2010

In-frame Mutagenesis Of Genes Encoding A Selenium-dependent Molybdenum Hydroxylase And Putative Accessory Proteins In Enterococcus Faecalis

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IN-FRAME MUTAGENESIS OF GENES ENCODING A SELENIUM-DEPENDENT MOLYBDENUM HYDROXYLASE AND PUTATIVE ACCESSORY PROTEINS IN *ENTEROCOCCUS FAECALIS*

by

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B.S. University of Central Florida, 2008

A thesis submitted in partial fulfillment of the requirements
for the degree Master of Science
in the Department of Biomedical Sciences
in the College of Medicine
at the University of Central Florida
Orlando, Florida

Fall Term
2010

Major Professor: William T. Self

ABSTRACT

Enterococcus faecalis is a well known nosocomial drug resistant pathogen that is responsible for urinary tract infections, bacteremia, wound infections and endocarditis through the formation of biofilms. It has been shown that 68 genes present within the core genome of *E. faecalis* are upregulated in biofilm formation. One of those 68 genes is a putative selenium-dependent molybdenum hydroxylase (SDMH). Adjacent to this gene are a series of open reading frames that have been postulated to play a role in the maturation of a labile selenium cofactor. The biosynthesis of this labile cofactor has yet to be studied at either the genetic or biochemical level. The addition of selenium to growth medium caused a significant increase in biofilm density and extracellular hydrogen peroxide by wild type *E. faecalis*. By site-directed mutagenesis gene products encoded in the SDMH operon were shown to be necessary for the selenium-dependent biofilm formation as well as extracellular hydrogen peroxide production. This biofilm and peroxide phenotype is inhibited both by tungsten or auranofin in wild type *E. faecalis* suggesting the SDMH is a necessary enzyme for selenium-dependent biofilm and peroxide formation. These results show that the gene products encoded within the SDMH operon are necessary for a selenium-dependent biofilm formation as well as extracellular hydrogen peroxide production. These mutants will provide the basis for defining the synthesis of the labile selenium cofactor and allow for an expanded understanding of the biological use of selenium.

I would like to dedicate this thesis to my wife Eryn Mallard for her support through this entire experience.

ACKNOWLEDGMENTS

pLTO6 vector was a generous gift from Dr. Lynn Hancock (Kansas State University Manhattan, KS), and pAT28 was obtained from Dr. Janet Manson (Schepens Eye Research Institute Boston, MA). This work was supported in part by the NSF grant CBET 0753068 to William T. Self.

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INTRODUCTION

Enterococci are well-characterized gram positive opportunistic pathogens that are routinely recovered from hospitalized patients. These patients have either had multiple antibiotic treatments or hospitalized for extended periods of time. The focus of this study is on *Enterococcus faecalis,* a well-known nosocomial bacterium also known as a vancomycin resistant enterococcus (VRE). VRE has been shown to have a negative impact on mortality and cost of hospitalization(24), and accounts annually for 110,000 urinary tract infections, 25,000 cases of bacteremia, 40,000 wound infections and 1,100 cases of endocarditis(15).VRE infection has been shown to increase the duration of stay in the hospital by six days at an average additional cost of $27,000(5). Infection within certain groups such as those with weakened immune systems are most common among those hospitalized. A 2006 study at MD Andersen Cancer Center at the University of Texas found that 29% of all immunosuppressed patients developed bacteremia(26).

The use of vancomycin as an antibiotic has increased dramatically. This is due in part to methicillin-resistant staphylococci, prosthetic device related infection, *Clostridium difficle* colitis, and misuse of the drug itself. The vancomycin resistance of *Enterococcus faecalis* comes from presence of *vanH, vanA, vanX, vanS* and *vanR* genes that are similar to a gene cluster on Tn*1546* transposon that confers high level vancomyicin resistance. These genes change peptidoglycan precursors from D-Alanine-D-Alanine (D-Ala-D-Ala) in susceptible bacteria to D-alanine-D-lactate (D-Ala-D-Lac). This change in precursor prevents the formation of a complex between vancomycin and the peptidoglycan precursors which inhibits cell wall

synthesis. The *vanH* gene reduces pyruvate to D-lactate; the *vanA* gene is responsible for the ligation of D-Ala to D-Lac, while the *vanX* gene is responsible for the hydrolysis of D-Ala-D-Ala. These proteins are transcriptionally regulated by the VanR-VanS two-component system in the presence of vancomycin (2, 27)

Infection by VRE is caused by the formation of a biofilm, which has been implicated in bilary stents, tissue surfaces, urinary catheters, and heart valves (30, 35). Biofilms occurs through surface attachment as the cells begin to encase themselves in an exopolymeric coat formed from DNA, proteins and polysaccharides. Cell that are present in a biofilm are known to respond poorly to antibiotics. Biofilms not only pose a an infection problem for patients but also increase the transfer of resistance genes to other bacterial species within the biofilm(25). Understanding biofilm physiology is important because more than 60% of bacterial infections treated by clinicians involve biofilm (8). Mature biofilms can tolerate up to a 1000 times higher concentration of antibiotic than their planktonic counterparts and are also resistant to phagocytosis(29).

E. faecalis biofilms have been characterized by the need for specific genes in biofilm formation which include *gelE,* and the *fsr* locus(29). The *fsr* (*E.* faecalis regulator) locus contains the *fsrA, fsrB,* and *fsrC* genes which are involved in quorum sensing. The FsrB protein encodes a peptide lactone at its C-terminus which when accumulated in the extracellular space is recognized by FsrC a histidine kinase. The FsrC protein then leads to the activation of FsrA a response regulator and transcription factor. FsrA leads to the transcription of *gelE* (gelatinase) and *sprE* (serine protease). This quorum sensing system controls biofilm formation through the production of the gelatinase protein since mutation of the *fsr* operon and lack of gelE production resulted in prevention of biofilm formation (11). The *fsr* gene locus is also responsible for

regulation of numerous genes in late exponential and early stationary phase growth. Specifically it has been shown that the *fsr* locus regulates the *bopABCD* gene cluster (4), and the disruption of gene cluster via transposon mutagenesis lead to a significant decrease in glucose dependent biofilm formation(6). In a recent article, Ballering et al identified 68 genes upregulated during biofilm production in the core genome of *E faecalis*. One of those 68 genes identified is present in this study is EF2570 the putative selenium-dependent molybdenum hydroxylase (SDMH) (1).

