxen-polydimethylsiloxane (CAR-PDMS)
were purchased from Supelco (Bellfonte, PA, USA). SPME was used to concentrate the
headspace gases in order to bring the concentration of the analyte gases into a detectable
range for GC/MS analysis. Larger volume bacterial cultures (250–900 mL) incubated in
Schott® Flasks using a specially-designed enclosure cap for liquid bacterial cultures
(Stalder et al., 1995) were used in order to increase the concentration of headspace gases
for both manual syringe and SMPE collection methods. The SPME absorption times used
ranged between 15 and 60 min. SPME collected samples were also analyzed by GC with
fluorine-induced chemiluminescence detection.

**Gas chromatography with fluorine-induced chemiluminescence detection (SCD)**

The gas chromatograph used for SCD analysis was a Hewlett Packard Model
5890 Series II. The capillary column used was DB-1 with a 5.0-μm chromatographic film
(30 m x 0.32 mm i.d., 100 % dimethyl polysiloxane film) from J&W Scientific (Folsom,
CA, USA). With this column, cryogenic trapping is not required because of the ability of
the thick film to trap and separate low boiling point compounds even at relatively high
initial chromatographic program temperatures. The detector was a sulfur
chemiluminescence detector Model 300 (Ionics Instruments, Boulder, CO, USA). This
detector uses gas-phase fluorine ($F_2$) to induce chemiluminescence by its reaction with organo-metalloid compounds. The analog signal from the detector was processed by a Hewlett Packard 3396 Series II integrator. Helium was the carrier gas with a flow rate of 1 mL/min. The GC injector temperature was 275° C and the initial oven temperature was 30° C. All samples were analyzed using splitless injection. The initial temperature was held for 2 min and then ramped to 250° C at 15 degrees/min. The final temperature was held for 1 minute. Syringe checks were run using a ramp of 15 degree/min from 30 to 225° C.

Calibration and gas phase concentrations

When necessary a calibration curve (Linear Least Squares, LLS) was created in order to calculate the gas phase concentration (ppbv) of the organo-metalloids for which standards were available. Standards used were concentrated forms of the compound of interest. One in forty-one dilutions (1:41, 25 μL of previous dilution added to 1 mL solvent) were used in order to create calibration standards that fell within the linear range of the instrument. The dilution consisted of taking 25 μL of the standard and adding it to 1 mL of acetonitrile. Further serial dilutions, in a small-volume glass vial (5 mL), involved taking 25 μL of that diluted standard and adding it to 1 mL of acetonitrile. Other dilution ratios were also used in order to create concentrations that fell within the linear range of the instrument. A minimum of 5 standards were used for each LLS plot. Calibration standards were only used for GC-SCD analysis. Calibration using SPME was not performed. To find the gas phase concentration of an organo-chalcogen in the headspace above bacteria, a headspace gas sample was injected into the GC and a signal was produced by the SCD corresponding to the organo-chalcogen (see above). The
intensity of this signal (a numerical value, integrated peak area) was correlated with the known standards by the LLS equation. The injected mass (in picograms) of the organo-chalogen was known at this point for the 1 mL of headspace injected into the gas chromatograph. Headspace concentrations were reported in gas phase mixing ratios (parts per billion by volume, ppbv) assuming ideal gas conditions but at the temperature of the sample’s headspace.

Gas chromatography/mass spectrometric analysis

A Hewlett Packard 5890 gas chromatograph coupled with a Hewlett Packard 5973 Mass Selective Detector (90-eV electron impact) was used for MS analysis. Ultra high purity helium was the carrier gas with a flow rate of 1 mL/min. The chromatographic column used was an Agilent DB-624 (1.4-µm film thickness, 30-m length; Agilent Technologies, Palo Alto, CA USA). Because of the chromatographic column used, liquid nitrogen was required as a cryogen to achieve substantially lower than ambient initial oven temperatures. Various temperature programs were used for the GC/MS runs; an example chromatographic program was -20° C degrees initial temperature for 3 min then 5 degrees/min to a final temperature of 250° C. All injections were splitless. SPME was used for all GC-MS results reported here. All mass spectra reported were reproduced from extracted data collected from the gas chromatograph-mass spectrometer.
Chapter 3

Data and Results

Part 1: Aerobic/Anaerobic Bioreactor Experiments

*Pseudomonas fluorescense* K27 was amended with 1 mM selenite in a 3.0-L bioreactor and was exposed to alternating aerobic/anaerobic cycling. Figure 4 shows the % saturation of dissolved oxygen and the concentration of oxygen (mg/L) in the liquid phase of the bacterial culture over time. The bacteria were allowed to grow for 72 hours. The alternating cycles were as follows: 12 hours of nitrogen then 6 hours of air purging at 50 mL/minute for both gases. Figure 5 shows data from the same bacteria with the same parameters except the flow rate of the gases was 250 mL/minute. The concentration of oxygen in the liquid media was determined by measuring the percent saturation of dissolved oxygen and comparing that with the concentration of dissolved oxygen provided from a dissolved oxygen probe.
Figure 4. Alternating anaerobic/aerobic purge cycling in a 1 mM selenite amended culture of *P. fluorescens* K27. The alternating cycles were 12 h N\textsubscript{2} then 6 h air purging at 50 mL/min.
Figure 5. Alternating anaerobic/aerobic purge cycling in a 1 mM selenite amended culture of *P. fluorescens* K27. The alternating cycles were 12 h N\textsubscript{2} then 6 h air purging at 250 mL/min.
Part 2: Analysis of Headspace Gases Produced by Genetically Modified *E. coli* Amended with Tellurium Salts

Recombinant bacteria were amended with both tellurite and tellurate salts with final solution phase concentrations of 0.01 mM or 0.2 mM. The cultures were grown into stationary phase, 48 hours. Headspace gases were sampled from the *E. coli* clone 1VH. Figure 6 shows the fluorine-induced sulfur chemiluminescence chromatogram of the headspace gases collected by gas syringe from a 2.7-L culture amended with 0.01 mM tellurite while Table 2 shows the retention times of various organo-chalcogen compounds and the gas phase concentration of compounds for which there were available standards. Gas chromatography-mass spectrometry was used to confirm the identity of compounds produced by these bacteria. The GC/MS total ion chromatogram is shown in Figure 7 that duplicates the growth conditions from Figure 6. Figure 7 shows the production of CH$_3$TeCH$_3$, CH$_3$SSCH$_3$, and CH$_3$SSSCH$_3$ for a similar culture. Previously unreported compounds of interests are CH$_3$TeH (from GC-SCD, Figure 6) and CH$_3$TeSCH$_3$ and CH$_3$TeTeCH$_3$ (from GC/MS, Figure 7). The mass spectrum of CH$_3$TeTeCH$_3$ is shown in Figure 8 and the mass spectrum of CH$_3$TeSCH$_3$ is shown in Figure 9.

