# Differential analysis of unique genes expressed in *Stenotrophomonas maltophilia* strain OR02 in response to selenite

by

Nathan Moffo

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**Differential Analysis of unique genes expressed in *Stenotrophomonas maltophilia* strain OR02 in response to selenite**

Nathan R. Moffo

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Nathan R. Moffo, Student Date

Approvals:

_____

Dr. Jonathan J. Caguiat, Thesis Advisor Date

_____

Dr. David Asch, Committee Member Date

_____

Dr. Xiangjia Min, Committee Member Date

_____

Dr. Salvatore A. Sanders, Dean of Graduate Studies Date

Abstract

*Stenotrophomonas maltophilia* Oak Ridge Strain OR02 (*S. maltophilia* 02), which was isolated from a heavy metal contaminated site in Oak Ridge, TN, grows in the presences of toxic levels of heavy metals, including selenite. Selenium exists in four different valences as selenate (+6), selenite (+4), elemental selenium (0) and selenide (-2). Selenate ($SeO_4^{2-}$) and selenite ($SeO_3^{2-}$) are soluble, uncolored forms of selenium and elemental selenium forms an insoluble red precipitate. Microbes that use selenium, import it into the cell as selenite, reduce it to selenide and incorporate it into selenocysteine. When environmental selenite concentrations are higher than required, some bacteria reduce it to insoluble elemental selenium and methylate it to form volatile methyl selenide. When *S. maltophilia* 02 is growing in the presence of 0.5 mM sodium selenite, it produces an insoluble red precipitate and a stale garlic odor, which are presumable elemental selenium and methyl selenide, respectively. A subtractive hybridization technique was used in an attempt to identify genes that are expressed in response to high concentrations of selenite. *S. maltophilia* 02 was grown to early log phase and exposed to 0.5 mM selenite. After two hours of selenite exposure, RNA was extracted from untreated and treated cultures. The RNA was converted to double-stranded cDNA and cut with the four-base cutter, restriction endonuclease, *Sau3*AI. After ligating linkers to the digested cDNA, it was amplified by PCR. The PCR reaction on the untreated cDNA was labeled with dCTP and dUTP biotin. The PCR reaction on the treated cDNA did not contain biotin labeled nucleotides. PCR products from both reactions were mixed, heated at 98°C to denature the DNA and allowed to hybridize at 68°C for 24 hours. The hybridization mixture was added to streptavidin magnetic beads and exposed to a magnetic stand to remove the cDNA from RNA that was present under

both the treated and untreated conditions. The remaining cDNA from RNA transcribed in response to selenite was PCR amplified and used in further hybridization and subtraction steps. After 3 rounds of subtraction, the final product was cloned and sequenced. If successful, this method was expected to possibly detect genes for proteins involved in glutathione synthesis and maintenance since many bacteria detoxify selenite using glutathione. It was expected to detect genes for proteins involved in methylation, as methylation is another form of detoxification.

The subtractive hybridization procedure employed to find these unique genes however encountered difficulties in separating rRNA from mRNA, and due to complications from the amount of rRNA in total RNA, no unique genes were identified. If repeated, exhaustive efforts must be made to ensure that rRNA encoding genes are completely removed before sequencing.

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| | |
|---|---|
| mM | milliMolar |
| mg | milligram |
| kg | kilogram |
| µg | microgram |
| ng | nanogram |
| l | liter |
| µl | microliter |

# INTRODUCTION

## Oak Ridge

As part of the Manhattan Project during WWII, four facilities were constructed in Oak Ridge, Tennessee to produce enriched uranium (Widner, 1996). One facility code-named Y-12 utilized an electromagnetic method of enrichment until 1948, then switched to lithium enrichment for the development of thermonuclear weapons. This was achieved by a process called Colex, which involves massive amounts of mercury. Thus, tons of mercury was lost to the nearby East Fork Poplar Creek river and environment. Additionally, waste products from the other facilities were disposed of in poorly constructed unlined impoundments known as the S-3 ponds (Brooks, 2001 and Revis, 1989). The S-3 ponds contained liquid wastes from the uranium operations, primarily acidic uranium nitrate, but also included additional amounts of calcium, magnesium, sodium, potassium, and aluminum. These wastes likely leaked into the East Fork Poplar Creek. In 1983 efforts to treat the site led to the eventual treating, filling of, capping of and paving over the S-3 ponds to make a parking lot.

## *Stenotrophomonas maltophilia*

*S. maltophilia* is a Gram-negative, aerobic, non-fermentative bacterium (Morrison, 1886). It is a member of the *Proteobacteria* phylum, the *Gammaproteobacteria* class, *Xanthomonadales* order, and the *Xanthomonadaceae* family (Swings, 1983). *S. maltophilia* is a common inhabitant in soil, on plant roots, and in aqueous environments. *S. maltophilia* can possess a wide range of resistances to heavy metals, such as mercury (Hg), cadmium (Cd), lead (Pb), zinc (Zn), cobalt (Co), and selenium (Se). This ability has led it to be researched for potential uses in bioremediation

efforts or in biogenesis of nanoparticles and bioactive compounds for industrial use (Mukherjee, 2016). In addition to heavy metals, *S. maltophilia* shows resistance to a wide range of antibiotics, such as beta-lactams, aminoglycosides, carbapenems, and quinolones, and thus may cause nosocomial infections in immunocompromised patients, although its level of invasively is expected to be low. (Pages, 2008 and Falagas, 2008). Genomic analysis has revealed the possession of genes encoding ABC transporters, RND family efflux pumps, and type I, II, IV, V, and twin arginine translocation (Tat) system proteins (Zhu, 2012).

*Stenotrophomonas maltophilia* Oak Ridge Strain OR02 (S02) was isolated from Poplar Creek sediment. This strain of *S. maltophilia* exhibited the ability to grow in the presence of toxic levels of heavy metals and metalloids, using a method of detoxification that converts the metal salts into insoluble products (Revis, 1989, and Holmes, 2009).

**Selenium**

Selenium (Se) is a trace element and like hydrogen (H), oxygen (O), carbon (C), nitrogen (N), phosphorous (P), and sulfur (S) is an essential element to living organisms. Selenium is a metalloid that can exist in four oxidation states: elemental selenium (Se [0]), selenate ($SeO_4^{2-}$ [+VI]), selenite ($SeO_3^{-}$ [+IV]), and selenide ($Se^{2-}$ [-II]). The selenium oxyanions, selenate and selenite, are highly soluble in water, though are the most toxic, while elemental selenium is insoluble (Ranjard, 2002). The oxyanions are capable of being taken up by cells for metabolism and are reduced to less toxic forms. Selenium enters the environment from both geochemical and anthropogenic sources (Fernández-Martínez, 2009). Much of environmental selenium comes from agricultural usage or selenium dioxide produced by burning coal and other fossil fuels, but it is also

naturally occurring in sedimentary rock where it erodes or leaches into the water sources (Winkel, 2012). Microorganisms play an important role in selenium's biogeochemical cycle, as several have the ability to reduce or oxidize selenium species. Selenium has several manufacturing uses such as in the production in glass, electronics, pigments, or certain pharmaceuticals (Kessi, 1999).

Selenium is an essential cofactor in several selenoproteins. These selenoproteins usually have antioxidant functions, due their reactive ability with free radicals (Staicu, 2017). Selenoproteins are found in all three domains of life, but not in all lineages (Oremland, 2004). Selenocysteine is a cysteine analogue that replaces sulfur with selenium and is found in the active site of selenoproteins. Trace amounts of selenium are therefore essential to one's diet. The difference between essential and toxic levels of selenium has a very low margin, in matters of parts per million (ppm) or even less (Lens & Lenz, 2009).

Selenium is toxic for two main reasons. One is due to similar properties it shares with sulfur that allow it to replace sulfur in thiol-containing proteins (Turner, 1998). When selenium replaces a sulfur atom in cysteine or methionine, conformational changes of a protein can occur and lead to loss of function. Areas contaminated with selenium oxyanions such the Kesterson Reservoir in California have shown selenium pollution can lead to bioaccumulation in the food web that eventually lead to deformations and death in fish and waterfowl (Nancharaiah, 2015). The second reason is due to sulfur-containing proteins that react with selenite that can create reactive oxygen species (ROS). These reactive oxygen species harm the cell in several ways including damaging DNA/RNA, proteins, and lipids (Kessi, 2004). Biotic reduction of selenium oxyanions is an

increasing area of research, as a method to detoxify polluted environments as well as to produce industrially useful selenium nanospheres to be used in emerging nanotechnology (Pearce, 2008).

## Metabolism

### Import

It has been proposed that some bacteria utilize permeases, specifically the sulfate permease CysA, to transport selenate or selenite into the cell (Turner, 1998). In this model, selenite competes with sulfate for entry, which can lead to a reduced effect of selenite's toxicity when sulfate is present. In a paper by Jasenec *et al* (2009), concentrations of 40 mM selenite and 1 mM sulfate appears to have led to increased transport of selenite through the sulfate transport system into the cells. This pathway has been shown to be inhibited by the presence of L-cysteine in the medium. L-cysteine was revealed to provide selenite resistance by inhibiting the expression of sulfate transport genes (Jasenec). In *E. coli* however, inhibiting this system has been proved to be insufficient to prevent selenite entry, indicating another pathway must be used. Studies have shown in plants, algae, and fungi that phosphate's presence inhibits selenite uptake, possibly indicating a role phosphate transporters may have (Lazard, 2010). Using an inhibiter of nitrite uptake in *S. maltophilia* strain SEITE02, a decrease in selenite reduction was observed, suggesting selenite enters the cell via the nitrite absorption pathway (Antonioli, 2007). Research in specific selenite transporters is still inconclusive, but a reported protein, DedA, observed in *R metallidurans* is indicative to be either a direct transporter or helper (Ledgham, 2005). In *E. coli* a gene identified as *gutS* was shown to be upregulated by selenite. *GutS* shares homology and structure to membrane-

located permeases and transport proteins, suggesting it serves a role in selenite assimilation, although its exact role is not clear (Guzzo, 2000). Gonzalez-Gil *et at.* (2016) suggests outer membrane porin proteins have a role in transport into the periplasm.