Selenium utilization in both bacteria and humans comes from three separate but interconnected pathways. All three start with the activation of selenium to selenophosphate by the selenophosphate synthetase (*selD* in *E. coli*). However, it is after activation where the pathway diverges. The first branch is the incorporation of selenium into the 21st amino acid selenocysteine. The second being a modified tRNA, selenouridine, and finally a labile selenium cofactor present in a small class of molybdoenzynes (33). These enzymes have been termed "selenium-dependent molybdenum hydroxlases" (SDMH), and include nicotinic acid hydroxylase (NAH), purine hydroxylase (PH) and xanthine dehydrogenase (XDH). All have a labile selenium cofactors attached the molybdenum center that is poorly understood. These SDMHs are characterized by the conjugation of molybdenum to a pterin ring complex forming the molybdopterin cofactor (Mop). The Mop is a necessary feature of an active molybdenum hydroxylase. These SDMHs are involved in the hydroxylation of aromatic of aliphatic substrates with the use of water as the oxygen source to generate reducing equivalents. The transfer of electrons occurs through both iron/sulfur clusters and flavin (FAD) that are essentially an intramolecular electron transport chain in the enzyme (12). The location of the selenium atom in these enzymes has been shown to be associated with the pterin cofactor directly attached to the

molybdenum and not as the stable amino acid selenocysteine as in the formate dehydrogenase in *E. coli.*

The best characterized SDMH is the nicotinic acid hydroxylase (NAH) first identified by Earl Stadtman's group (13). The NAH's function is catalyze the breakdown of nicotinic acid to ammonia, propionate, acetate and carbon dioxide through the addition of water to the double bond of nicotinic acid subsequently followed by reduction of $NADP^+$ to NADPH(34). This observation that the selenium is not found as selenocysteine is due to the release of selenium from the cofactor by urea, SDS or heating. Electron paramagnetic resonance (EPR) studies have shown the selenium is bound to the molybdenum based on hyperfine interactions between Mo (V) centers and ^{77}Se(9).

A bioinformatic analysis of all bacterial genomes suggested that *E. faecalis* had the genes necessary to activate selenium, however the genes necessary to incorporate selenium into selenocysteine or selenouridine were not present (10, 37). This provided a unique opportunity to learn about the labile cofactor insertion without the background of the two well known uses of selenium in biology. Aside from characterizing a third selenium utilization trait, the gene encoding the putative SDMH is also upregulated during biofilm formation(1). Previous work in our laboratory suggests the putative SDMH in *E faecalis* is a xanthine dehydrogenase (XDH), since under specific growth conditions an increase in xanthine dependent reduction of 2, 6 dichloroindolphenol (DCPIP) was observed.

SDMH enzymes are known to be involved in purine and pyrimidine metabolism. XDH hydroxylates xanthine to form uric acid and this leads to the release of electrons that can be transferred to NAD^+, or to molecular oxygen forming superoxide anions(28). Uric acid can also be used as a nitrogen source (32), and xanthine could be broken down into carbon and energy

sources(23). Xanthine can also be converted into the DNA base guanine, or converted to hypoxanthine, a precursor to adenine.

This study aims to determine whether mutations of the genes in the SDMH operon found in *E. faecalis* V583 are necessary for the synthesis of an active SDMH.

METHODS

Bacterial strains

The bacterial strains used in this study are derivatives of *Enterococcus faecalis* v583. *Escherichia coli* XL-1 Blue was used for cloning experiments.

Materials

The standard 20 amino acids, pantothenic acid, sodium selenite, sodium molybdate, and sodium tungstate were purchased from ICN Biomedicals Inc. (Aurora, OH). Thiamine and biotin were purchased from Sigma-Aldrich (St. Louis, Mo). Riboflavin and nicotinic acid were purchased from Arcos Organic (Geel, Belgium). Magnesium sulfate was purchased from Alfa Aesar (Ward Hill, MA). Tryptic soy broth minus dextrose was purchased from Difco (Sparks, MD). Pyridoxine, KH_2PO_4, K_2HPO_4, sodium citrate, $(NH_4)_2SO_4$, and glucose were purchased from Fisher Scientific (Fair Lawn, NJ). Uric acid was purchased from Avocado Research Chemicals (Lancashire, England). Auranofin was purchased from Alexis Biochemica (San Diego, CA). All restriction enzymes were purchased from New England Biolabs (Ipswich, MA).

Bacterial growth conditions

Media used for growth of *E. faecalis* strains was either a defined media consisting of the following: the 20 standard amino acids (20 mg/L), pantothenic acid (20 mg/L), folic acid (200 μg/L), thiamine (100 mg/L), biotin (20 mg/L), nicotinic acid (2 mg/L), riboflavin (2 mg/L), pyridoxine (20 mg/L), KH_2PO_4 (2 g/L), K_2HPO_4 (7 g/L), sodium citrate (0.5 g/L), $(NH_4)_2SO_4$ (1 g/L), glucose (2 g/L), and $MgSO_4$ (120 mg/L), or a rich medium tryptic soy broth (TSB) or

tryptic soy broth plus glucose (1% glucose, TSBG). Additives to the growth media were as follows: sodium selenite (50 nM), sodium molybdate (1μM), sodium tungstate (1μM), uric acid (1mM) and Auranofin (1μM). Modified Luria broth (tryptone 10g/L, yeast extract 5g/L and NaCl 5g/L) was used for the culture of *E. coli* XL-1 Blue competent cells.

Construction of pLT06 derivatives

A schematic diagram of the construction of pLTO6 derivatives can be seen in Figure 1. Oligonucleotide primers for polymerase chain reaction (PCR) were designed so that approximately 50% of the target gene would be deleted after double homologous recombination. For each gene target two separate PCR reactions (four primers) were used. All primers were designed using Clone Manager from Sci-Ed Software (Cary, NC). A forward primer roughly 1kb 5' to the start codon (UR F) and a reverse primer roughly 25% 3' to the start codon (UR R) were designed. The second pair of primers were designed so that the forward primer would amplify 25% 5' to the stop codon (DR F) and the reverse primer would amplify 1kb 3' to the stop codon (DR R) leading to two separate PCR products per gene. Primers were designed with terminal restriction sites specific for insertion into the integration plasmid pLTO6. The UR F primer had an EcoRI site at the 5' end, the UR R and DR F primers had an XhoI site added that when ligated together it would bring the two PCR products together in frame deleting roughly 50% of the coding sequence of the target gene. An XbaI site was engineered to the DR R primer at the 3' end. PCR was performed on the *E. faecalis* v583 genomic DNA as a template using Finnzymes High Fidelity Phusion DNA Polymerase (Woburn, MA). Melting temperature was determined using the Finnzymes T_m calculator. PCR products were analyzed by 1% agarose gel electrophoresis to confirm the length of the products. PCR products of the correct length were