The chromatogram of the headspace gases collected from the solution phase reaction of CH$_3$SSCH$_3$ and CH$_3$TeTeCH$_3$ with sulfuric acid and zinc metal is shown in Figure 10. This reaction was used to produce CH$_3$TeSCH$_3$ in order to establish a GC-SCD retention time and to estimate the boiling point of CH$_3$TeSCH$_3$. The boiling point of DMTeS was estimated by comparing the boiling point and retention times of other well-known organo-chalcogen compounds and using their linear least squares relationship to approximate CH$_3$TeSCH$_3$’s boiling point based on that compound’s retention time. The LLS plot is shown in Figure 11.
Table 2. Retention times, boiling points, and gas phase concentrations of organo-chalcogen compounds that are found in the headspace above recombinant *E. coli* cultures.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Formula</th>
<th>Boiling Point (°C)</th>
<th>Retention Time (min)</th>
<th>Headspace Concentration at 48 h (ppbv)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanethiol</td>
<td>CH$_3$SH</td>
<td>6</td>
<td>2.28</td>
<td>—</td>
</tr>
<tr>
<td>Methanetellurol</td>
<td>CH$_3$TeH</td>
<td>57</td>
<td>5.61</td>
<td>—</td>
</tr>
<tr>
<td>Dimethyl telluride</td>
<td>CH$_3$TeCH$_3$</td>
<td>83</td>
<td>7.38</td>
<td>94</td>
</tr>
<tr>
<td>Dimethyl disulfide</td>
<td>CH$_3$SSCH$_3$</td>
<td>110</td>
<td>8.75</td>
<td>1300</td>
</tr>
<tr>
<td>Dimethyl tellurenyl sulfide</td>
<td>CH$_3$TeSCH$_3$</td>
<td>161</td>
<td>11.81</td>
<td>#</td>
</tr>
<tr>
<td>Dimethyl trisulfide</td>
<td>CH$_3$SSSCH$_3$</td>
<td>170</td>
<td>12.62</td>
<td>—</td>
</tr>
<tr>
<td>Dimethyl ditelluride</td>
<td>CH$_3$TeTeCH$_3$</td>
<td>196</td>
<td>14.09</td>
<td>#</td>
</tr>
</tbody>
</table>

— Standards for these compounds are not commercially available.
# These compounds were only detected with SPME.
Figure 6. Fluorine-induced chemiluminescence chromatogram of 1 mL of headspace gas from a 2.7-L culture of *E. coli* 1VH amended with 0.01 mM tellurite.
Figure 7. Reconstructed total ion chromatogram of SPME extract of headspace gas from a 250-mL culture of *E. coli* 1VH amended with 0.01 mM tellurite.
Figure 8. Mass spectrum of GC/MS peak at 24.69 min, dimethyl ditelluride from the sample, analyzed in Figure 5.
Figure 9. Mass spectrum of GC/MS peak at 19.96 min, dimethyl tellurenyl sulfide from the sample analyzed in Figure 5.
Figure 10. Fluorine-induced chemiluminescence chromatogram of headspace above an aqueous solution of H$_2$SO$_4$ + Zn + DMDS + DMDTe.
Figure 11. Linear least squares plot comparing compounds’ GC retention times in a standard temperature program to their boiling point. This plot can be used to determine the boiling point of a compound that has a known retention time.
Part 3: Analysis of Headspace Gases Produced by Genetically Modified *E. coli* Amended with Selenium Salts

BLASTn analysis (Altschul et al., 1990) of the nucleotide sequence of the cloned insert allowed the identification of three main open reading frames (ORFs) of 780, 600 and 399 bp which exhibited the same transcription orientation. The predicted protein products of these ORFs were most similar to a *Bacillus megaterium* uroporphyrin-III C-methyltransferase (71% identity/85% similarity) (Raux et al., 1998), *Bacillus anthracis* A2012 UbiE methyltransferase (63% identity/78% similarity) (Read et al., 2002), and a *Bacillus megaterium* BtuR protein (60% identity/80% similarity) (Raux et al., 1998), respectively. The nucleotide sequence of both strands of the 3,824 bp *Hind*III chromosomal DNA fragment from *G. stearothermophilus* V has been deposited in the GenBank database under GenBank Accession Number AY426747.

Recombinant bacteria were amended with both selenate and selenite. *E. coli* 1VH was grown for 48 hours and headspace analysis was performed. Figure 12 shows the SCD chromatogram of the headspace gases produced when 1VH was amended with 0.2 mM selenite. Cultures were grown in 16-mL tubes with a culture volume of 10 mL. This organism produced MeSH, DMS, DMSe, DMDS, DMSeS, DMDSe, DMTS, and DMSeDS. Figure 13 shows the chromatogram of the same organism but amended with 0.2 mM selenate. The headspace gases produced were the same as the gases produced by the selenite-amended cultures. GC/MS was used to confirm the identity of the compounds found in Figure 13 and the mass spectrum of DMTS is shown in Figure 14 and the mass spectrum of DMSeDS is shown in Figure 15. One-liter Schott flasks were used for the bacterial growth.
Figure 12. Fluorine-induced chemiluminescence chromatogram of 1 mL of headspace gas from a 10-mL culture of *E. coli* 1VH amended with 0.2 mM selenite.
Figure 13. Fluorine-induced chemiluminescence chromatogram of 1 mL of headspace gas from a 10-mL culture of *E. coli* 1VH amended with 0.2 mM selenate.
Figure 14. Mass Spectrum of dimethyl trisulfide from *E. coli* cultures amended with 0.2 mM selenate or selenite. SPME was used as the extraction method for GC/MS.
Figure 15. Mass Spectrum of dimethyl selenodisulfide from E. coli cultures amended with 0.2 mM selenate or selenite. SPME was used as the extraction method for GC/MS.
Part 4: Analysis of Headspace Gases Produced by Genetically-Modified *E. coli* Amended with Both Selenium Salts and Tellurium Salts