## Reduction

Several pathways for selenite reduction have been proposed. These include: 1) via Painter-type reactions 2) a thioredoxin-reductase system 3) siderophore-mediated reduction 4) sulfide-mediated reduction and 5) dissimilatory reduction. Depending on the species and amount of selenium, the microbial reduction of selenite is used in the following processes: assimilation into selenocysteine, dissimilation by reductases under anaerobic conditions to generate energy, or as detoxification. Regardless, when selenium oxyanions are reduced to elemental selenium, they can be exported out of the cell in the form of selenium nanoparticles (SeNPs), or further reduced to selenide. In the periplasm, reductases (such as quinone and flavin oxidoreductases) may work to reduce selenite, but at excessive amounts, selenite will enter the cytoplasm. Here reduction is mediated mainly by thiols, which is discussed later.

## Anaerobic Respiration

Several bacteria, such as *Bacillus selenitireducens* and *Shewanella oneidensis*, have been observed to grow anaerobically through dissimilatory reduction of selenite (Stolz, Oremland,1999). Alcohols, sugars, organic acids, and more can be used as electron donors to create metabolic energy (Eswasyah, 2016). Reductases of anaerobic respiration like nitrite reductase and sulfite reductase are active in selenite reduction in certain bacteria and have been studied in *S. oneidensis* MR-1. *Stenotrophomonas*

*maltophilia* has not been observed to use selenite in anaerobic respiration (Dungan, 2003). Dissimilatory reductions can sometimes reduce selenium species all the way to selenide. The sulfate-reduction pathway is one proposed method that reduces selenium oxyanions to selenide, but additional pathways are hypothesized. Selenide has additionally been observed to have been produced by selenodiglutathione's reaction with the enzymes glutathione reductase of yeast and thioredoxin reductase of mammals, though in *E. coli*, reduction was achieved via selenodigluthione's reaction with thioredoxin, which, now oxidized, gets reduced by thioredoxin reductase (Turner, 1998).

Detoxification Reduction

As mentioned previously, *S. maltophilia* has yet been observed to possess the capability to use selenite as a terminal electron acceptor during anaerobic growth. This indicates that selenite reduction is likely a method of detoxification. The use of reductases is not limited to anaerobic respiration and may additionally be used in detoxification reduction. Several enzymes catalyzing selenite reduction to elemental selenium have been studied. These include the aforementioned nitrite reductase and sulfite reductase but additionally include the enzymes glutathione reductase, arsenate reductase, fumarate reductase, and hydrogenase I, which have shown selenite reduction capabilities (Antonioli, 2007). Pathways involved in sulfur metabolism have also been proposed to be used in selenite reduction (Kessi, 2005).

Experiments by Antonioli *et al*. with the *S. maltophilia* strain SeITE02, a highly Se-resistant strain, have shown that glutathione plays a major role in the selenite reduction mechanism while also showing that the enzyme nitrite reductase may not be involved. Antonioli estimates ninety percent of initial selenite (0.5 mM) added was

transformed by glutathione. Reduction occurs in the cytoplasm, where NADPH and glutathione are readily available. An initial test that involved cell exposure to nitrite before being exposed to selenite showed no difference in pre-induced and non-induced growth, indicating nitrite reduction is independent from selenite reduction, which is relatively uncommon in selenite reducing bacteria. To find glutathione's impact on selenite reduction, a glutathione inhibitor, S-n-butyl homocysteine sulfoximine (BSO), was initially supplemented in the culture and selenite was added during various growth curve phases. When selenite was added before cells entered the log phase, reduction rates plummeted. Interestingly, the inhibitor did not lead to a decrease in selenite reduction when selenite was added in either the log phase or stationary phases of grown, possibly meaning the cell can metabolize selenite by the time it is in the log phase.

Once inside the cell, selenite rapidly reacts with glutathione or other -SH groups in peptides of proteins. In proteobacteria, glutathione is the most abundant thiol and thus is utilized most frequently. Originally proposed by Painter, then modified by Ganther, and again modified by Kessi and Hanselmann, a pathway utilizing glutathione (GSH) is believed to be the major process in selenite detoxification. This pathway outlined by Kessi *et al.* is described below. Once selenite enters the cell, it reacts with GSH spontaneously without the need for enzymes to produce selenodiglutathione (GS-Se-SG). This step leads to a rapid increase of $O_2^-$:

$$6\ GSH + 3\ SeO_3^{2-} \rightarrow 3\ GS\text{-}Se\text{-}SG + 2\ O_2^- + 5\ H_2O$$

The superoxide ($O_2^-$) then undergoes a cascade of degradation reactions catalyzed by superoxide dismutase, catalase, peroxidase, and likely cytochrome(s), whose products include oxygen and hydrogen peroxide. This is done to limit oxidative damage caused by

free radicals. The intermediate selenodiglutathinone acts as a substrate to glutathione reductase which reduces it into a selenopersulfide ($GS\text{-}Se^-$) in the following reaction:

$$3GS\text{-}Se\text{-}SG + 3NADPH \rightarrow 3GSH + 3GS\text{-}Se^- + 3NADP^+$$

Finally, the selenopersulfide then dismutases into $Se^0$, due to its instability:

$$3GS\text{-}Se^- + 3H^+ \rightarrow 3GSH + 3Se^0$$

The production of toxic superoxide anions is a side effect of reduction by this method. As previously mentioned, the cell will synthesize superoxide dismutase and catalase to protect itself from oxidative stress, however free radicals still may form and cause cellular damage.

Proteomic analysis showed that selenite exposure led to significant modulation of proteins involved in cell division, oxidative stress, cell wall synthesis, damaged-protein catabolism, and synthesis of carbohydrates, amino acids, and nucleotides (Antonioli *et al*.). Their findings support this method of reduction that leads to the production of superoxide anions, triggering cellular damage response. Specific induced proteins included 1) catalase, which converts hydrogen peroxide to oxygen and water, 2) a glutamate-cysteine ligase precursor, which is involved in the biosynthetic pathway of glutathione, and 3) glutathione synthase. The presence of these enzymes suggest that selenite reduction generates ROS which triggers a cellular response to the free radical formation.

When glutathione reductase is not present or is at its maximum saturation, abiotic degradation of selenodiglutathione occurs with oxidized gluatathione (GSSG) and selenopersulfide created as products:

$GS\text{-}Se\text{-}SG + GSH \Leftrightarrow GS\text{-}Se^{-} + H^{+} + GSSG$

Because reduced glutathione is not being continuously regenerated by glutathione reductase, GSSG build up slows down the reaction rate. In the absence of antioxidative enzymes like SOD, selenodigluathione reacts with the superoxide anion, $O_2^{-}$, as shown in the following equation:

$GS\text{-}Se\text{-}SG + 2\ O_2^{-} + 4\ H_2O + H^{+} \rightarrow GS\text{-}Se^{-} + GSH + 4\ H_2O_2$

This buildup of hydrogen peroxide will lead to macromolecule damage if antioxidant protections are not available, leading to cell death.

Methylation

Methylation is a part of assimilatory metabolism, since selenide is the form of selenium that is incorporated, and the methylated forms of selenium, dimethyl selenide and dimethyldiselenide, contain selenide. (Nancharaiah, 2015)

In addition to reduction, methylation is a method that microbes use to detoxify selenite. Methylated selenium species are volatile and therefore diffuse from the bacterium's environment. Methylation of selenium species is associated with a strong stale, garlic-like odor. There are several methylation pathways that transform selenite into volatile compounds, such as dimethyl selenide (DMSe) and dimethyl diselenide (DMDSe). Methyltransferase appears to use S-adenosylmethionine (SAM) as a methyl donor. This reaction has been shown to be catalyzed by a bacterial thiopurine methyltransferase (bTPMT) and by a tellurite methyltransferase (TehB). The first pathway of methylation was proposed by Challenger (1944), which involves two reduction steps and DMSe as a final product. In this pathway, selenite is first methylated,

then ionized, and reduced (forming intermediate products including methaneselenonic acid and the ion of methaneseleninic acid) methylated again (forming dimethyl selenone), and finally undergoes reduction to form DMSe. Modifications were made to this pathway to account for other final products such as DMDSe and dimethyl selenone, which were additionally observed as final products in selenite-contaminated areas. In the modified pathway, selenite is initially hydrolyzed before being methylated and reduced to form an intermediate, a methanselenic ion, which is either methylated to form DMSe or is reduced to form DMDSe. An alternate pathway to Challenger has been proposed by Doran (1982) in which selenite is completely reduced to selenide, then methylated to give DMSe. Dungan *et al*. (2003) was able to determine volatile Se forms produced by *Stenotrophomonas maltophilia* by collecting gas samples above cultures grown on TSA plates containing selenite. Expectedly the alkylselenides DMSe, DMSeS (dimethyl seleneyl sulphide), and DMDSe were detected, with DMSe and DMDSe as the most abundant.

*The metK* gene

*MetK* is the gene that encodes SAM synthetase. SAM synthetase converts L-methionone and ATP into S-adenosyl-L-methionone (SAM), an essential metabolite. SAM has a vital role in methyl transfer reactions, serving as the major methyl donor, but additionally has use as a source of methylene groups, amino groups, ribosyl groups, and aminopropyl groups (Fontecave, 2004). SAM can additionally be utilized in the production of glutathione (Perez-Leal, 2011). Deletion of *metK* has shown to be lethal in *E. coli*, which lacks the ability to take up SAM from the environment (Zhao, 2015). As

previously discussed, *S. maltophilia* methylates selenium during detoxification. Thus, selenite is proposed to induce an increased expression of *metK.*

Incorporation into amino acids

As previously mentioned, cells utilize selenium to create selenomethionine and selenocysteine. Incorporation is believed to occur via two mechanisms: a specific path, and a nonspecific path. Before incorporation, selenite must be reduced to selenide. The specific transport pathway has been defined by Lacourciere *et al.* (2002) in which selenium is directly incorporated in polypeptide chains as selenocysteine. This is directed by a UGA codon in association with the genes, *selA*, *selB*, *selC*, and *selD*. Once selenide is available, SelD catalyzes a reaction between selenide and ATP. The product is selenophosphate, which SelA uses to convert serine to selenocysteine. Selenocysteine is carried by SelC, a modified tRNA, to SelB which inserts the amino acid at a UGA stop codon. In a nonspecific pathway of incorporation, selenium can become unintentionally substituted for sulfur. This probably occurs only when the concentration of selenium oxyanions is higher than the concentration of sulfur oxyanions. The sulfate transporter is probably more specific for sulfate, but when there is more selenite than sulfate, selenite is imported and unintentionally incorporated into cysteine and methionine using the sulfate pathway. This is responsible for selenomethionine's synthesis and is another way of selenocysteine synthesis.