purified using the IBI Gel/PCR DNA Fragment Extraction Kit (Midsci St. Louis, MO). Purified PCR products and pLTO6 were then digested with EcoRI and XhoI for UR F, UR R products and XbaI and XhoI for DR F, DR R products overnight at 37 °C. Digested PCR products and pLTO6 were then analyzed by agarose gel electrophoresis to determine if there was any star activity. Digested PCR products were subsequently gel. After purification the 3' phosphate ends of pLTO6 were removed by incubation with alkaline phosphatase (NEB). The concentration of DNA for each component of the ligation reaction was determined using a Nanodrop UV-Visible Spectrophotometer (Thermo Scientific Wilmington, DE). The ligation reaction was as follows: 30 µL reaction containing 200 ng of pLTO6 and 12 ng of each insert (UR F, UR R and DR F, DR R). The ligation was subsequently transformed into *E. coli* XL-1 Blue competent cells. Since the permissive temperature of pLTO6 is 30°C all incubations of the vectors were performed at this temperature. Selection of plasmid containing transformants was carried out at 30°C for 48 hours on modified Luria plates supplemented with 15 µg/mL chloramphenicol (Arcos Organics Geel, Belgium). Colonies were inoculated into 1 mL of modified Luria broth supplemented with 15 µg/mL chloramphenicol. Plasmids were then isolated using a hurricane mini prep kit (Gerard Biotech Oxford,OH), and double digested to release a fragment of the insert (UR F, UR R and DR F, DR R). The size of the released fragment was confirmed using agarose gel electrophoresis. Sequencing primers for the pLTO6 derivatives were constructed using clone manager so that the forward primer would encompass the UR F and DR RE restriction sites as well as roughly 100 nucleotides of the vector itself. Sequencing primers were forward 5' AACCGTGTGCTCTAC 3' and reverse 5' GGCGCTTCATAGAGT 3'. Plasmids were sequenced by Genewiz (South Plainfield, NJ). Sequence data was aligned using clone manager to determine correct in frame insertion of the PCR products resulting in a gene deletion.

Table 1: Primers for pLTO6 derivatives

HP1 DR R	5’ ATATCTAGACACTTCTGTGGGCATACC 3’
HP1 DR F	5’ GTTCTCGAGCCTCTGACAGGGACTTTG 3’
HP1 UR R	5’ AACCTCGAGCCGAATGGCCAGAGGACA 3’
HP1 UR F	5’ GCTGAATTCCAACAATTAGCGGAGAAG 3’
HP2 DR R	5’ CCCTCTAGACCAACTGCACGCTACAGA 3’
HP2 DR F	5’ TTGCTCGAGGAGAGCCAAGGCCAACGA 3’
HP2 UR R	5’ GTTCTCGAGAGCGGTTCCTGCTAAAGT 3’
HP2 UR F	5’ CCAGAATTCTCTGGCGGACTGTTAATT 3’
nifS DR R	5’ TTGTCTAGATGGCAGAACCTCGCCAAT 3’
nifS DR F	5’ GGGCTCGAGGCAACTGGCACCTTACGT 3’
nifS UR R	5’ CGTCTCGAGAATCTGAGCCGCACTGGG 3’
nifS UR F	5’ TATGAATTCGCTGCTGAAGTAGCTATT 3’
selD DR R	5’ CAATCTAGAGACGCGAACAGCCACCTT 3’
selD DR F	5’ CTGCTCGAGAGTGTCGCTGCTGACGAA 3’
selD UR R	5’ ATGCTCGAGTCCGGTTACCGCTAGACC 3’
selD UP F	5’ TATGAATTCTATGCAGCGACTTCCTAT 3’
sirA DR R	5' TCGTCTAGACGCCCCCTCCGCGAACAA 3’
sirA DR F	5’ CAACTCGAGGCGGAGAAGGGAACCACG 3’
sirA UR R	5’ GACCTCGAGCTCGATAGTGCCTCCAGC 3’
sirA UR F	5' CTAGAATTCGAAGGAGCGGACAGCATG 3’
gene X DR R	5’ AGCTCTAGAAATCGGTGCCATTCCTCG 3’
gene X DR F	5’ GTGCTCGAGGACCTTGGGGAAAGGAGG 3’
gene X UP R	5’ ACCCTCGAGACAGCCATCCGTCAATGA 3’
gene X UR F	5’ GCTGAATTCGCTCGATCCTACGCAGTA 3’
XDH DR R	5’ TGTTCTAGATCGCCAAGCTCGCTGAAT 3’
XDH DR F	5’ GGACTCGAGAACGCCACTCAAGTACCA 3’
XDH UP R	5’ TAACTCGAGACATGCACCGCAAGAGCC 3’
XDH UP F	5’ AGAGAATTCGTTTCCGCCGAAGGTAGA 3’
XDHc DR R	5’ CATCCGAGCCACTTCCCG 3’
XDHc DR F	5' ATTCTCGAGACGGCGGACGATTATGGT 3'
XDHc UR R	5’ AACCTCGAGGGCTATTCTACCTTCGGC 3’
XDHc UR F	5’ GTAGAATTCTTTACGCTTGTGGTGATG 3’

Biofilm Assay (20-22)

200μL cultures (1:4 inoculum) of the seven different media conditions (TSBG +/- sodium selenite, sodium molybdate, sodium tungstate, uric acid and auranofin) in a 96 well plate was used to determine biofilm density. The cultures were incubated at 37℃ for 48 hours. Planktonic cells were removed by inverting the plates. Biofilms were then washed with 200 μL of dH_2O by inverting and gently shaking. Biofilms were dried at 37 °C for 30 minutes. Biofilms were stained with 200μL of 0.1% safranine and incubated at 37 °C for 30 minutes. The safranine stain was discarded by inverting and gently shaking, and the stained biofilms were washed with 200 μL of dH_2O three times. Any leftover dH_2O within the wells was removed by carefully pipetting out the dH_2O while not disturbing the stained biofilm. The stained biofilms were then dried at 37 °C for 30 minutes. The safranine stain was dissolved using 200 μL 80:20 100% ethanol: acetone followed by addition of 50 μL of 10% SDS. The plates were allowed to stand for 5 minutes at room temperature. Biofilm density was measured using UV-visible spectrophotometry (absorbance at 490nm) using a SpectraMax 190 (Molecular Devices Sunnyvale, CA).