In order to investigate whether dimethyl tellurenyl selenide (DMTeSe) would be produced by this bacterium a reasonable experiment to explore this would be to amend this metalloid resistant bacterium with both selenium *and* tellurium salts. 1VH cultures were amended with either 0.2 mM selenite and tellurite or 0.2 mM selenate and tellurate. Figure 16 is the SCD chromatogram of a 1VH culture amended with 0.2 mM selenite and tellurite. The bacteria produced MeSH, DMS, DMSe, DMDS, DMSeS, DMDSe, and DMTS. Figure 17 shows the SCD chromatogram of a 1VH culture amended with 0.2 mM selenate and tellurate. The gases produced by this organism were MeSH, DMS, DMSe, DMTe, DMDS, DMSeS, DMDSe, and DMTS. The reaction of DMDTe and DMDSe with sulfuric acid and zinc was used to produce DMTeSe. Gas chromatography was used to separate the headspace products and the mass spectrum of DMTeSe is found in Figure 18.
Figure 16. Fluorine-induced chemiluminescence chromatogram of 1 mL of headspace gas from a 10-mL culture of *E. coli* 1VH amended with 0.2 mM selenite and tellurite.
Figure 17. Fluorine-induced chemiluminescence chromatogram of 1 mL of headspace gas from a 10 mL culture of *E. coli* 1VH amended with 0.2 mM selenate and tellurate.
Figure 18. Mass spectrum of DMTeSe from the reaction of DMSe and DMTe with sulfuric acid and zinc metal.
Chapter 4

Discussion

Part 1: Aerobic/Anaerobic Bioreactor Experiments

This work involved an effort to switch the facultative anaerobe *Pseudomonas fluorescense* K27 back and forth between anaerobic and aerobic metabolism by changing the oxygen content in gases purged through liquid cultures. Various purge cycles were attempted but usually involved a complete cycle of 18 or 24 hours made up of 2 to 1 anaerobic to aerobic purge times. Data in Figure 4 show the extent of oxygen saturation during alternating anaerobic/aerobic purging of a 1 mM selenite-amended culture with 50-mL purge rates for alternating nitrogen and air cycles. While the N\textsubscript{2} purge cycles were clearly successful from the point of view of establishing anaerobic conditions, only the initial air purge cycle (hours 12-18, Figure 4) achieves what appears to be O\textsubscript{2} saturation. And even during that first air purge cycle as the bacterial growth moved into lag phase, this low air purge rate does not supply enough oxygen to establish complete aerobic growth (at least as measured by D.O.). This temporal microaerobic condition (Zeng and Deckwer, 1996; Stephenson et al., 1999) is even more obvious in the second and third air purges (Figure 4) where the bacterial population has become so large that only approximately 5 to 10% of O\textsubscript{2} saturation is achieved.

The execution of 250-mL aerobic purge rates runs (Figure 5) was our response to the probability that bacterial metabolism was not significantly being switched to aerobic growth in the 50-mL/min air runs detailed above. The choice of the amount of the higher purge rate of 250 mL also had a more mundane explanation: The liquid cultures’ headspace foam production increased with gas purge rate and 250 mL/min was the most
we could purge the cultures without foam—and entrained culture components—being swept out of the culture headspace via the purge exit tubing. We did not try antifoam agents as a means to decrease the foam production in these experiments (Burschäpers et al., 2002).

The determination of the establishment of aerobic conditions for a significant amount of the time during aerobic runs was clear from the D.O. readings which hovered around 0% during nitrogen purging and achieved approximately 100% during the 250 mL/min air purging cycles. Our handheld D.O. meter reported these as 0.15 mg/L and 6.8 mg/L dissolved O$_2$ respectively (Figure 4 and Figure 5). Though the lower reading of this meter probably has significant error, the 6.8 mg/L O$_2$ at 100% saturation is reasonable given the ionic strength of our medium (YSI meter manual, United State Geological Society, 2005). While completely aerobic conditions were established for the first two aerobic purge cycles—which covers almost all the log phase for this microbe under these conditions—the third aerobic purges apparently only approached 50% O$_2$ saturation. The final air purge cycle’s O$_2$ content moved higher, probably because death phase was well under way and the cells were lysing. And while the average yield of elemental selenium was higher in these runs than any of the other conditions, the increase was not statistically significant, again at neither 95% nor 90% confidence levels (Hapuarachchi, 2002).

In an effort to compare the effects of strictly anaerobic and sequential anaerobic/aerobic conditions, a microaerobic study was achieved as well. During the later part of the aerobic low air purge rate runs (Figure 4 after 30 hours) and possibly the last purge cycle in the high air purge runs (Figure 5), conditions of substantially less than 100% oxygen saturation were achieved. Even given our poorly responding D.O. probe
which displayed O$_2$ content above 100% saturation, the general trends are still clear: microaerobic conditions were present during a substantial part of the low purge and later part of the high air purge runs (Isik and Sponza, 2002). Only the 250-mL min$^{-1}$ purge rates achieved substantial aerobic growth during the early log phase, and therefore the high air purge rate runs represent mixed anaerobic/aerobic and microaerobic conditions (Figure 5).

**Part 2: Analysis of Headspace Gases Produced by Genetically-Modified *E. coli* Amended with Tellurium Salts**

The highly reduced compounds methanetellurol and dimethyl tellurenyl sulfide were synthesized as described in Materials and Methods. Reduction reactions were carried out with two different reducing agents 1) Zn + HCl or H$_2$SO$_4$ (Noller, 1965; Virtue and Lewis, 1934) or 2) dithiothreitol, DTT (Singh and Kats, 1995):

$$\text{CH}_3\text{SSCH}_3 + \text{CH}_3\text{TeTeCH}_3 + \text{HCl} + \text{Zn} \rightarrow \text{CH}_3\text{SH} + \text{CH}_3\text{TeH} \rightarrow \text{CH}_3\text{TeSCH}_3$$

$$\text{CH}_3\text{SSCH}_3 + \text{CH}_3\text{TeTeCH}_3 + \text{DTT} \rightarrow \text{CH}_3\text{SH} + \text{CH}_3\text{TeH} \rightarrow \text{CH}_3\text{TeSCH}_3$$