Export

Reduced selenium species not methylated or assimilated following reduction into $Se^0$ will either accumulate within the cell and form nanoparticles or be secreted.

Elemental selenium particles found outside the cell are due to either release by cell lysis, or through a membrane efflux pump being located near membrane-associated reductases (Tomei, 1995)

These nanoparticles are often characterized as being spherical or ovoid, and between 10-400 nm in dimension (Tan, 2018). SeNPs are defined as being photoelectric, semiconducting, X-ray-sensing, and possessing antimicrobial activity. (Lampis, 2017). In the extracellular space, they increase in size and number over time. A bacterium identified in 2003 by Dungan *et al.* as *Stenotrophomonas maltophilia* isolated from seleniferous agricultural evaporation pond sediment was found to produce Se deposits about 270 nm in diameter. These deposits were found near the cell surface and free in the medium. EDX spectroscopy showed that the Se deposits also contained calcium and potassium, whose significance is unknown. This testing however did not determine if there were any intracellular deposits of selenium, but Dungan determined that selenium oxyanions were reduced either on the outer cell membrane or in intracellularly, followed by expulsion, but did not cause cell lysis.

As previously mentioned and observed in SeITE02, SeNPs appeared primarily in the extracellular space and in the cytoplasm after a delay in selenite reduction. Since reduction occurs in the cytoplasm, a method of extracellular transport is sought. The detection of 'empty ghost cells' signals that SeNPs produced by SeITE02 probably triggered lysis, contrary to Dungan's findings. Lampis *et al.* observed that SeNPs enlarged over time, due to a nucleation mechanic similar to Ostwald ripening where the Se particle aggregate. SeNPs contained an organic layer of carbohydrates, lipids, and proteins on their surface that may help form and stabilize the SeNP or limit its size.

Analysis of the organic layer taken from a granular sledge revealed the most abundant proteins included outer membrane porins and the elongation factor Tu, although Tu's association with Se particles maybe only be due to its ionic charge, and not due to a role it has in the sphere's assembly (Gonzalez-Gil, 2016). Gonzalez-Gil *et al* additionally proposes a mechanism where selenite reduced in the cytoplasm by glutathione leads to very large Se particles that eventually damage and lyse the cell membranes. But as for SeITE02, a defined mechanism is unclear.

Additionally, an alcohol dehydrogenase (AdH) homologue is believed to be associated with SeNP synthesis and stabilization, was bound to SeNP. This enzyme has oxidoreductase activity and is involved in alcohol metabolism. It may be bound due to a high affinity to selenium caused by its amino acid composition. SefA, a protein identified by Debieux *et al*. (2011), plays a role in nanosphere formation of $Se^0$ in *T. selenatis*. Cloning of *sefA* into *E. coli* revealed that its protein product is likely to be involved in protein export.

**Representational Difference Analysis**

Representative Difference Analysis (RDA) is a technique developed for the identification of genes uniquely expressed in one population under a given condition (Bowler). This method relies on hybridization of cDNA fragments in two samples due to complementary base-pair sequences. Subtractive hybridization is a procedure utilized to report genes upregulated in one cell population compared to another. Advantages of subtractive hybridization include requiring only a small amount of mRNA, detecting rare mRNA (representing as little as ~ 0.01% of total mRNA present), and allowing the ability to clone novel genes. Genes expressed 5-10 times more in the tester will likely be

detected. Successful procedures can remove 95% of starting sequences. Disadvantages include its technically demanding nature, the potential loss of rare RNA due to the kinetics of subtraction, and the inability to completely remove cDNA common to both driver and tester (Byers, 2000). Figure 1 illustrates a modified RDA technique which uses streptavidin's and biotin's well documented affinity for one another to identify uniquely expressed genes in response to selenite (Weber). cDNA fragments from the untreated population are referred to as the driver, and cDNA obtained from the treated population are referred to as the tester. Specific adaptors are ligated to *Sau3A1* digested cDNA fragments. The adaptors serve as complementary sequences to primer binding sites. Both the driver and tester cDNA will be amplified by PCR using different primers, creating representative cDNA profiles of each population. In addition, the driver cDNA will contain biotinylated dCTP and dUTP in the PCR reaction and the tester cDNA PCR reaction will not. Then when the two populations are mixed together they are denatured and allowed to hybridize. Tester fragments also present in the driver will renature with strands from the driver, producing double-stranded DNA that has one strand labeled with biotin. Fragments that are uniquely expressed in response to selenite will produce double-stranded DNA that is free of biotin. The addition of streptavidin beads will attract the biotin, and a magnet is used to pull out the streptavidin-biotin-DNA complex, leaving the unique cDNA fragments in solution. The utilization of biotin and streptavidin to achieve separation of tester and driver DNA fragments is a modification from the RDA procedure originally outlined by Bowler. In the original paper by Bowler, the biotin/streptavidin method of removal was not utilized, but kinetic enrichment of the target sequences was achieved by using mung bean nuclease to degrade single stranded DNA belonging to the

driver while tester-tester hybrids amplified exponentially due to their ligated adaptors used for PCR. Fragments obtained after subtraction can be amplified by PCR, cloned and sequenced. A BLAST can then be used to determine if our differentially expressed genes are predicted to be involved in selenite resistance.

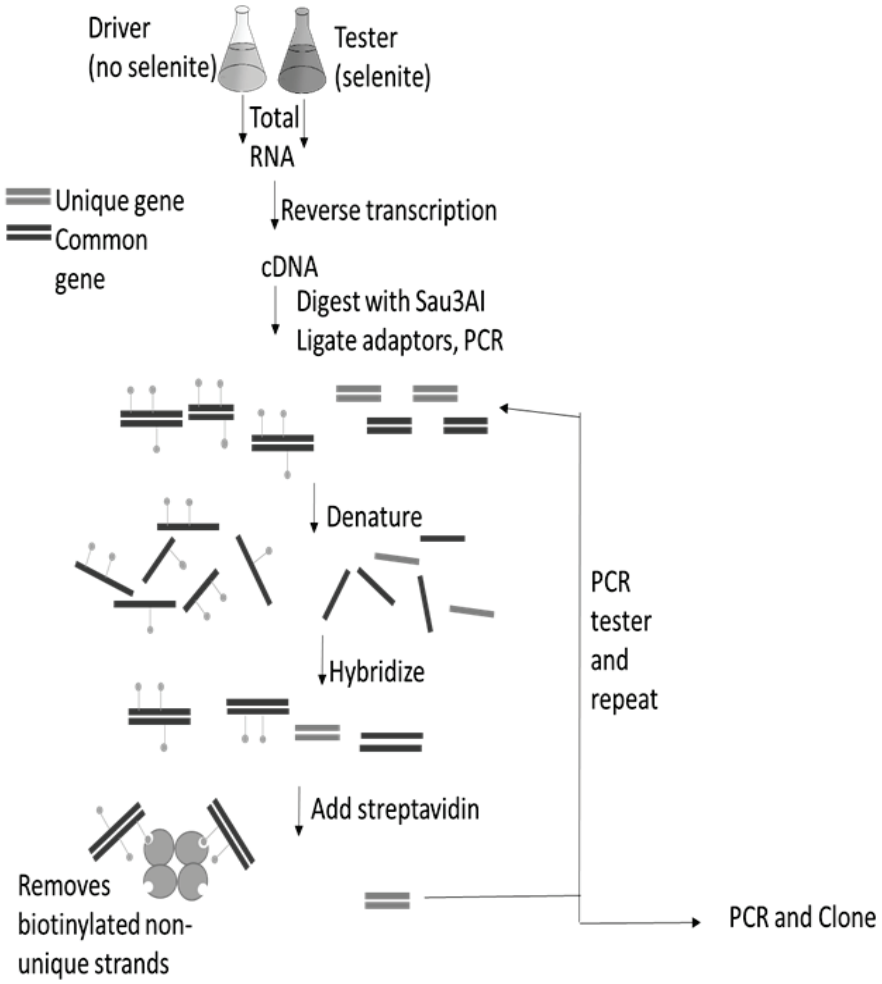


**Figure 1: Overview of RDA and subtractive hybridization method.** Total RNA is collected from culture in exponential growth. cDNA is produced by reverse transcription using random primers. The resulting cDNA is digested by *Sau*3AI to

produce a 5’ overhang that the 12-mer oligonucleotides hybridize to. The 24-mer oligonucleotide hybridizes to overhanging nucleotides of the 12-mer and is ligated to the 5’ end of the cDNA by T4 DNA ligase. *Taq* polymerase fills in the sequence complimentary to the 24-mer, thus creating the primer binding site used in PCR reactions. Biotin-labeling PCR is utilized to mark the driver cDNA in biotin and generate a suitable amount for the hybridization steps. Tester DNA is also amplified, but not with biotin labeling. The adaptors are removed from the driver by digestion with *Sau*3 AI prior to hybridization. Excess driver DNA is mixed with tester DNA, denatured, and left to rehybridize. Only tester sequences can rehybridize with their complementary tester sequences. Sequences from the tester also present in the driver will rehybridize with the excess sequences from the driver. To eliminate driver DNA and tester-driver hybrids, the hybridization solution is mixed with streptavidin beads. The streptavidin will attract the biotin-labeled strands. If the mixture is placed against a magnetic rack, the streptavidin-biotin-DNA complex will be pulled to the wall of the test tube, and the biotin-free solution can be extracted. A PCR reaction using the tester’s primers and subtracted solution as template will produce tester DNA that can be used in additional hybridize/subtraction steps. Once subtraction is complete, the PCR produce can be cloned using a StrataClone Kit and used in sequencing.

RNA in bacteria

Total RNA in bacteria contains more than 95% rRNA (Peano, 2013). Therefore, it is crucial to remove this excess rRNA for successful transcriptome profiling and detection of unique mRNA. Prokaryotic mRNA contains short or no polyA tails, leading

to difficulties separating mRNA from the abundant rRNA in total RNA samples. In eukaryotes, the polyA tails of mRNA can be used to isolate the mRNA by way of oligo (dT)s as primers in cDNA first strand synthesis. With prokaryotic first strand synthesis, random primers must be used which do not distinguish the mRNA from the rRNA, leading to high amounts of cDNA coding rRNA. To overcome this obstacle an additional driver consisting of biotinylated 16S and 23S rDNA is used to eliminate the rDNA from the tester population. This strategy removes background ribosomal RNA cDNA, allowing for enrichment of unique cDNA fragments.