Hydrogen peroxide determination in culture media(3)

Cultures (5 mL) were grown in TSBG +/- 50nM selenite in a 6 well culture dish for 48 hours at 37 °C. Extracellular hydrogen peroxide was determined using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen Carlsbad, CA). The culture medium (500 μL) was clarified by centrifugation for 5 minutes at 5000 rpm. Culture free media was then diluted in reaction buffer until the level of peroxide obtained was within the standard curve (0 μM to 5 μM hydrogen peroxide). Fluorescence of resorufin dye was determined using a Cary Eclipse

RESULTS

Biofilm production by Enterococcus faecalis v583 is increased by selenium supplementation.

Selenium supplementation of *E. faecalis* v583 cultures was utilized in order to determine the potential role of gene products encoded within the SDMH operon in biofilm formation. The gene encoding the molybdoenzyme has been shown to be specifically upregulated in cells that are within the biofilm(1) . We observed using a modified safranine staining that the addition of selenite leads to an increase in biofilm formation in *E. faecalis* v583 (Figure 2). In defined media (DM), the addition of selenite (DM-Se) leads to an approximately 1.5 fold increase in biofilm density as compared to defined media alone. However the addition of tungstate with selenite in defined media caused biofilm levels to decrease below defined media alone. This is likely due to tungsten impairing molybdenum transport and thus incorporation into the molybdoenzyme(18). The addition of auranofin in a defined media plus selenite also causes a decrease in biofilm formation to defined media levels. This is likely due to the formation of a stable auranofin-selenium product in culture media inhibiting selenium transport into the cell (16).

In tryptic soy broth (TSB) the density of the biofilm produced by *E. faecalis* v583 is decreased slightly relative to defined media alone. The addition of selenite provides no gain in biofilm density in tryptic soy broth (TSB-Se). The addition of tungsten or auranofin to TSB-Se medium produces no difference in biofilm formation as compared to TSB. This shows that some nutrient is missing in the TSB medium that is essential for biofilm formation in the presence of added selenite.

Tryptic soy broth plus glucose (TSBG) provides a more robust basal biofilm than DM or TSB. The addition of selenite (TSBG-Se) leads to an approximately 2.5 fold increase in biofilm density. This robust biofilm that is seen in the presence of TSBG-Se is inhibited with the addition of tungsten or auranofin. This robust biofilm and its subsequent inhibition correlate with xanthine dependent reduction of dichloroindolphenol, suggesting the molybdoenzyme plays a role in biofilm formation.

Gene products encoded within the SDMH operon are essential for selenium-dependent biofilm formation

Based on growth data of wild type *E. faecalis* (Figure 2) and the potential role of the SDMH in biofilm formation(1) we decided to isolate in-frame deletions of genes within the SDMH operon to deduce their role in SDMH synthesis and potentially biofilm formation. Selection of in frame deletions was performed as described in the methods section using a temperature sensitive base vector pLTO6(36) . pLTO6 is a temperature sensitive suicide vector that can replicate in *Escherichia coli* and *Enterococcus faecalis*. It contains a temperature sensitive replication of origin that allows for vector replication outside the chromosome at 30 °C, and thus facilitates recombination into the chromosome at 42 °C. pLTO6 contains a positive selection resistance allele for chloramphenicol (*cat)* as well as the *lacZ* gene encoding for β-galactosidase that will cleave 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) a modified sugar leading to blue colonies. A negative selectable marker $pheS^{*}$ is present in pLTO6 that changes the specificity of the phenylalanine tRNA synthetase to allow for the charging of the phe-tRNA with 4-chloro-DL-phenylalanine. Cells containing the pLTO6 vector after the

temperature shift are plated on media with 4-chloro-DL-phenylalanine result in death due to the insertion of this modified amino acid that disrupts protein folding and protein:protein interacts by changing weak forces within the proteins themselves(19). Mutant strains were constructed by inserting two PCR products that amplified upstream and downstream of the target gene but only amplified approximately 25% of the target gene. These PCR primers were linked together using a novel XhoI site that was engineered into the PCR products and inserted into the vector through pLTO6's intrinsic EcoRI and XbaI sites. This lead to the construction of pLTO6 derivatives: pCM1 (Δ*selD*), pCM2 (Δ*xdh*), pCM3 (Δ*hp1*), pCM4 (Δ*hp2*), pCM5 (Δ*nifS*), pCM6 (Δ*gene X*), and pCM7 (Δ*sirA*).

To date, five of the deletion constructs have been integrated and selected in *E. faecalis* v583, however only the Δ*selD,* Δ*hp2,* Δ*gene X,* and Δ*sirA* were isolated thus far. The Δ*nifS* strain might be an essential gene in *E. faecalis*, based on the 22 clones that have been isolated (wild type revertant) without obtaining a mutant allele. This may due to role of NifS as a desulfurase enzyme that removes sulfur atoms from cysteine for iron-sulfur cluster formation, in addition to its likely role in the removal of selenium from selenocysteine. It could be that the NifS protein in *E. faecalis* is needed for construction of many of the iron-sulfur clusters in this organism(17).

In tryptic soy broth plus glucose (TSBG) deletion of the *selD* gene (Figure 3) has no effect on glucose dependent biofilm density. However the selenium-dependent increase in biofilm density was abolished in the Δ*selD* strain. The addition of uric acid or molybdate did not enhance biofilm density in the wild type nor did it affect biofilm density in the Δ*selD* mutant. Likewise addition of tungstate or auranofin also did not affect the Δ*selD* mutant. This result leads to the inference that the activation of selenium to selenophosphate by the selenophosphate synthetase (SelD) is essential for selenium-dependent biofilm formation. We presume the SDMH

encoded within this operon is nonfunctional in a *selD* mutant. However the role for the SDMH in biofilm formation is not yet known.

The functions of the other gene products within the SDMH operon are unknown. Thus the deletion of the genes could elucidate whether they are required for SDMH maturation or selenium-dependent biofilm formation. Mutations in *gene X* (Figure 4), *sirA* (Figure 5), or *hp2* (Figure 6) have the same phenotype as a *selD* mutant. This leads to conclusion that these proteins are either involved in the synthesis of a mature SDMH or have a novel function for the increased selenium-dependent biofilm density.