The formation of CH$_3$TeSCH$_3$, when using HCl and Zn, requires an oxidant after DMDS and DMDTe are reduced. Oxygen located in the headspace of the reaction mixture is the source of the oxidation (1 mL solution phase, 15 mL gas phase). The results of these experiments allowed us to identify the retention times of compounds for which there are no commercially available standards. Table 2 lists all of the organometalloids determined in this work along with their retention times in the chemiluminescence chromatography. While the Zn/acid reduction experiments (HCl or H$_2$SO$_4$) produced the expected products as detected by injections of the sample headspace gas from the reaction, dithiothreitol apparently did not have enough reducing power in the aqueous system we employed to yield the mixed S/Te compound we were trying to synthesize.
However, experiments using DTT as a reducing agent did yield detectable amounts of dimethyl sulfide, dimethyl telluride, dimethyl disulfide, and trace amounts of dimethyl ditelluride in the headspace above the reaction mixture, but no CH$_3$TeSCH$_3$. Detection of DMS and DMTe could be explained by the mechanism by which DTT reduces DMDS and DMDTe which is different from the mechanism when HCl and Zn are used as the reducing agents.

Figure 10 displays the chromatogram of the fluorine-induced chemiluminescence headspace above a stronger reducing mixture: H$_2$SO$_4$/Zn with DMDS and DMDTe added. Methanetellurol (CH$_3$TeH) was apparently so unstable in this system—or oxidized so quickly upon sampling—that its gas phase presence was below detection limits (although its retention time is indicated in Figure 10 based upon previous samples); however, the product of methanethiol and methanetellurol in the presence of oxygen, CH$_3$TeSCH$_3$, is clearly present. SPME sampling of the H$_2$SO$_4$/Zn reaction headspace produced extensively overloaded chromatography for DMDS peaks (data not shown); however, methanetellurol was clearly detected. For some of the headspace components commercially available standards allowed us to determine their gas phase concentrations (reported in parts per billion by volume, ppbv, at the temperature of the headspace of the culture of 37°C) for samples analyzed by injection of a known volume followed by GC with fluorine-induce chemiluminescence detection. For the mixed metalloid/sulfur species we averaged the appropriate integrated response of the dimetalloidal compound with the dimethyl disulfide compound and used that estimate to determine the headspace concentration. Response (calibration sensitivity) is defined by the slope of the calibration
curve in the linear range. Table 2 also shows the concentrations of these compounds in the headspaces of *E. coli* 1VH cultures amended with 0.01 mM sodium tellurite.

These *E. coli* cells are able to grow in the presence of low millimolar concentrations of tellurite and tellurate (Araya et al., unpublished results). Within a few hours of tellurite amendment, the culture darkens as elemental Te is generated via bioreduction; analogously, selenium reducers like *P. fluorescens* K27—amended with selenite—produce red, elemental Se (Hapuarachchi et al., 2004). In addition, reduction and methylation also lead to organo-Te production and the release of these relatively volatile compounds in the culture headspace. Figure 6 shows the fluorine-induced chemiluminescent chromatogram of 1.0 mL of headspace gas collected in the headspace of *E. coli* 1VH grown into stationary phase and 48 hours after amendment with 0.01 mM sodium tellurite. Chromatographic peaks were identified using the retention time of known standards for DMTe and DMDS and GC/MS (using SPME sampling). DMTS was identified via GC/MS. Detection of organo-sulfur compounds in bacterial headspace is common in the cultures of metalloid-resistant microorganisms that have been examined in the past (Van Fleet-Stalder and Chasteen, 1998; Stalder et al., 1995; Chasteen, 1993). Methanetellurol was tentatively identified via its retention time, its boiling point (correlated to its retention time) and the reduction experiments described below. CH₃TeH, DMTe, or DMDTe were not detected in the headspaces of Te-free cultures. Wild type *E. coli* amended in identical control experiments did show some growth but produced only organo-sulfur species; no organo-tellurium could be detected because the wild type bacteria did not contain the genetic material to reduce and methylate tellurium.
The mass spectra of many of the metabolic products found in the headspaces of *E. coli* are widely available in mass spectral data bases. Spectra of organo-sulfur compounds may be found online, for example (NIST, 2003); however, the organo-metalloidal compounds are much less common and so the most unusual are included here. Figure 7 is the reconstructed total ion chromatogram (Sparkman, 2000) of the SPME extracted headspace (~150 mL) above 250 mL of 1VH culture at 37°C. The SPME extraction time was 45 min. For this run the GC/MS temperature program was slowed down to 3°C/min. CH₃TeCH₃ has been found earlier as a component in bacterial and fungal headspace (Basnayake et al., 2001; Fleming and Alexander, 1972) but CH₃TeTeCH₃ has been reported in fungal headspace only in a single report (Chasteen et al., 1990). The experiments with recombinant *E. coli* reported here have provided detectable amounts of DMTe (Figures 4 and 5) and DMDTe (Figure 7, mass spectrum found in Figure 8) using either 1 mL gas sampling or SPME. Figures 8 and 9 are the mass spectra of dimethyl ditelluride (peak at 24.69 min) and dimethyl tellurenyl sulfide (peak at 19.96 min) respectively. This latter compound, CH₃TeSCH₃, a mixed tellurium/sulfur molecule, has not been reported in the literature. The SPME technique concentrates gas components as they are collected, allowing detection of dimethyl tellurenyl sulfide and increasing injected on-column amounts for GC/MS analysis (Figure 7). Based on its chromatographic retention time, the boiling point of CH₃TeSCH₃ can be estimated to be 161°C (Figure 11). Recently a CAS registry number has been assigned for this compound, 762268-67-7 with a CAS name of methanesulfentelluroic acid, methyl ester.

The mass spectrum of dimethyl tellurenyl sulfide (Figure 9) shows an isotope cluster around 160 m/z (158, 160, 162) corresponding to $^{126}\text{Te}^{32}\text{S}$, $^{128}\text{Te}^{32}\text{S}$, and $^{130}\text{Te}^{32}\text{S}$. 
This cluster excludes the possibility of confusing this mass spectrum with that of 
CH₃TeO₂CH₃, dimethyl tellurone, a mistake made in earlier work with the selenium analog to CH₃TeSCH₃, CH₃SeSCH₃, which has also been detected in bacterial headspace (Chasteen, 1998; Reamer and Zoller, 1980). Dimethyl tellurone would have an isotope cluster of fragment ions at 157, 159, and 161 corresponding to CH₃¹²⁶Te¹⁶O, CH₃¹²⁸Te¹⁶O, and CH₃¹³⁰Te¹⁶O, and these are not present. This volatile headspace molecule elutes with an approximate boiling point of 161°, again reasonably precluding the possibility of its being dimethyl tellurone. Dimethyl sulfone's boiling point (this is the sulfur analog to the tellurone) is 238°; dimethyl selenone's melting point is 153°.