## HYPOTHESIS

The cellular processes *Stenotrophomonas maltophilia* exhibits when exposed to selenite in the environment includes reduction, methylation, and incorporation. Reduction of selenite into elemental selenium likely involves glutathione. Therefore, genes that maintain glutathione such as glutathione reductase and glutathione synthase may be selectively expressed. Since reduction can generate damaging ROS, enzymes such as catalase, may be induced. The *tpm* gene which is involved in the methylation of selenium is another gene I expect to be differentially expressed. Homologous genes to *dedA* and *gutS* may also be identified which may provide evidence of how *Stenotrophomonas maltophilia* takes up selenite from its environment. The presence of the *sefA* gene would further associate it with the export of selenium nanospheres.

## METHODS

### Bacterial Strains

*Stenotrophomonas maltophilia* strain OR02 (ATCC 53510) was obtained from the American Type Culture Collection (Manassas, VA). Competent StrataClone SoloPack Competent Cells (*Escherichia coli*) were acquired from Agilent Technologies (Cedar Creek, TX).

### Growth Media

Lennox Broth (LB) contained of 10 g/l tryptone, 5 g/l sodium chloride, and 5 g/l yeast extract, and was purchased through Molecular Biologicals International, Inc. (Irvine, CA). When required, growth medium was supplemented with 50 μg/ml of kanamycin, 100 μg/ml ampicillin or 40 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside **(**X-gal). Stock solutions of X-gal were dissolved in N,N-dimethylformamide (Thermo-Fisher Waltham, MA).

### Growth Curves

An overnight culture of *S. maltophilia* 02 grown at 30ºC in LB medium was diluted 1:50 into two side-armed flasks containing fresh LB medium. The flasks were incubated at 30ºC with shaking at 150 rpm and growth was followed by measuring turbidity every half hour using a Klett Colorimeter. Then, 2.5 hours after starting the culture, sodium selenite was added to a concentration of 0.5 mM to one of the cultures and an equal volume of sterile water was added to the second culture. Turbidity was continued to be measured up to 8 hours.

**Genomic Preparations**

The Wizard® Genomic DNA Purification Kit was purchased from Promega (Madison, WI). 1 ml of overnight culture was centrifuged at 10,000 x RMP for 2 minutes. The supernatant was poured off and 600 µM of Nuclei Lysis Solution was added with gentle pipetting to resuspend. Following a 5-minute incubation at 80ºC, 3 µl of RNase solution (4 mg/ ml) was added with gentle inversion to mix. Samples were then held at 37ºC for 60 minutes. After a return to room temperature, 200 µl of Protein Precipitation Solution was added and vortexed for 20 seconds at maximum speed. Samples were moved on ice for 5 minutes and then centrifuged for 3 minutes at 10,000x RPM. Supernatant was transferred to a 1.5 ml microcentrifuge tube containing 600 µl of isopropanol. After gentle inversions samples were centrifuged for 2 minutes. Supernatant was poured off and left to air dry for 15 minutes. 100 µl of DNA Rehydration Solution was added to submerge the pellet. Samples were held at 4ºC refrigerator overnight.

**PCR of 16S and 23S rRNA**

PCR reactions for 16S and 23S rRNA contained 10 µl of 2X GoTaq® Green Master Mix (Promega; Madison, WI), 0.2 µM forward primer, 0.2 µM reverse primer and 1 µl of genomic DNA from *S. maltophilia* 02 in a total volume of 20 µL. All primers were purchase from Integrated DNA Technologies (Coralville, IA). The mixture was then incubated at 90ºC for 2 minutes, followed by 30 cycles of 90ºC for 1 minute, 56ºC for 1 minute, and 72ºC for 2 minutes. After a final incubation at 72ºC for 10 minutes, the

reactions were held at 25ºC. Results were then run on an 1% agarose gel using electrophoresis, and visible bands were viewed under UV light.

**Cloning using StrataClone**

PCR products were cloned using the StrataClone PCR Cloning Kit (Agilent, Santa Clara, CA). A ligation reaction, containing 3 µl of cloning buffer, 2 µl of the PCR product, and 1 µl of the vector mix, was mixed gently by pipetting, incubated for 5 minutes at room temperature and then placed on ice. A StrataClone SoloPack tube of competent cells was thawed on ice for each ligation. 1 µl of the reaction mixture was added to thawed competent cells and mixed gently. The reaction was incubated on ice for 20 minutes. The reaction was heat-shocked at 42ºC for 45 seconds and put back on ice. Then, 250 µl of LB medium warmed to 42ºC was added to the cells, which were shaken at 37ºC for 1.5 hours. 100 µl of cells were spread on LB-ampicillin plates containing X-gal. White colonies were then inoculated in 5 mL of LB supplemented with 50 µg/ml kanamycin and grown at 37ºC in a rotating incubator.

**Plasmid Preparations**

The Wizard® Plus SV Miniprep DNA Purification System was purchased from Promega (Madison, WI). Overnight cultures were centrifuged at 7000x RPM for 10 minutes. The supernatant was poured off and the pelleted cells were resuspended in 250 µl of resuspension buffer. After adding 250 µl of lysis buffer, the cells were mixed by gentle inversion and incubated at room temperature for 5 minutes. 10 µl of alkaline protease solution was added to the lysate which was mixed by gentle inversion and again

incubated for 5 minutes. The lysate was then mixed with 350 µl of neutralizing buffer and centrifuged for 10 minutes at 14,000 RPM. The supernatant was pipetted into a spin column and centrifuged for 1 minute. The flow-through was discarded, and 750 µl of wash buffer was added. The spin column was centrifuged for 1 minute, and the flow-through was discarded. An additional 250 µl of wash buffer was added. After centrifuging it through the spin column and discarding the flow-through, the spin column was centrifuged an additional 1 minute to remove any remaining buffer. The spin column was transferred to a clean 1.5 ml microcentrifuge tube, and the plasmid DNA was eluted by centrifuging 100 µl of nuclease free water through the spin column for 1 minute.

**RNA isolation**

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). During the growth curve procedure, at approximately 3.5 hours of incubation after starting the culture, 100 µl of bacterial culture was mixed with 200 µl RNAprotect Bacteria Reagent (Qiagen, Hilden, Germany) and briefly vortexed. Samples were then centrifuged for 10 minutes at 5000 x g at 25°C. Supernatant was poured off and residual supernatant was removed by gentle dabbing over a paper towel. 10 µl of Proteinase K (Qiagen, Hilden, Germany) was mixed with 100 µl of TE buffer (30 mM tris, 1 mM EDTA, pH 8.0, [Amresco, Solon, OH]) containing 15 mg/ml lysozyme. The pellet was resuspended by gentle pipetting in 110 µl of this mixture. Samples were then vortexed and held at room temperature for 10 minutes, with 10 second vortexes every 2 minutes. 350 µl of RLT buffer containing 10 µl β- mercaptoethanol (Sigma-Aldrich, St. Louis, MO) was then added to each sample. A brief centrifugation to create a pellet was

performed and supernatant was transferred to a RNeasy Mini spin column placed in a 2 ml collection tube. 250 µl of 95% ethanol was then added to each sample and centrifuged with the flow-through being discarded. The column was then loaded with 700 µl Buffer RW1 and incubated for 5 minutes before being centrifuged for 15 seconds at ≥ 8000 x g. After removing the flow-through, 500 µl of RPE buffer were centrifuged through the column for 15 seconds at ≥ 8000 x g. The flow-through was discarded and the spin column was placed in a new 2 ml collection tube. Another 500 µl of RPE buffer was added to the column and centrifuged at ≥ 8000 x g for 2 minutes. The flow-through was discarded, and the spin column was centrifuged again for 1 minute to dry the membrane. The spin column was placed in a new 1.5 ml collection tube, and 30 µl of nuclease free water was placed on the membrane. Following a 1-minute centrifugation, this step was repeated with an additional 30 µl of nuclease free water. Elimination of residual DNA was performed using a TURBO DNA-free Kit (Thermo-Fisher Waltham, MA). 6 µl of 10X TURBO DNase buffer and 1 µl TURBO DNase was added to each sample and incubated at 37°C for 20 minutes. After adding 7 µl of DNase Inactivation Reagent and mixing by pipetting, the solution was incubated for 5 minutes at room temperature. Finally, the sample was centrifuged at 10,000 x g for 1.5 minutes, and the supernatant was moved to a new 1.5 ml tube and stored at -80°C. The RNA concentration was measured by using a Nanodrop spectrophotometer (Thermo-Fisher Waltham, MA).

**Synthesis of cDNA**

The first strand of cDNA was synthesized using the ProtoScript First Strand cDNA kit (New England BioLabs, Beverly, MA). 100 ng of RNA was mixed with 2 µl of

random primer (50 μM) and nuclease free water for a final volume of 8 μl. After incubating the reaction at 70°C for 5 minutes, it was briefly spun in a microcentrifuge and placed on ice. Then, 10 μl of M-MuLV Reaction Mix and of 2 μl M-MuLV Enzyme Mix was added. A negative control tube containing the same amount of Reaction Mix but with 2 μl of water instead of Enzyme Mix was also created. The reaction was incubated in a thermocycler at 25°C for 5 minutes, 42°C for 1 hour, 80°C for 5 minutes. The reaction was stored at -20°C overnight. The second strand of cDNA was synthesized using the Protoscript Second Strand cDNA Synthesis Kits (New England BioLabs, Beverly, MA). The first strand cDNA reaction was thawed on ice and mixed with 8 μl Second Strand Synthesis Reaction Buffer, 4 μl of Second Strand Synthesis Enzyme Mix, and nuclease-free water for a final volume of 80 μl. Gentle pipetting was done to mix the reagents, and the reaction was incubated at 16°C for 2.5 hours.

The second strand cDNA reaction was cleaned-up using a MinElute PCR Purification Kit (Qiagen). After adding 5 volumes of PB buffer to 1 volume of the second strand reaction, the reaction was mixed with 10 μl of 3 M sodium acetate, pH 5.0 and added to a MinElute column. The column was centrifuged for 1 minute at 13,000 rpm, and the flow-through was discarded. 750 μl of PE buffer was added and centrifuged through the column for 1 minute. The flow-through was discarded and the column was centrifuged for 2 minutes to completely remove excess buffer. The MinElute column was placed in a clean 1.5 ml centrifuge tube, 30 μl of EB Buffer (10 mM Tris-Cl, pH 8.5) was added to the column and the column was centrifuging for 1 minute. The flow-through, which contained the eluted cDNA, was stored at -20°C.