Extracellular hydrogen peroxide production is decreased in the presence of SDMH operon mutants.

It is well know that xanthine oxidase, a well studied molybdenum hydroxylase, has the ability to produce both superoxide and hydrogen peroxide in the presence of xanthine and oxygen by directly reducing the oxygen though its flavin (FAD) cofactor(7). Extracellular hydrogen peroxide production is not well understood in bacteria. However it is know that *E. faecalis* does produce large amounts of superoxide that dismutes into hydrogen peroxide but the mechanism of this ROS production is unknown(14).

In wild type *E. faecalis* v583 the addition of selenite led to a drastic increase in hydrogen peroxide levels in the culture medium (Figure 7). This effect of selenium supplementation on extracellular peroxide production was abolished when the culture medium was supplemented with tungstate or auranofin.

A *selD* deletion mutant of *E. faecalis* did not exhibit increased extracellular hydrogen peroxide when selenite was added to the culture medium (Figure 9). However wild type revertant

strains under the same condition exhibited a nearly ten-fold increase in extracellular hydrogen peroxide levels. The addition of tungsten or auranofin did not reduce basal levels of peroxide in the *selD* deletion strain. This leads to the conclusion that the activation of selenium is necessary for the increase in extracellular hydrogen peroxide production. Given the well established ability of molybdenum hydroxylases to produce superoxide, it is likely that the SDMH is responsible for the extracellular hydrogen peroxide production.

As stated previously, the function of *hp2* (Figure 8), *gene X* (Figure 10), and *sirA* (Figure 11) are unknown at this time. However, deletion of these genes exhibits the same phenotype as the *selD* mutant with respect to extracellular hydrogen peroxide production. Thus it can be inferred that these genes that are within the SDMH operon are involved directly in the maturation of the SDMH, the enzyme most likely responsible for the increase in extracellular hydrogen peroxide.

DISCUSSION

In this study we set out to determine whether deletion of the gene products in the SDMH operon in *Enterococcus faecalis* lead to a discernable phenotype. Previous work on *E. faecalis* biofilms shows that the addition of glucose causes an increase in biofilm density (31). In our study we show that the addition of both glucose and selenite to the culture medium leads to a substantially denser biofilm than with glucose alone. We believe that this increase in biofilm density can be attributed to the putative xanthine dehydrogenase (XDH) encoded within the SDMH operon due to previous work done by Ballering et al(1). This hypothesis is strengthened by the addition of selenite leading to a significantly enhanced biofilm density, since the xanthine dehydrogenase is a selenium-dependent molybdenum hydroxlyase (SDMH). Thus the addition of selenite correlates with the biofilm data presented and supports previous work stating that this putative XDH is upregulated in biofilm formation.

The bioinformatic analysis of the *E. faecalis* genome showed that the genes clustered around the XDH are within an operon. We show in this study that deletion of genes within the SDMH operon specifically the *hp2, gene X, sirA* and *selD* have a negative effect on selenium-dependent biofilm density as compared to their wild type revertant counterparts. This leads to the conclusion based on the previous data of *E. faeclais* v583 biofilm density that these genes are likely involved in the maturation of the labile selenium cofactor. The function of the *selD* gene is known in that it is a selenophosphate synthetase and its role in activating selenium for insertion into labile selenoenzymes has never been established. This work shows that the activation of selenium to selenophosphate os required for the production of an active SDMH. The functions of

the other genes at this time are still unknown however their deletion leads to a phenotype of abolishing the selenium-dependent biofilm and thus must be important in the maturation of the SDMH.

Production of superoxide is a known characteristic of this class of molybdoenzymes. Thus the amount of extracellular hydrogen peroxide, the breakdown product of superoxide, was shown to correlate with selenium-dependent biofilm formation. Wild type *E. faecalis* v583 and wild type revertant strains all produced substantial amounts of extracellular hydrogen peroxide in the presence of added selenium. This extracellular hydrogen peroxide correlated with higher biofilm density. This extracellular hydrogen peroxide production was again abolished in the deletion strains for *hp2, gene X, sirA* and *selD*. As stated previously the function of the *hp2, gene X,* and *sirA* at this time are unknown. However this study shows that they are required for increased extracellular hydrogen peroxide formation in selenium-dependent biofilm formation. We speculate that the SDMH is responsible for hydrogen peroxide production (14), and therefore involved in the fratricidal mechanism of biofilm formation of this nosocomial pathogen(35). These results and mutant constructs, establish a foundation for long term study of the of SDMH in biofilm physiology.