Proceeding down the periodic table in this same group, it's reasonable to assume (CH₃)₂TeO₂ has a boiling point substantially above 161°C, and this therefore acts as a further means of eliminating it as the compound that has been detected here. Dimethyl tellurone's mass spectrum has not been reported in the literature, and the compound has not been successfully synthesized; however, an earlier worker in this research group attempted this synthesis using microwave-induced oxidation with sodium periodate or classical solutions phase oxidatation using 3-chloroperoxybenzoic acid as the oxidant (Akpolat, 1999).

Also interesting was the detection of the very reduced molecule methanetellurol, CH₃TeH (Figure 6) [tellurium oxidation state of -2, Te(-2)]. Although the selenium analog of this molecule, methaneselenol, CH₃SeH [Se(-2)], has been detected in microbial headspace in earlier work with the fluorine-chemiluminescence detector surveying headspaces of selenate-amended bacterial cultures of *Citrobacter freundii*, *Pseudomonas aeruginosa*, and *Pseudomonas cepacia* (now *Burkholderia cenocepacia*)
(Chasteen, 1990), headspace examination work that has been carried out subsequently has not detected that compound. *E. coli* 1VH appears to be able to reduce and methylate sulfur and tellurium along the entire oxidation continuum from methanethiol to dimethyl trisulfide (individual sulfur oxidation state varies) and from methanetellurol to dimethyl ditelluride [Te(-1)]. Careful examination of even 60 min SPME extractions using GC/MS did not yield detection of dimethyl tritelluride; however, the boiling point of this compound, while not reported in the literature, is estimated to be approximately 287°C, given the predictable trends in this alkylated metalloid family. At 37°C the gas phase concentrations of compounds with this sort of low volatility might preclude their detection even if they were present in bacterial culture. Solution phase sampling was not undertaken in this project.

While fluorine-induced chemiluminescence detection of *E. coli* 1VH headspace, carried out using a 1.0-mL headspace gas injections, did detect CH₃TeH, GC/MS runs based on the longest sampling-time SPME extractions showed no detectable amounts of methanetellurol. In fact, 1-mL headspace gas injections produced no detectable amounts of metalloid-containing compounds at all at the concentrations present after 72 hr of growth; this is the reason why SPME was used for the GC/MS chromatography. The response of our GC/MS is, approximately, 15 times less sensitive than our chemiluminescent system for the compounds under study (ratio of GC/MS detection limit to chemiluminescence detection limit, calculated as peak producing 3 times the noise, 3S/N). Therefore if easily oxidizable CH₃TeH were microbially produced in the reducing conditions of the anaerobic culture, SPME extraction of GC/MS detection—which necessarily exposed the collection fiber to atmospheric oxygen during transfer to the GC
injector—might not show detectable amounts of CH$_3$TeH. The oxidation product of methanetellurol is dimethyl ditelluride (Tarbell, 1961; Irgolic, 1974), and this might in part explain the source of DMDTe in these cultures headspaces. Since both methanetellurol and DMDTe were detected in headspace gas samples sampled via SPME but methanetellurol was only found in 1-mL headspace samples which were not exposed to atmospheric oxygen, the CH$_3$TeH to DMDTe oxidation probably plays some part in DMDTe presence in these bacterial headspaces. DMDTe was never detected in headspace samples without using the SPME fiber. The fluorine-induced chemiluminescence detection limit for DMDTe (3S/N) is 350 ppbv in a 1-mL gas injection (Swearingen Jr. et al., 2004).

The source of CH$_3$TeSCH$_3$ in bacterial headspace is also almost certainly either by direct produced by bacterial metabolism or by interaction of reduced and slightly more oxidized tellurium and sulfur metabolic compounds in liquid culture or headspace gas. Metathesis (Guryanova, 1970; Killa and Rabenstein, 1988) or displacement processes (Kostiner et al., 1968) could both yield CH$_3$TeSCH$_3$ in these cultures (Chasteen, 1993; Noller, 1965):

**Metathesis**

\[
\text{CH}_3\text{SSCH}_3 + \text{CH}_3\text{TeTeCH}_3 \rightleftharpoons 2\text{CH}_3\text{TeSCH}_3
\]

**Displacement**

\[
\text{CH}_3\text{SSCH}_3 + \text{CH}_3\text{TeH} \rightleftharpoons \text{CH}_3\text{TeSCH}_3 + \text{CH}_3\text{SH}
\]

\[
\text{CH}_3\text{TeTeCH}_3 + \text{CH}_3\text{SH} \rightleftharpoons \text{CH}_3\text{TeSCH}_3 + \text{CH}_3\text{TeH}
\]
Since all these chemical species are present in the amended-culture solution and
headspace, their original sources are hard to determine. In addition and despite the great
efforts made for decades to unveil the mechanism(s) of bacterial tellurite resistance, there
are still few satisfactory explanations for it. In this context, some housekeeping genes
have been found that seem to play a rather unspecific role in protecting the cell against
tellurite damage (Vásquez et al., 2001; Tantaleán, 2003; Araya et al., unpublished
results).

In this work it has been determined that genes coding for functions associated with
ubiquinone (ORF 600) and vitamin B\textsubscript{12} biosynthesis (ORF 780 and ORF 399), which are
vitally important in the redox structure of cell metabolism, are responsible for the
evolution of the volatile tellurium derivatives identified here, and these Te-containing
compounds are released into culture headspace and can be determined by gas phase
sampling using gas syringe and/or solid phase microextraction followed by GC/F\textsubscript{2}-
induced chemiluminescence or GC/mass spectrometry. Figures 6 and 7 are
chromatograms displaying the presence of those volatiles in 1VH headspace. 1VH was
the clone which contained ORF 600, ORF 780, and ORF 399. Since tellurite
detoxification mechanisms do not exclude or support the participation of specific
methyltransferases that catalyze the evolution of tellurium gaseous compounds, the
results presented here strongly support such a mechanism; thus 1VH generates the less-
toxic volatile Te derivatives. Others have reported on the importance of methyl
transferases in tellurite resistance (Cournoyer et al., 1998; Liu et al., 2000) and thiopurine
methyltransferase has been specifically implicated (Cournoyer et al., 1998; Warner et al.,
1995); however, Te-resistance of a Te-resistant *E. coli* strain (TehB) is not always
accompanied by the production of detectable amounts of volatile Te-containing compounds (Liu et al., 2000).