**Digestion of cDNA**

*Sau3*AI digestions contained 2 µl of 10X Cut Smart buffer, 1 µl of *Sau*3AI (New England BioLabs), 3 µl of nuclease-free water and 14 µl of purified cDNA. The reactions were incubated at 37°C for 3 hours. The reaction was heated at 80°C for 20 minutes to inactivate the enzyme.

**Linker Ligation**

Tester ligations contained 2 µl 10X T4 DNA ligase buffer (New England BioLabs), 4 µl nuclease-free water, 9 µl digested cDNA, 2 µl of 0.25 mM R-Bgl-12 and 2 µl of 0.5 mM R-Bgl-24. Driver ligations contained 2 µl of 0.25 mM J-Bgl-12, and 2 µl of 0.5 mM J-Bgl-24, instead of R-Bgl-12 and R-Bgl-24. In a thermocycler, the reaction was heated to 50°C for 2 minutes, then cooled 1°C every minute until the temperature reached 10°C. 1 µl of T4 DNA ligase (New England BioLabs) was added, and the reaction was incubated at 4°C overnight.

**PCR amplification with and without biotin**

The tester strain was amplified without using biotin, while the driver strain and rDNA utilized biotin-labeling PCR. The PCR reactions were set up by creating a mix of 10 µl GoTaq, 2 µl nuclease-free $H_2O$, 2.5 µl 25 µM Biotin-11-dUTP, 2.5 µl 25 µM Biotin-11-dCTP, 5 µl J-Bgl-24 primer (4 µM), and 3 µl template (driver) DNA. In tester reactions, in place of biotin, nuclease free water was used, and 5 µl R-Bgl-24 (4 µM) primer was used instead of the J-Bgl-24. The PCR reaction was run at 95°C for 2 minutes, 95°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute, and repeated 16-32

times, then set to 72°C for 10 minutes, and held at 10°C. The number of cycles was determined by running pilot reaction of various cycle amounts and determining the optimal number that generate enough but not too much. For the tester population, 16 cycles was determined as ideal, while for the driver, 24 cycles was needed.

PCR reactions were cleaned using the MinElute purification Kit, as described above.

**Subtractive Hybridization**

A 50 µl solution containing 100 ng of tester cDNA and 5000 ng of biotin labeled 16S rDNA and 5000 ng of biotin labeled 23S rDNA in 0.5X SSC was allowed to hybridize over 24 hours at 68°C. The following day, 6 tubes of 700 µl of magnetic streptavidin beads (Promega, Madison, WI) were pipetted into 1.5 mL tubes placed in a magnetic separation stand. Buffer was removed, leaving only magnetic streptavidin beads on the walls of the tube. The beads were washed twice by resuspending them in 200 µl of 0.5X SSC buffer (Amresco), followed by placing the microfuge tubes containing them in the magnetic stand and removing the buffer with a pipette directly prior to the addition of the hybridization mix. The reaction was then added to the washed streptavidin beads and incubated at 25°C for 10 min with shaking at least every 2 minutes. The streptavidin magnetic beads were collected using a magnetic stand and solution was pipetted into the next tube containing the beads. This was repeated for all 6 bead-containing tubes. A 3 µL aliquot of the final solution (referred to as DP1) was used as template in a 30 cycle PCR reaction with the R-Bgl primers run at 95°C for 2 minutes, with 95°C for 1 minute, and 72°C for 4 minutes. This product was cleaned and added in a 50 µl, 0.5X SSC, second hybridization mix containing 1500 ng 16S, 1500 ng 23S, and 500ng driver cDNA, and

allowed to hybridize overnight as previously. This second subtraction step yield the second difference product (DP2). DNA from DP2 was amplified in a PCR reaction and used in a third and final hybridization. The last hybridization contained 1500 ng 16S, 1500 ng 23S, and 500ng driver cDNA, and ~137 ng DP3 was used as the tester. The solution from the final subtraction was amplified and used for cloning and sequencing.

**DNA Sequencing**

Sequencing reactions were carried out using a Genome Lab™ Dye Terminator Cycle Sequencing with Quick Start Kit (AB Sciex, Concord, Ontario, Canada). 50 fmol of plasmid DNA was diluted in a volume of nuclease-free water to a final volume of 10 µl. This was heated at 96°C for 1 minute. Then, 2 µl of 1.6 µM forward or reverse M13 primer and 8 µl master mix were added and mixed. The sequencing reaction was run at 96°C for 20 seconds, 50°C for 20 seconds, and 60°C for 4 minutes for 30 cycles. Then, the reactions were held at 4°C. 5 µl of a mix containing 2 µl 3M sodium acetate (pH 5.2), 2 µl 0.5M EDTA (pH 8.0) and 1 µl glycogen per sample was added to each DNA sample and mixed by gentle pipetting. 60 µl of cold 95% ethanol was added to each sample. Samples were centrifuged at 14,000 rpm at 4°C for 15 minutes. Supernatant was removed without disturbing the pellet. 200 µl of ice cold 70% ethanol was added, and the samples were centrifuged for 2 minutes. The supernatant was removed and an additional wash with 200 µl 70% ethanol was performed. Samples were then dried in a CentriVap for 10 minutes to eliminate residual ethanol. Finally, 40 µl of sample loading buffer was added to every sample, which were separated on a Beckman Coulter CEQ™ 2000 XL DNA Analysis System.

Table 1. Primers used in this study

| Primer | Application | Nucleotide Sequence |
|---|---|---|
| 8F | 16S rRNA amplification | 5'-AGAGTTTGATCCTGGCTCAG-3' |
| 1492R | 16S rRNA amplification | 5'-GGTTACCTTGTTACGACTT-3' |
| SO2-23F | 23S rRNA amplification | 5'-CAAGCGAATAAGCGCACAC-3' |
| SO2-23R | 23S rRNA amplification | 5'-CCGCACGGATCATTAGTATCA -3' |
| R-Bgl-12 | RDA | 5'-GAT CTG CGG TGA-3' |
| R-Bgl-24 | RDA | 5'-AGC ACT CTC CAG CCT CTC ACC GCA-3' |
| J-Bgl-12 | RDA | 5'-GAT CTG TTC ATG-3' |
| J-Bgl-24 | RDA | 5'-ACC GAC GTC GAC TAT CCA TGA ACA-3' |
| M13 R | Sequencing | 5'-GGA AAC AGC TAT GAC CAT G-3' |
| M13 F | Sequencing | 5'-GTA AAA CGA CGG CCA GT-3' |
| Met K1 | MetK | 5’-ATC AAG ATG CTG GAC CTG GT-3’ |
| Met K2 | MetK | 5’-TTA CTT CAG CTT CGA ATC GG-3’ |

## RESULTS

### Growth of *S. maltophilia* in selenite

Growth curves of *S. maltophilia* 02 in the presence and absence of 0.5 mM defined the selenite-resistance phenotype. An overnight culture of *S. maltophilia* 02 was diluted 1:50 into fresh LB medium and growth was followed by measuring turbidity every half hour up to 8 hours using a Klett colorimeter. After 2.5 hours of incubation, the culture was split into equal volumes and either 0.5 mM sodium selenite or an equal amount of deionized water was added. To identify genes expressed in response to selenite using Representative Difference Analysis (RDA), cells samples were removed for total RNA purification 2 hours after selenite was added at time point 4.5 hours. Figure 2 shows the average growth from seven cultures. The initial lag phase appeared to last about 1 hour before the cultures entered log phase. After the selenite was added, both cultures remained in log phase for an hour. The untreated culture shifted to stationary phase at 4.5 hours, and the selenite treated culture shifted to stationary phase at 4 hours. The turbidity of the selenite treated culture during stationary phase was consistently lower that the turbidity of the untreated culture during stationary phase. The selenite-exposed cells most likely sacrificed some growth for selenite detoxification. The death phase is not included, since the experiment was monitored for only the first 8 hours. At this final time point, the selenite induced culture had a red tint to it, and both cultures had a cloudy appearance, signaling cell growth. The following day, the selenite-induced culture was dark red, contained a red precipitate, and produced a strong scent of stale garlic. This red

precipitate interfered with turbidity measurements which did not accurately reflect cell growth.