APPENDIX: FIGURES

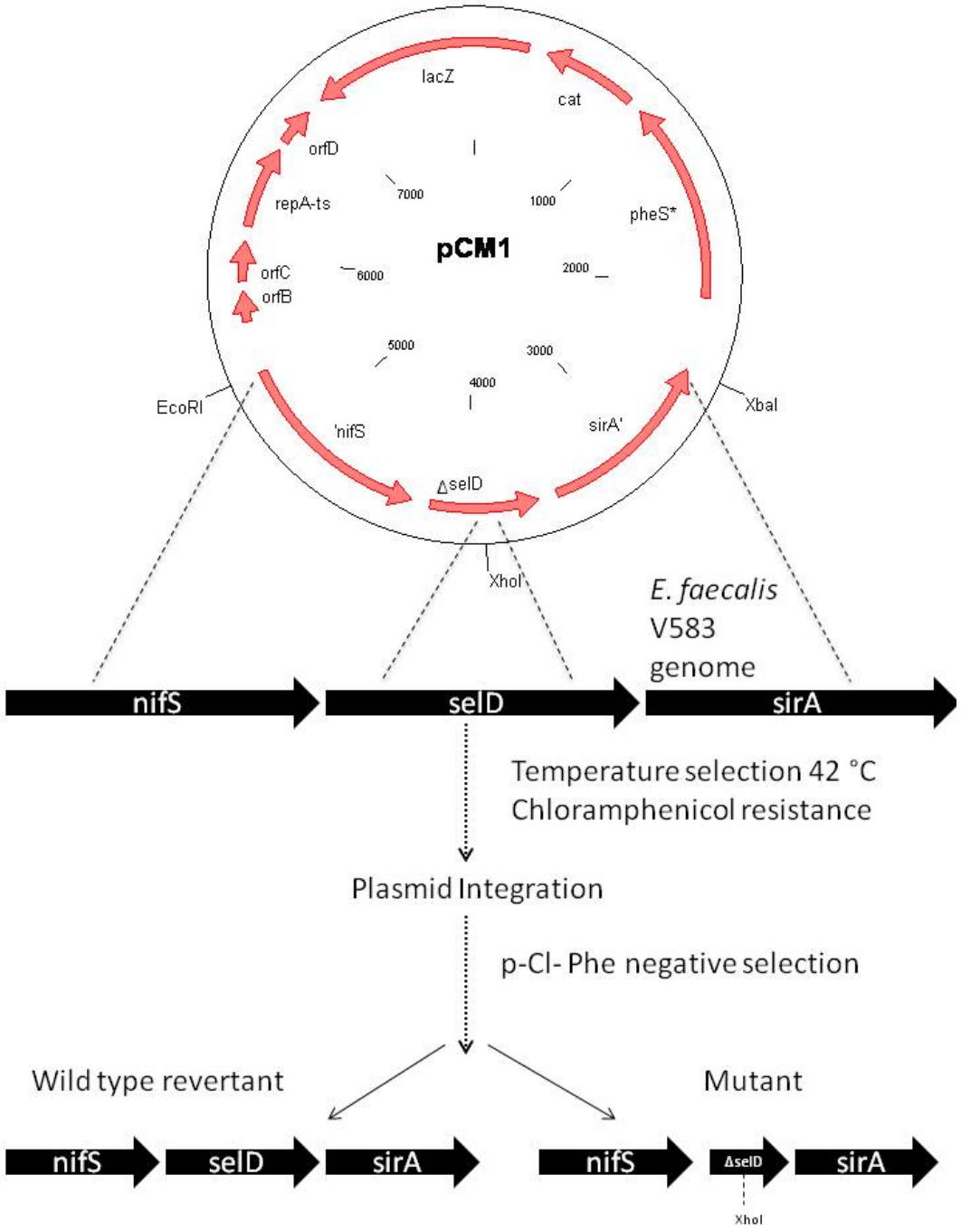

Figure 1: Selection of pLTO6 derived mutant strains of *E. faecalis* v583

The construct pCM1, which targets and in-frame deletion of the *selD* gene is shown as a representative example.

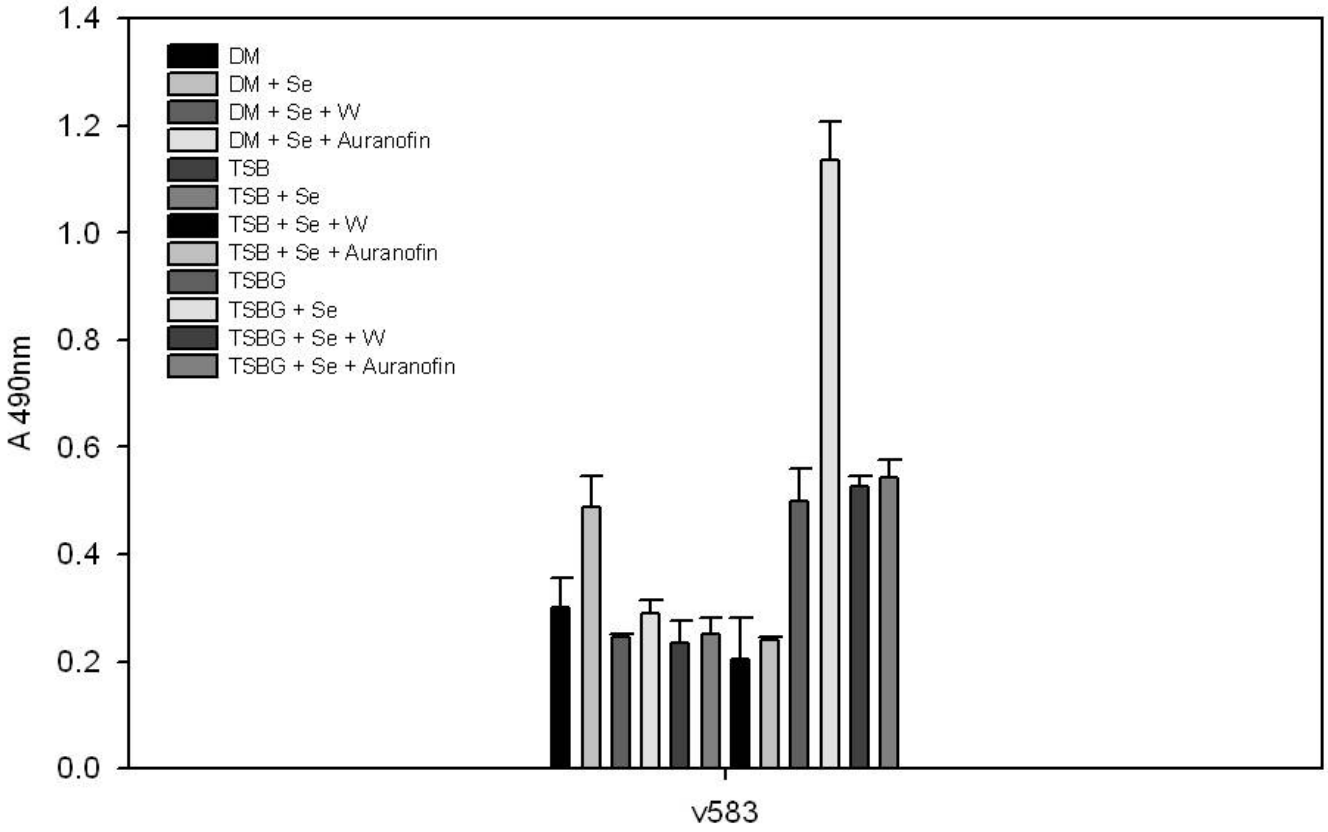

Figure 2: Selenium supplementation leads to enhanced biofilm production by E. faecalis v583

E. faecalis v583 were cultured for 48 hours at 37°C in a 96 well plate in defined media (DM), tryptic soy broth (TSB) or tryptic soy broth with glucose (TSBG). Selenite was added to a final concentration of 50 nM, tungsten 1 μM, and auranofin 1 μM. Biofilm density was determined using a safranine staining method, see methods for details.