Experiments were designed to see if there was a statistical difference between the organo-tellurium production of cells amended with different oxidation states of tellurium, tellurite and tellurate. Ten samples of 0.01 mM tellurite-amended and ten samples of 0.01 mM tellurate-amended *E. coli* JM109 with ORF 600 (ubie methyltransferase) were grown independently for 48 hours. Nine out of ten tellurate-amended cultures produced detectable headspace amount of DMTe with an average headspace concentration of 68 ppbv DMTe. The detection limit (3S/N) for our detector system is 19 ppbv DMTe. No organo-tellurium was detected above cultures that were amended with tellurite. Therefore the reduction and methylation mechanism expressed in this clone appears to be specific to tellurite and not to tellurate (Araya et al., 2004).

**Part 3: Analysis of Headspace Gases Produced by Genetically-Modified *E. coli* Amended with Selenium Salts**

As described above, *E. coli* 1VH cultures were also amended with 0.01 mM and 0.2 mM of either sodium selenite or sodium selenate. Figures 12 and 13 show the headspace chromatograms of cultures of 1VH amended with 0.2 mM selenite (Figure 12) and 0.2 mM selenate (Figure 13). Organo-sulfur compounds detected were MeSH, DMS, DMDS, and DMTS and these compounds eluted in order of increasing boiling point given the chromatographic column’s stationary phase. Organo-selenium compounds that were detected include dimethyl selenide and dimethyl diselenide.

Dimethyl selenenyl sulfide, a mixed selenium/sulfur compound, was also detected above both types of Se-amended cultures. An unknown chromatographic peak was detected in the headspace gases of both cultures at late elution times and therefore high
compound boiling point. It was theorized that this was a result of the presence of dimethyl selenodisulfide (CH$_3$SeSSCH$_3$). This was based on the late elution of the peak and its proximity to the DMTS peak in both the chemiluminescence and MS chromatography, always after DMTS (whose boiling point is 170 °C). Gas chromatography/mass spectrometric analysis was used in order to identify the unknown chromatographic peak. 1-mL gas injections used with the chemiluminescent system proved too low in concentration for our GC/MS (all organo-chalcogen concentrations were below GC/MS detection limits for 1-mL gas injections), and therefore SPME was used to concentrate the sample in order to bring the concentration of the analytes above the poorer detection limits of the GC-MS. Dimethyl selenenyl sulfide, DMDS, and DMTS were detected using GC/MS analysis (data not shown). Lower boiling point compounds such as methanethiol and dimethyl selenide were not substantially retained on the GC/MS chromatographic column because of the temperature program’s relatively high initial temperature. In fact the GC-MS ionization filament was not even turned on until these components had eluted.

Figure 14 contains the mass spectrum of DMTS, the compound eluting at 19.4 min in the total ion chromatogram (not shown) of a 1VH culture amended with selenium. This is presented for comparison to the mass spectrum of the subsequent compound which eluted at 23.9 min in that chromatography. Interpretation of the DMTS mass fragments are as follows: The $m/z$ 126 is the molecular ion CH$_3$SSSCH$_3^+$. The $m/z$ 111 fragment represents CH$_3$SS$^+$; $m/z$ 79 is the CH$_3$SS$^+$ fragment; $m/z$ 64 is SS$^+$; and $m/z$ 45 is the CHS$^+$ fragment. There is a $m/z$ 47 ion, most probably CH$_3$S$^+$. Figure 15 is the mass spectrum of the peak eluting after DMTS in both SCD and GC/MS.
chromatography, dimethyl selenodisulfide. Fragments at $m/z$ 174 and 172 represent the molecular ions of $\text{CH}_3\text{SS}^{80}\text{SeCH}_3^+$ and $\text{CH}_3\text{SS}^{78}\text{SeCH}_3^+$ respectively and their relative intensities mimic the relative abundance of these Se natural abundances. $^{80}\text{Se}$ (49.82%) and $^{78}\text{Se}$ (23.52%) are the two most abundance isotopes of selenium (CRC Handbook). Fragments at $m/z$ 93 and 95 are logically $\text{CH}_3^{78}\text{Se}^+$ and $\text{CH}_3^{80}\text{Se}^+$. Note that these $m/z$ 93 and 95 fragment intensities do not match the natural, relative abundance of selenium isotopes as do the $m/z$ 174 and 172 molecular ions, that is, the relative intensities do not mimic the Se isotopic abundance. An explanation of this can be that the stability of the $\text{HC}≡^{80}\text{Se}^+$ fragment is greater than that of $\text{CH}_3^{80}\text{Se}^+$. Fragments $\text{HC}≡^{78}\text{Se}^+$ and $\text{CH}_3^{78}\text{Se}^+$ are not shown in the mass spectrum to confirm this trend. A similar trend can be found in the $\text{CH}_3\text{S}^+$ fragment which has a $m/z$ of 47, however the most prominent spectral peak found in that region of the spectrum is $m/z = 45$ which is the fragment $\text{HC}≡\text{S}^+$.