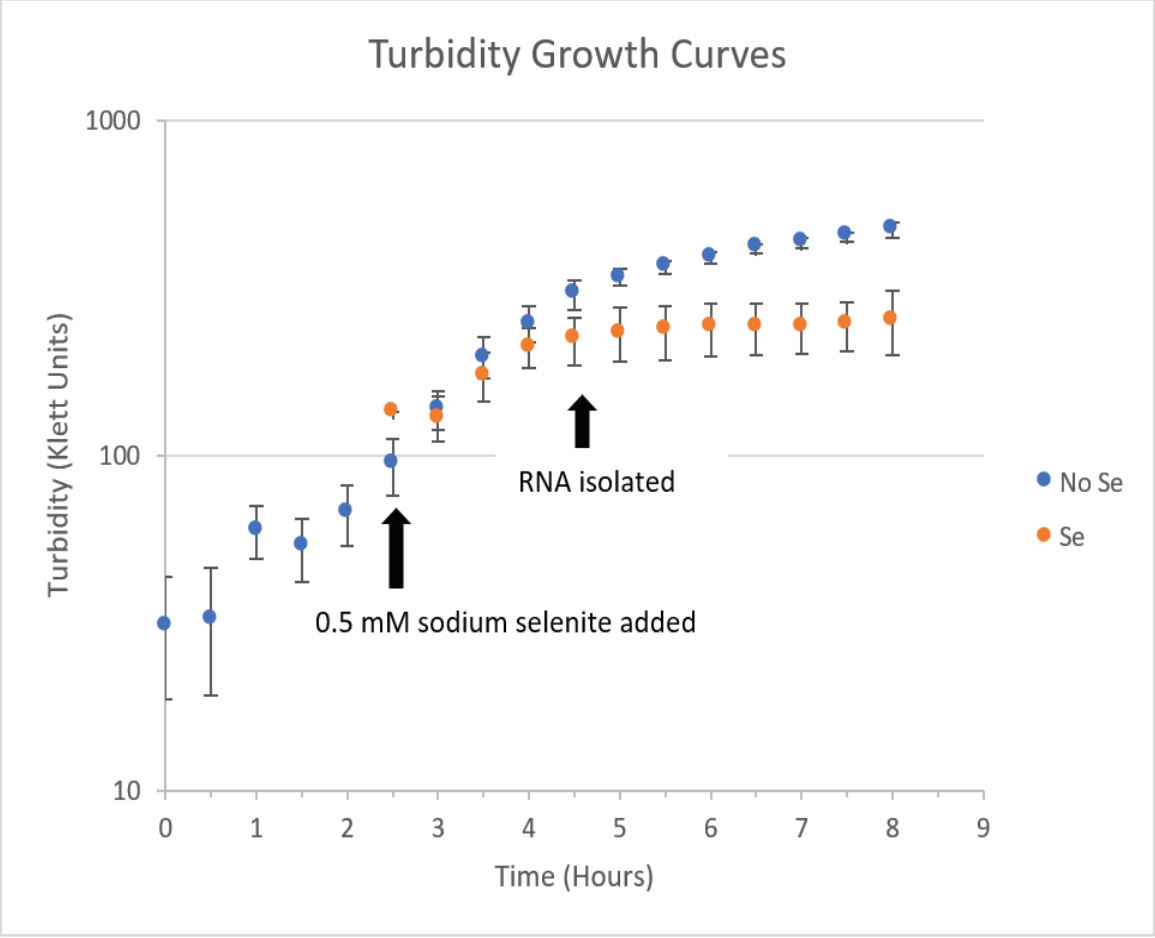


**Figure 2: Turbidity growth curves.** An overnight culture of S. *maltophilia O2* was diluted 1:50 in LB medium and grown at 37°C. At 2.5 hours the culture was split into equal volumes and either water or selenite was added. Growth was measured every 30 minutes for 8 hours by taking recordings using a Klett colorimeter. Values are the average of 7 experiments.

Cloning 16S and 23S rDNA

The 16S and 23S rRNA genes from *S. maltophilia* were cloned so that they could be labeled with biotin and used as driver DNA to remove 16S and 23S cDNA from tester DNA. Gel electrophoresis of the PCR amplified 16S and 23S genes were approximately 1.5 kb and 2.8 kb respectively as expected (Fig. 3A). The PCR fragments were then

cloned into plasmid pSC-A-amp/kan from the StrataClone PCR cloning kit. The recombinant plasmids were purified, digested with *Eco*R I and separated on a 1 % agarose gel (Fig. 3B). The profile in lane 3 shows that the digestion of the 16S recombinant plasmid was incomplete and that *Eco*R I cut in the middle of the 16S rDNA insert. The second band from the top in this lane is the 16S rDNA insert, and the two bottom bands are the digested insert. The profile in lane 5 shows that *Eco*R I also cut in the middle of the cloned 23S rDNA insert. The cloned fragments were then partially sequenced to verify their identities. Figure 4 shows the BLAST results confirming the 16S plasmid insert shared a 99% identity with a *S. maltophilia* 16S rRNA gene, and the 23S plasmid insert shared a 98% identity with a 23S rRNA gene from another strain of *S. maltophilia*. Both rRNA genes were PCR amplified using biotin-labeled dCTP and dUTP for use in subtractive hybridization.

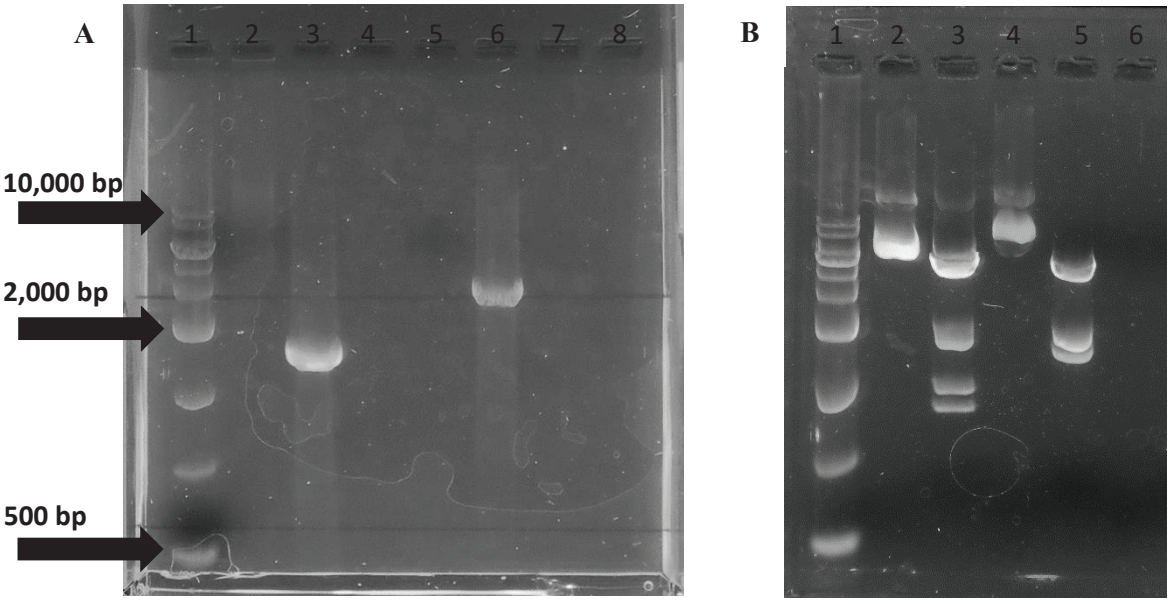


**Figure 3:** 1% agarose gels. **(A)** Amplified 16S and 23S PCR products. 1 Kb ladder (lane 1), 16S PCR (lane 3), 23S PCR (lane 6)**.** (B) *Eco* RI digestions of the rDNA recombinant plasmids. 1 Kb ladder (lane 1), 16S-containing plasmid (lane 2), *Eco* RI-digested 16S plasmid (lane 3), 23S-containing plasmid (lane 4), *Eco* RI-digested 23S plasmid (lane 5).

A

Stenotrophomonas maltophilia strain Ap-6 16S ribosomal RNA gene, partial sequence

Sequence ID: MK641664.1 Length: 1508 Number of Matches: 1

Range 1: 879 to 1508 GenBank Graphics ▼ Next Match ▲ Previous Match

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 1133 bits(613) | 0.0 | 625/631(99%) | 1/631(0%) | Plus/Minus |

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Query  1     GGTTACCTTGTTACGACTTCACCCCAGTCATCGGCCACACCGTGGCAAGCGCCCTCCCGA  60
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Sbjct  1508  GGTTACCTTGTTACGACTTCACCCCAGTCATCGGCCACACCGTGGCAAGCGCCCTCCCGA  1449

Query  61    AGGTTAAGCTACCTGCTTCTGGTGCAACAAACTCCCATGGTGTGACGGGCGGTGTGTACA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1448  AGGTTAAGCTACCTGCTTCTGGTGCAACAAACTCCCATGGTGTGACGGGCGGTGTGTACA  1389

Query  121   AGGCCCGGGAACGTATTCACCGCAGCAATGCTGATCTGCGATTACTAGCGATTCCGACTT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1388  AGGCCCGGGAACGTATTCACCGCAGCAATGCTGATCTGCGATTACTAGCGATTCCGACTT  1329

Query  181   CATGGAGTCGAGTTGCAGACTCCAATCCGGACTGAGATAGGGTTTCTGGGATTGGCTTAC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1328  CATGGAGTCGAGTTGCAGACTCCAATCCGGACTGAGATAGGGTTTCTGGGATTGGCTTAC  1269
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B

Stenotrophomonas maltophilia strain R551-3 23S ribosomal RNA gene, complete sequence

Sequence ID: NR_076594.1 Length: 2880 Number of Matches: 1

Range 1: 4 to 693 GenBank Graphics ▼ Next Match ▲ Previous Match

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 1190 bits(644) | 0.0 | 677/692(98%) | 5/692(0%) | Plus/Plus |

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Query  1     CAAGCGAATAAGCGCACACGGTGGATGCCTTGGCGGTCAGAGGCGATGAAGGACGTGGCA  60
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Query  61    GCCTGCGAAAAGTATCGGGGAGCTGGCAACAAGCTTTGATCCGGTAATGTCCGAATGGGG  120
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Sbjct  64    GCCTGCGAAAAGTATCGGGGAGCTGGCAACAAGCTTTGATCCGGTAATGTCCGAATGGGG  123