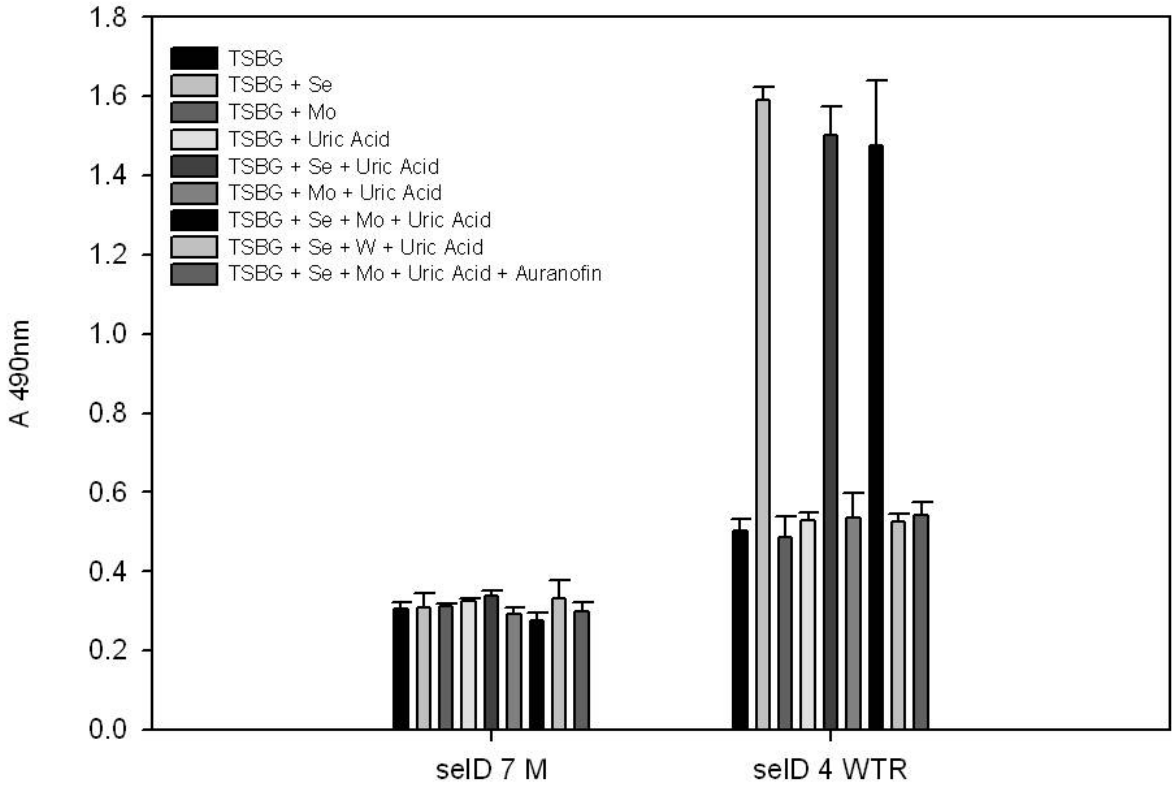

Figure 3: Mutation of the *selD* gene abolishes selenium-dependent biofilm formation

Δ*selD* and wild type revertant strains were cultured for 48 hours at 37°C in a 96 well plate in tryptic soy broth + 1% glucose (TSBG). Selenite was added to a final concentration of 50 nM, tungsten 1 μM, and auranofin 1 μM. Biofilm density was determined using a safranine staining method as described in the methods section.

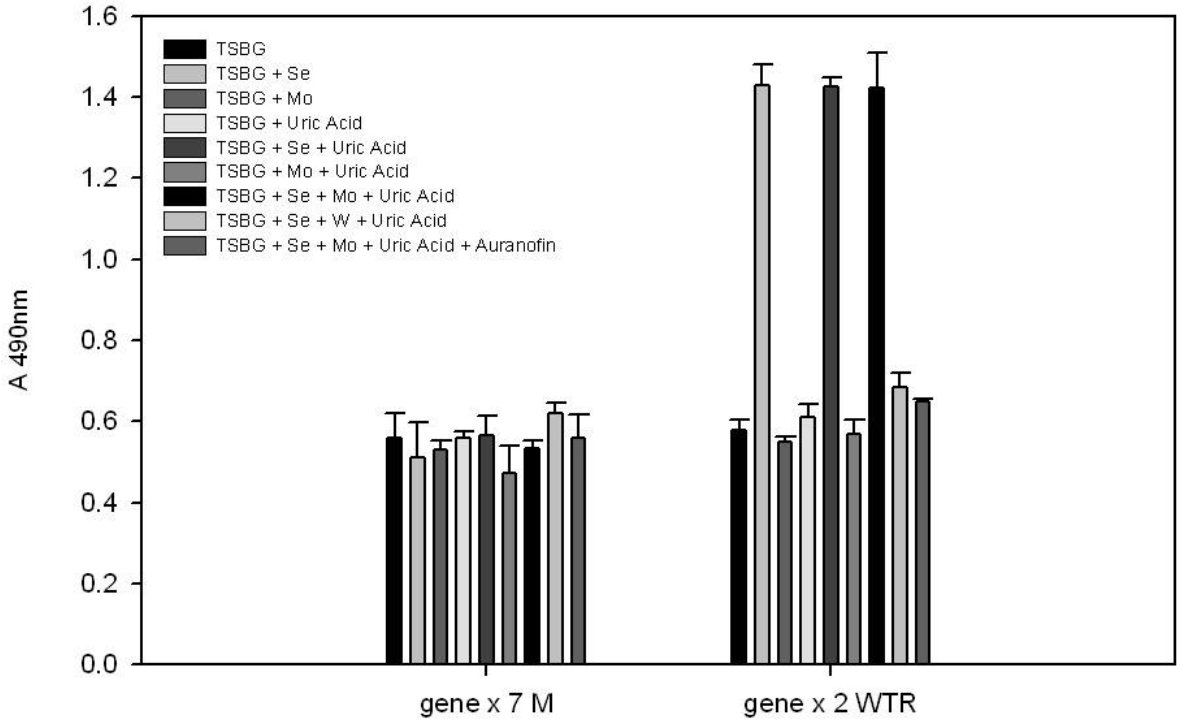

Figure 4: Mutation of *gene x* abolishes selenium-dependent biofilm formation

Δ*gene x* and wild type revertant strains were cultured for 48 hours at 37°C in a 96 well plate in tryptic soy broth + 1% glucose (TSBG). Selenite was added to a final concentration of 50 nM, tungsten 1 μM, and auranofin 1 μM. Biofilm density was determined using a safranine staining method as described in the methods section.