The presence of the very prominent $m/z$ 79 fragment in the mass spectrum of this compound (Figure 15) is the first bit of evidence that the structure of this compound can be designated as $\text{CH}_3\text{SeSSCH}_3$ as opposed to $\text{CH}_3\text{SSeSCH}_3$; however, we feel there’s more: the mixed Se/S compound bis(methylthio)selenide, $\text{CH}_3\text{SSeSCH}_3$, has been synthesized and its mass spectrum recorded (Cai et al., 1994), and there are significant differences between that published spectrum for $\text{CH}_3\text{SSeSCH}_3$ and that of Figure 15. Most importantly, if the compound eluting after DMTS in the bacterial headspace sampled in the work reported here were in fact $\text{CH}_3\text{SSeSCH}_3$ our mass spectrum would—based on the published spectrum—display fragments at $m/z$ 144 and $m/z$ 127. These correspond, respectively, to $\text{S}^{80}\text{Se}^+$ and $\text{CH}_3\text{S}^{80}\text{Se}^+$ and these are not seen in the mass spectrum in Figure 15. And while Cai et al.’s spectrum of $\text{CH}_3\text{SSeSCH}_3$ displays both of
these, their m/z 144 peak is quite small and so might be below the intensity threshold in our spectrum in this trace analysis. The three heteroatom fragment is also missing in the mass spectrum of DMTS (Figure 15) taken with our GC-MS and in the mass spectrum of DMTS published on the NIST website (2003). SSS⁺ has a m/z of 96, a fragment which is absent from both mass spectra of DMTS. Therefore the presence or absence of m/z 144 in our spectrum sheds no light on this discussion. However, the CH₃S⁸⁰Se⁺ m/z 127 ion is quite prominent in the published spectrum (~70% of molecular ion intensity) and completely missing from our spectrum in Figure 15. Table 3 shows the difference in the mass spectrum of this work and that of Cai et al. The fragments are listed in order of most intense to least intense.

Table 3. Comparison of the Mass Spectrum of CH₃SeSSCH₃ in Figure 15 with the Mass Spectrum of CH₃SSeSCH₃ from Cai et al.

<table>
<thead>
<tr>
<th>Mass Spectrum of CH₃SeSSCH₃</th>
<th>Mass Spectrum from Cai et al.</th>
</tr>
</thead>
<tbody>
<tr>
<td>m/z / Relative Intensity</td>
<td>Fragment</td>
</tr>
<tr>
<td>174 / 100</td>
<td>CH₃⁸⁰SeSSCH₃⁺</td>
</tr>
<tr>
<td>45 / 67</td>
<td>HCS⁺</td>
</tr>
<tr>
<td>79 / 59</td>
<td>CH₃SS⁺</td>
</tr>
<tr>
<td>172 / 37</td>
<td>CH₃⁷⁸SeSSCH₃⁺</td>
</tr>
<tr>
<td>93 / 24</td>
<td>HC⁸⁰Se⁺</td>
</tr>
<tr>
<td>95 / 21</td>
<td>CH₃⁸⁰Se⁺</td>
</tr>
<tr>
<td>47 / 18</td>
<td>CH₃S⁺</td>
</tr>
<tr>
<td>64 / 16</td>
<td>SS⁺</td>
</tr>
</tbody>
</table>
CH$_3$SSeSCH$_3$ was synthesized (Cai et al.) by reacting N-chlorobenzotriazole with dimethyl selenide. The product was later reacted with methanethiol to form CH$_3$SSeSCH$_3$. In Table 3 there are fragments of CH$_3$SSCH$_3^+$ ($m/z$ 94) and CH$_3$SS$^+$ ($m/z$ 79) from the Cai et al. mass spectrum. The logical origin of these fragments comes from the rearrangement of CH$_3$SSeSCH$_3$ within the MS; therefore these fragments are relatively low in intensity in that compound’s spectrum. Further rearrangement is also seen with the presence of CH$_2$SCH$_3^+$ at $m/z$ 61 again a lower intensity. From the Cai et al. mass spectrum there is no evidence of a carbon atom bonded to a selenium atom; however, the mass spectrum in Figure 15 does show evidence of a carbon bonded to selenium. This helps to confirm our belief that the compound that was detected eluting after DMTS is dimethyl selenodisulfide with the structure CH$_3$SeSSCH$_3$.

The GC-chemiluminescence chromatogram and GC/MS total ion chromatogram were also compared in an effort to estimate the boiling point for dimethyl selenodisulfide. A linear least squares (Figure 11) analysis was performed using data from both chromatograms using the boiling points of the known analytes and their retention times as the opposing axes. Based on the LLS plot, the boiling point of CH$_3$SeSSCH$_3$ estimated from the GC/MS chromatogram was 187º C, while the boiling point from the GC-chemiluminescence chromatogram was 192º C, a reasonable correlation given different columns and temperature programs. The compound reported in Cai et al. eluted with the boiling point of 203º C based on comparisons of known compounds with established boiling points and retention times from their chromatogram. Since the estimated boiling points of CH$_3$SSeSCH$_3$ and CH$_3$SeSSCH$_3$ are different by 11º C, the difference in structure could account for this. Further, to evaluate whether this process
was valid, the boiling point of dimethyl trisulfide was extrapolated from the Cai et al. chromatogram and did not differ from the reported boiling point of 170° C (Klason, 1887). The estimated boiling point of DMTS from our GC/SCD was ~171° C. This establishes that the boiling point of CH$_3$SSeSCH$_3$ is different from the boiling point of CH$_3$SeSSCH$_3$ and not an artifact of the chromatography. This phenomenon can be seen while comparing boiling points of phenol derivatives that have the same empirical formula but with different ortho, meta, and para positions (Bowman and Stevens, 1950).

Since the instrument’s response to this compound was very near the detection limit of the GC-MS instrument, more work was carried out in order to increase the concentration of DMSeDS in the headspace gases above the recombinant cultures. This was achieved by heating cultures to higher temperatures before SPME sampling to increase headspace concentrations. When the temperature of the culture extraction was raised to 70° C, a higher concentration of DMSeDS was indeed detected based on the increase of signal from the total ion chromatogram; however, the mass spectra from these runs did not reveal any additional spectral peaks to help confirm the identity of this compound.

Molecular modeling was used to determine if there was any property of the molecules that would explain the difference in boiling point between CH$_3$SeSSCH$_3$ (192° C) and CH$_3$SSeSCH$_3$ (203° C). Optimized geometries for CH$_3$SeSSCH$_3$ and CH$_3$SSeSCH$_3$ were calculated using the Spartan Pro (Wavefunction, Inc.) computational software package. These geometries were determined by Semi-Empirical (PM3) methods and for each, the molecules dipole was derived by the software. The boiling point of a compound is related, in part, to the polarity (dipole) of that compound (Wade
Jr., 1999). The calculated polarity of CH$_3$SeSSCH$_3$ was 0.805 debye and the polarity of CH$_3$SSeSCH$_3$ was 1.35 debye. This difference in polarity can be attributed to the symmetric structure of CH$_3$SSeSCH$_3$. Because of the symmetry involved, the individual dipole moments within that compound do not cancel each other out. In the case of CH$_3$SeSSCH$_3$, an un-symmetric structure, individual dipole moments at the C-S bond and the S-Se bond partially cancel each other out resulting in a lower overall dipole (polarity) of the compound.