Query  121   AAACCCACCCGCTTGCGGGTATCCTGCAGTGAATACATAGCTGCTGGAAGCGAACCTGGT  180
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Sbjct  124   AAACCCACCCGCTTGCGGGTATCCTGCAGTGAATACATAGCTGCTGGAAGCGAACCTGGT  183
```

**Figure 4: BLAST results of sequenced DNA.** Plasmid insert DNA was sequenced and BLAST analyses were performed at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Fig 4A The 16S sequence shares 99% identity to a 16S rRNA gene. 4B. The is the 23S sequence shares 98% identity to a 23S rRNA gene.

## RNA isolation and cDNA generation

Total RNA was isolated from samples taken from selenite-treated and untreated cultures (Fig. 2) using a RNeasy Mini kit and treated with a TURBO DNA free kit to remove contaminating DNA. A PCR reaction using the 8F and 1492R primers was performed, and the products were separated on a 1% agarose gel to confirm removal. In

the gel (Fig. 5), no bands were present in lanes 2 and 3 containing the PCR product from the RNA extract, signifying that chromosomal DNA was removed and therefore did not amplify from PCR. The profile in lane 4 contains a positive control using the PCR product generated from S02 genomic template. The RNA concentration was measured by a Nanodrop spectrophotometer and first strand synthesis of cDNA was performed using Protoscript First Strand cDNA kit and random primers. No more than 1 µg of RNA was used as template, and when there was not enough for 1 µg the amount used from the tester was equal to the amount used from the driver. Then, the second strand was synthesized using the Protoscript Second Strand cDNA synthesis kit. The product was cleaned up using a MinElute PCR purification kit.

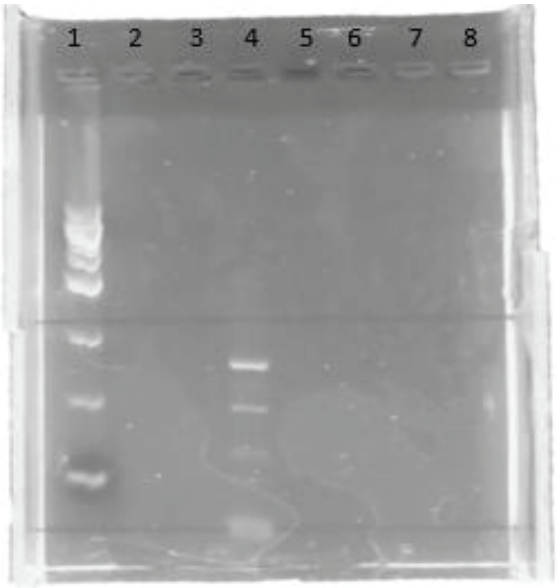


**Figure 5:** 1% agarose gel electrophoresis of PCR product from collected RNA. 1 kb ladder (lane 1), Tester-collected PCR (lane 2), Driver-collected PCR (lane 3), positive control S02 genomic DNA PCR (lane 4).

**Table 2:** Nanodrop measurements of RNA and DNA concentration.

| Sample | RNA concentration ng/µl | cDNA concentration ng/µl |
|---|---|---|
| Tester sample 1 | 95.3 | 3.9 |
| Tester sample 2 | 10.5 | 9.1 |
| Tester sample 3 | 113.7 | 17.2 |
| Driver sample 1 | 274.6 | 14.5 |
| Driver sample 2 | 77.7 | 3.2 |
| Driver sample 3 | 76.4 | 20.8 |

**Digestion with *Sau3*AI and generation of representatives**

The cDNA product was then digested by the *Sau3*AI enzyme to generate *Bgl* II sticky ends. The DNA was precipitated, pelleted, washed with 70% ethanol, dried and resuspended in nuclease free water, as outlined by Bowler. Then, the R-Bgl-12 adaptors were ligated to the sticky ends of the digested tester cDNA, and the J-Bgl-12 adapters were ligated to the sticky ends of the driver cDNA. The tester and driver cDNAs were PCR amplified using the R-Bgl-24 and J-Bgl-24 primers, respectively. Pilot reactions (Fig. 6) showed the tester had optimal amplification at 16 cycles (lanes 3 and 4) while driver representations were suitable at 24 cycles (lanes 10 and 11). Over amplification introduces bias in the representatives and under amplification provides insufficient material for subtraction. Using the optimal number of cycles, 3X driver PCR reaction were set up containing biotin-labeled dCTP and dUTP, while the tester PCR reactions did not contain the labeled nucleotides. The PCR reactions were cleaned-up by Qiagen's

purification kit and concentration was measured in by a Nanodrop spectrophotometer. Driver cDNA was then digested by *Sau3*AI to remove the J-Bgl adaptors.

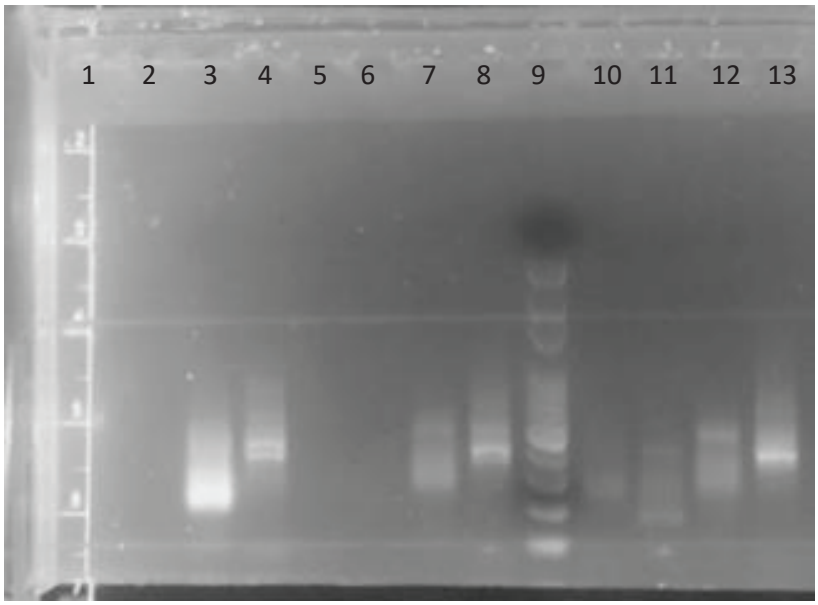


**Figure 6:** 1% agarose gel showing amplified cDNA of the tester and driver. Tester cDNA 16 cycles (lanes 3 and 4), tester cDNA 20 cycles (lanes 7 and 8), 100 bp ladder (lane 9), driver cDNA 24 cycles (lanes 10 and 11), and tester cDNA 24 cycles (lanes 12 and 13).

**Subtractive Hybridization**

Ribosomal DNA was removed from the tester DNA first. Tester DNA was mixed with biotin labeled 16S and 23S rDNA in a tester:16S rDNA:23S rDNA tester ratio of 1:50:50 or 5µg:5µg:0.1µg. After denaturing the DNA mixture at 95°C, it annealed at 68°C for 24 hours.

Magnetic streptavidin beads and a magnetic rack were used to remove the 16S and 23S rDNA from the tester DNA. Hybrids of the biotin labeled driver rDNA and tester rDNA bound to the streptavidin magnetic beads, which bound to the side of a tube exposed to

the magnetic rack. Nanodrop analysis of the remaining solution (DP1) showed about 1 µg was still present in the final solution, thus removal of all the driver did not occur (Table 3). A second hybridization reaction containing a 16S:23S:driver cDNA: tester DNA ratio of 15:15:5:1 or 1.5µg:1.5µg:0.5µg:0.1µg was set up and a subtraction step similar to the first was performed to produce the second difference product (DP2). The DNA remaining in DP2 was found to be around 0.8 µg, again suggesting not all driver was removed by the streptavidin beads. A final hybridization step was performed using the same ratio for DP2. This final product was called DP3, which contained around 0.3 µg of total DNA. Fig. 7 shows results of PCR reactions using 23S primers where the first subtraction eliminated 23S from the tester, since DP1 (lane 2) did not contain 23S DNA that was present before the subtraction (lane 1). Lanes 8-11 faintly show that the pre-digested cDNA of lanes 8 and 11 did have a small trace of 23S DNA present.

Following subtraction and before proceeding to the next, DP1, DP2 and DP3 were amplified by PCR in 30 cycles using the R-Bgl-24 primer. Any remaining driver DNA was not PCR amplified because the R-Bgl-24 primer only had primer binding sites on the tester fragments. The resulting PCR amplified DNA was cloned using the StrataClone PCR cloning kit. Four clones were selected, and digestion with *Eco*R I revealed three contained an insert of about 300 bp.

**Table 3:** DNA amounts used in subtractive hybridization steps.

| Subtraction step | Starting amount of biotinylated driver DNA (ng) | Starting amount of tester DNA (ng) | Final amount of total DNA after subtraction (ng) |
|---|---|---|---|
| 1 | 9680 | 100 | 1078 |
| 2 | 3362 | 100 | 817 |
| 3 | 3841 | 137 | 317 |

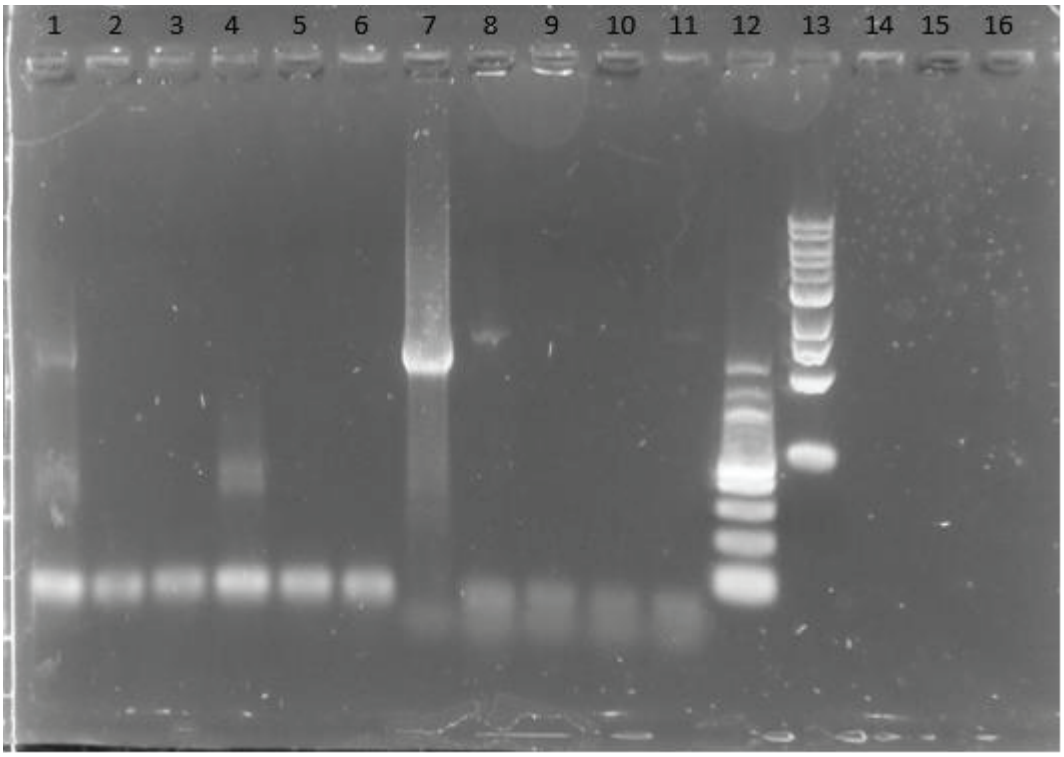


**Figure 7:** 23S PCR products from subtractions. Initial product from un-subtracted sample (lanes 1 and 4), after 1 round of subtraction (lanes 2 and 5), after second round of subtraction (lanes 3 and 6), from genomic template (lane 7), from tester cDNA (lanes 8 and 9), from driver cDNA (lanes 10 and 11), 100 bp ladder (lane 12), and 1 kb ladder (lane 13).

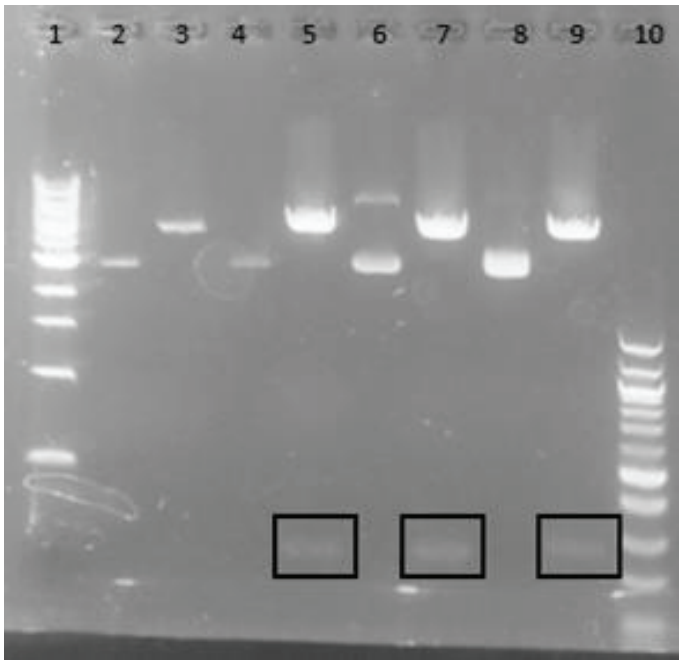


**Figure 8:** *Eco* R1 digestions of plasmids. Plasmids containing the PCR-amplified fragment of DP3 were digested to verify insertion. 1 kb ladder (lane 1), undigested plasmid (lanes 2, 4, 6, and 8), digested plasmid (lane 3, 5, 7, and 9), 100 bp ladder (lane 10). Boxed in lanes 5, 7, and 9 are the PCR inserts, whose size can range from 200 bp to 700 bp.

BLAST analyses of the sequenced products found that most fragments were either 16s and 23s rRNA genes, indicating insufficient subtraction or removal of the contaminating DNA fragments.