*E. coli* 1VH cultures amended with either 0.2 mM sodium selenite or selenite produced DMSeDS (4 cultures at each amendment concentration). Gas phase concentrations could not be calculated for DMSeDS because there is no available standard; however, peak area and relative standard deviation (RSD) between the 4 replicates can be determined (a statement of the reproducibility of the headspace analysis). The RSD of DMSeDS found above selenite amended cultures was 9.2%, and RSD of DMSeDS found above selenate amended cultures was 34.1%. This difference in variability can attributed to slight differences of bacterial growth within the culture tubes which can affect the overall production of volatile compounds being released into the headspace.

When comparing selenite-amended cultures with selenate-amended cultures the oxidation state of the metalloid did not affect the production of organo-selenium compounds in the headspace. Figure 12 and Figure 13 show the chemiluminescence chromatogram of headspace gases produced by *E. coli* 1VH which contains the genetic material for ubie methyltransferase plus two other enzymes. DMSe, DMDSe, DMSeS, and DMSeDS were detected in all cultures no matter what the oxidation state of selenium
was. Selenate-amended cultures along with the selenite-amended cultures were each independently grown with a total of 4 tubes for each oxidation state. All 8 culture tubes that were analyzed produced volatile organo-selenium compounds. A repeat of these experiments support these findings.

**Part 4: Analysis of Headspace Gases Produced by Genetically-Modified E. coli Amended with Both Selenium and Tellurium Salts**

Similar to methods mentioned above, *E. coli* 1VH cells were amended with both selenium and tellurium salts. Figure 16 and Figure 17 show the chemiluminescence chromatograms from headspaces of cultures amended with 0.2 mM selenite and tellurite (Figure 16) and 0.2 mM selenate and tellurate (Figure 17). Figure 16 shows the volatile selenium compounds DMSe, DMDSe and DMSeS. Figure 17 shows the production of DMSe, DMTe, DMDSe, and DMSeS. The objective of this experiment was to see if this modified organism could produce DMTeSe. If this compound had a similar response to the detector as DMDSe, the detection limit of DMTeSe in the headspace would be approximately 25 ppbv. DMDTe calibration could not be used to estimate the detection limit of DMTeSe because of the low purity and degradation of the available standard. Based on the data shown, no DMTeSe was produced by the organism in these mixed Se/Te amendments: however, since the boiling point of DMTS is 170° C and the boiling point of DMTeSe was estimated to be 173°C by analyzing the headspace of the reaction mixture of DMDSe, DMDTe, Zn, and sulfuric acid, the retention times of DMTS and DMTeS would be very close and that could cause there to be very little base line separation, i.e., possibly overlapping chromatographic peaks. In both Figures 16 and 17 DMTS is detected with little evidence to support the possibility of the close elution of DMTeSe. A sign would be two peaks seen together but the space in between those two
peaks would not be base line separated. Figure 18 shows the mass spectrum of synthetic DMTeSe from the reaction of DMDSe and DMDTe with sulfuric acid and zinc metal. This spectrum, along with the spectrum of biologically produced DMTeS (Figure 9), is included as a reference for future research in order to identify DMTeSe in headspace gases of biological cultures. Future research could involve creating a more favorable environment in order for the bacteria to produce the mixed selenium/tellurium species.

Mixed selenium and tellurium amendments still continue with the same metalloid oxidation state trends as established earlier with individual chalcogens amendment. Cultures amended with the +4 oxidation state of both selenium and tellurium produced DMSe, DMDSe, and DMSeS (Figure 16). Cultures amended with the +6 oxidation state of selenium and tellurium produced DMSe, DMTe, DMDSe, and DMSeS (Figure 17). This correlates with previous data showing that tellurate-amended cultures of this organism do produce DMTe while tellurite-amended cultures do not produce any DMTe (Araya et al., 2004). Amendments using both oxidation states of selenium not surprisingly produced a variety of organo-selenium compounds.
Chapter 5

Conclusions

*Pseudomonas fluorescens* K27 was grown under sequential aerobic/anaerobic growth conditions. During these experiments microaerobic activity was seen in the low purge runs and the last aerobic cycle of the high purge run. The high purge runs did create a mixed aerobic/anaerobic environment.

Methanetellurol, dimethyl tellurenyl sulfide, and dimethyl ditelluride were identified above genetically-modified bacterial cultures of *E. coli*. These compounds have not been previously detection in the headspace above bacterial cultures.

Tellurate-amended cultures expressing the gene for ubie methyltransferase produced dimethyl telluride while cultures amended with tellurite did not produce DMTe. Dimethyl selenodisulfide was identified above cultures amended with selenium salts. *E. coli* cultures expressing the ubie methyltransferase amended with both selenate and selenite amendments produced a variety of organo-selenium compounds.

Mixed bacterial amendments of selenite and tellurite only produced DMSe in the headspace gases above *E. coli* 1VH. Mixed amendments of selenate and tellurate produced organo-selenium and organo-tellurium compounds in the headspace gases above *E. coli* 1VH. Dimethyl tellurenyl selenide was not detected above cultures amended with both selenium and tellurium. The successful synthesis and mass spectrum of dimethyl tellurenyl selenide and dimethyl tellurenyl sulfide were reported in this work.
Bibliography


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## Appendix
### Chemical Abstract Service Registry Numbers

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Vita

Jerry Wayne Swearingen Jr. was born on August 19, 1980 in Houston, TX. He graduated from Conroe High School in the spring of 1998. Undergraduate studies began at Sam Houston State University with the initial major of Computer Science. In the Fall of 1999 Jerry changed his major to the ACS certified degree in Chemistry. During the summer of 2001 Jerry began to do research in the analytical chemistry laboratory of Dr. Chasteen. During his time in the lab he has published in *Process Biochemistry*, *Analytical Biochemistry*, and *Journal of Biological Inorganic Chemistry*. Jerry graduated in the spring of 2003 with a B.S. in Chemistry. He immediately began graduate school in the fall of 2003. Jerry received his Masters of Science in Chemistry in August of 2005. During his time at Sam Houston State Jerry taught various freshman, sophomore, and senior level labs. He also presented work at various conferences and meetings such as the American Chemical Society Regional Meeting, Texas Academy of Science, and the Sam Houston State University Graduate Research Exchange.