Detection of *metK* in post-subtraction mixture

Zachary Marinelli (2017) identified a selenite sensitive S02 mutant, which had a mutation in the promoter region of *metK*. To test for the expression of the *metK* gene, PCR reactions using the MetK 1 and MetK 2 primers were performed using template taken before and after the subtraction steps. The *metK* gene was also amplified from S02 genomic DNA, as well as from collected cDNA samples. The *metK* gene was expected to

be upregulated in the tester, due to methylation of selenium in the cell. However, of the cDNA amplified, only driver cDNA (Fig. 9, lanes 11 and 12) had a band present. The *metK* gene was present in the hybridization reactions though, and it appears it was partially subtracted as the bands in wells 3 and 6 (after 2 subtractions) appear less than those in wells 2 and 5 (after 1 subtraction) which are in turn less than those from wells 1 and 3 (hybridization mix before subtraction). If *metK* was upregulated in the tester, its presence in the driver would not be sufficient to remove it entirely from the population.

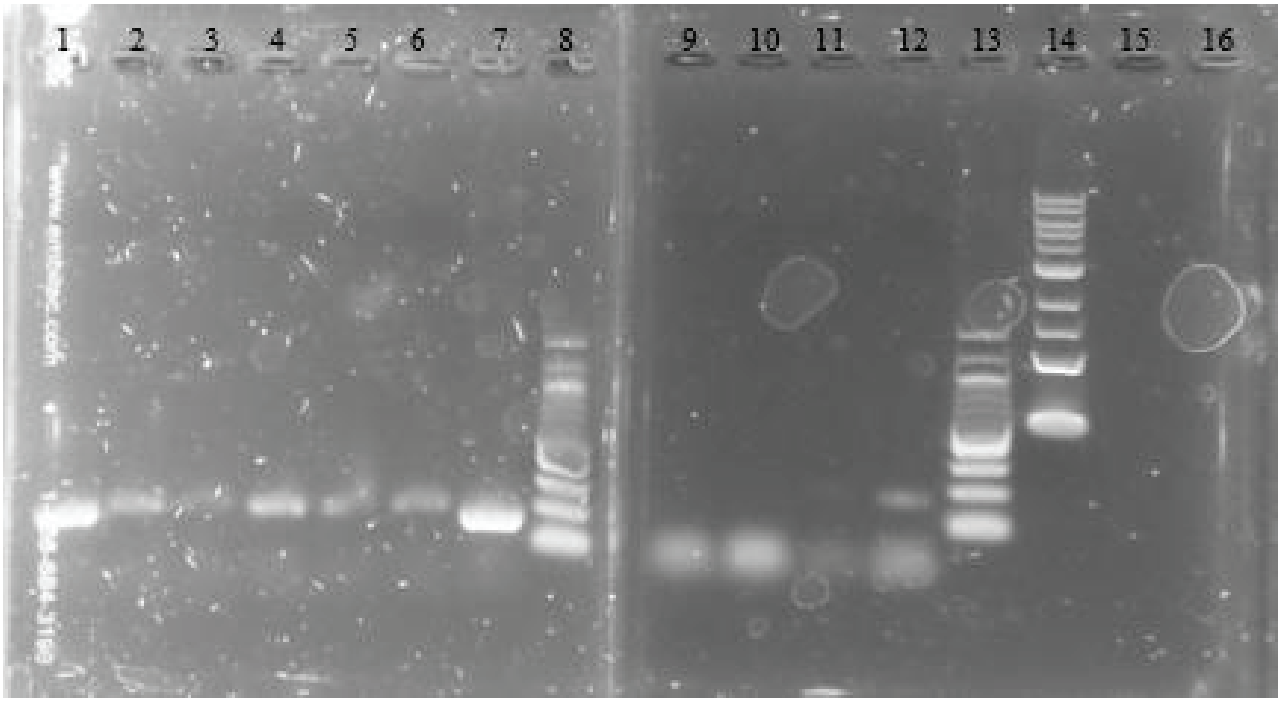


**Figure 9:** Detection of the *MetK* gene from PCR reaction. Initial product from un-subtracted sample (lanes 1 and 4), after 1 round of subtraction (lanes 2 and 5), after second round of subtraction (lanes 3 and 6), from genomic template (lane 7), from tester cDNA (lanes 9 and 10), from driver cDNA (lanes 11 and 12), 100 bp ladder (lanes 8 and 13), and 1 kb ladder (lane 14).

## DISCUSSION

*S. maltophilia* is able to grow in the presence of the toxic compound selenite by reducing it to elemental selenium, likely through the use of glutathione systems. The present research focused on identifying genes that are upregulated in *S. maltophilia* cultures that are exposed to 0.5 mM selenite, which may identify certain metabolic pathways involved in the process. Selenite slowed the growth of *S. maltophilia*, likely sacrificing growth to metabolize the toxic oxyanion. Previous research suggested that glutathione reductase, glutathione synthetase and genes expressed in response to oxidative damage would be upregulated in the presence of selenite exposure (Painter, 1941). A modified representational difference analysis (RDA) technique and subtractive hybridization were used to detect these genes. However, an inability to remove driver cDNA and rDNA interfered with the results.

An additional consideration for future employment of this procedure would to ensure that the cells are still in exponential phase when harvested. Here, RNA was collected 2 hours post-addition of selenite. It was observed that this was enough time for the cells to begin entering stationary phase. Collection at stationary phase rather than at exponential phase may lead to the collection of non-optimal RNA. When a cell culture enters stationary phase, the rate of growth is equal to the rate of death and replication does not continue exponentially. This leads to changes in RNA expression, as the cells may start producing stress-related genes, which are not ideal in this study, and works against the idea of using driver cDNA to eliminate unique tester cDNA. Harvesting the cells' RNA at 1 hour after adding selenite may give better results since both cultures are in exponential phase.

The use of the different primers R-Bgl-12 to PCR amplify the tester DNA and the J-Bgl-12 to PCR amplify the driver DNA may have contributed to error. Figure 6 showed that the tester cDNA required 16 cycles for optimal PCR amplification, whereas the driver cDNA required 24 cycles for PCR amplification. This difference in cycling could introduce bias to the final results. In the future, it may be better to use the R primers to amplify both the tester and driver cDNA and remove the linkers from the driver DNA before the subtraction step. Bowler originally had used the different adaptors to distinguish between DP1, DP2, and DP3, which was deviated in this experiment from but should be considered in the future.

To test whether 23S rDNA was removed from the tester, the subtracted product was amplified in PCR using the S02 23S primers, with the idea that if any was remaining, it would amplify and show up in a gel. However, digestion with *Sau3* AI cut at a spot in the primer binding site, meaning PCR would not occur. Therefore, from Figure 7 we can only conclude that the 23S driver was removed, but not if the tester's 23S fragments were removed.

The subtractive hybridization did not work as planned. As outlined in Table 3, the streptavidin did not remove all the driver DNA. The biotinylated DNA may have saturated the streptavidin magnetic beads, leaving some of the tester: driver hybrids behind. Adding a second subtraction step with magnetic beads may resolve this issue.

It may be possible to remove rRNA contamination using alternative methods. rRNA interference is not a new challenge to transcriptome studies. Several products have appeared in the market that claim the ability to remove >95% of the 16S and 23S rRNA from total RNA. The MICROB*Express* Bacterial mRNA Enrichment Kit (ThermoFisher)

works by utilizing a similar mechanism to the one that has been performed in this paper. The kit provides an 'optimized set of capture oligonucleotides' that have one end that targets conserved regions of the 16S or 23S rRNAs and the other end contains sequence complementary to a secondary probe coated on magnetic beads. Another commonly used method of rRNA depletion is the mRNA-ONLY Prokaryotic mRNA Isolation Kit (Epicentre). This process uses a 5'-phosphate-dependent exonuclease to digest RNA having a 5' monophosphate.

Also produced by Epicentre/Illumina is the Ribo-Zero kit. This kit provides rRNA specific probes that are biotinylated and able to be captured with streptavidin beads, in a similar method to what is outlined in this paper.

Of the methods described above, each have drawbacks that prevent complete and total elimination of rRNA; MICROB*Express*'s capture oligonucleotides have been designed for *E. coli*, and thus have limited success on the rRNA of other bacteria, and mRNA-ONLY only achieved about 70% of total rRNA removal among several species (Ciulla). Illumina has discontinued many of its Ribo-Zero kits but are developing new products.

Another factor to consider would be the conditions that the DNA was allowed to hybridize in. The RDA paper by Bowler does not involve removal by streptavidin and biotin, but rather relies on mung bean nuclease to degrade single stranded DNA that does not contain the tester-specific adaptors. Combining the steps Bowler proposed and the steps employed by other experiments using subtractive hybridization leaves room for optimization of a protocol to successfully tie one into the other. There was no clear consensus to certain conditions such as time for hybridization (ranging from 1 hour to 48)

or optimal ratio of tester: driver (ranging from 1:2 to 1:400). The 0.5X SSC buffer concentration used could additionally be examined to find if that is the ideal concentration to use.

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