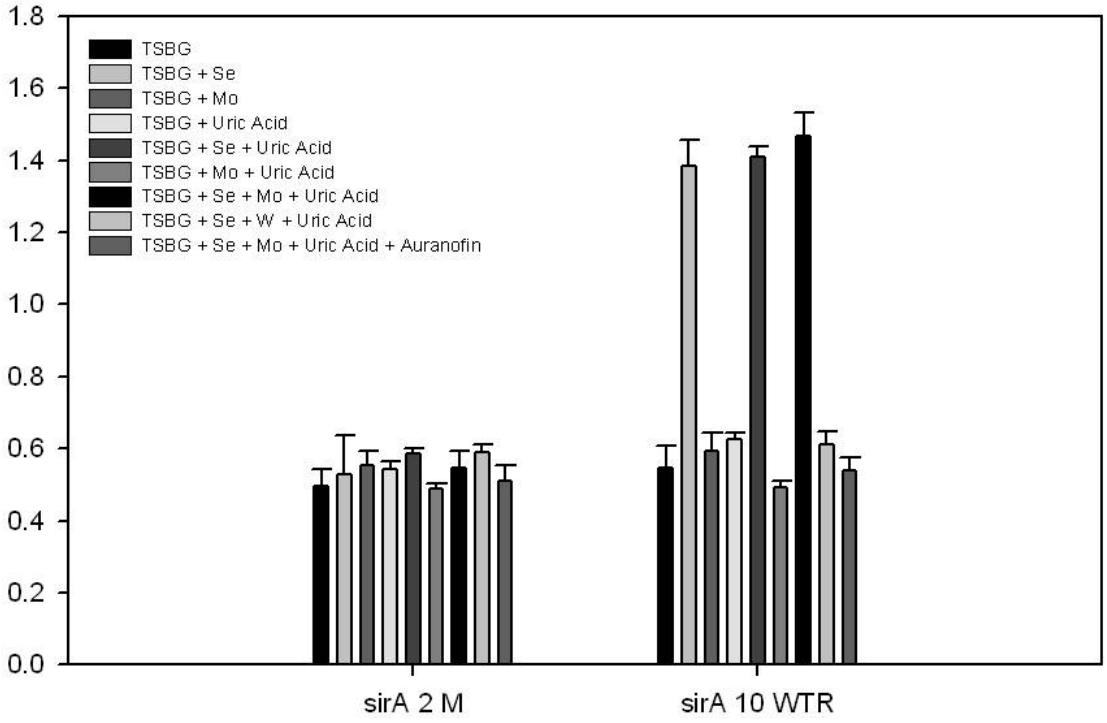

Figure 5: Mutation of the *sirA* gene abolishes selenium-dependent biofilm formation

Δ*sirA* and wild type revertant strains were cultured for 48 hours at 37°C in a 96 well plate in tryptic soy broth + 1% glucose (TSBG). Selenite was added to a final concentration of 50 nM, tungsten 1 μM, and auranofin 1 μM. Biofilm density was determined using a safranine staining method as described in the methods section.

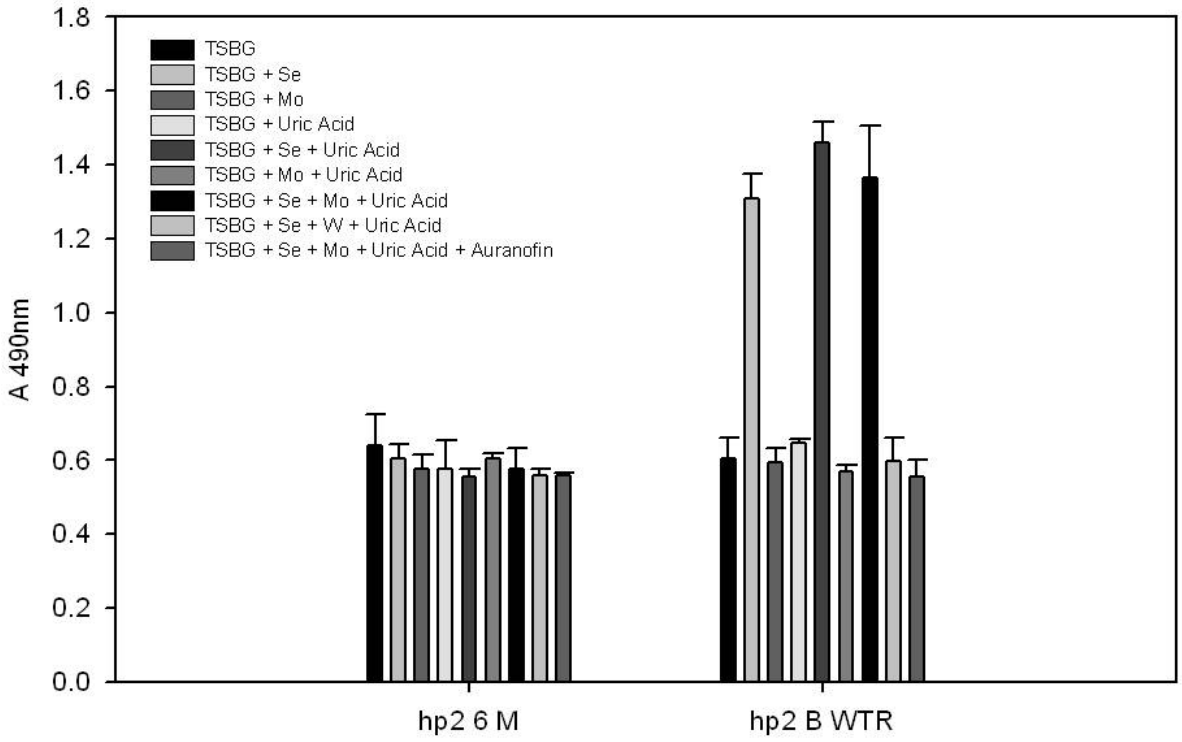

Figure 6: Mutation of the *hp2* gene abolishes selenium-dependent biofilm formation

Δ*hp2* and wild type revertant strains were cultured for 48 hours at 37°C in a 96 well plate in tryptic soy broth + 1% glucose (TSBG). Selenite was added to a final concentration of 50 nM, tungsten 1 μM, and auranofin 1 μM. Biofilm density was determined using a safranine staining method as described in the methods section.

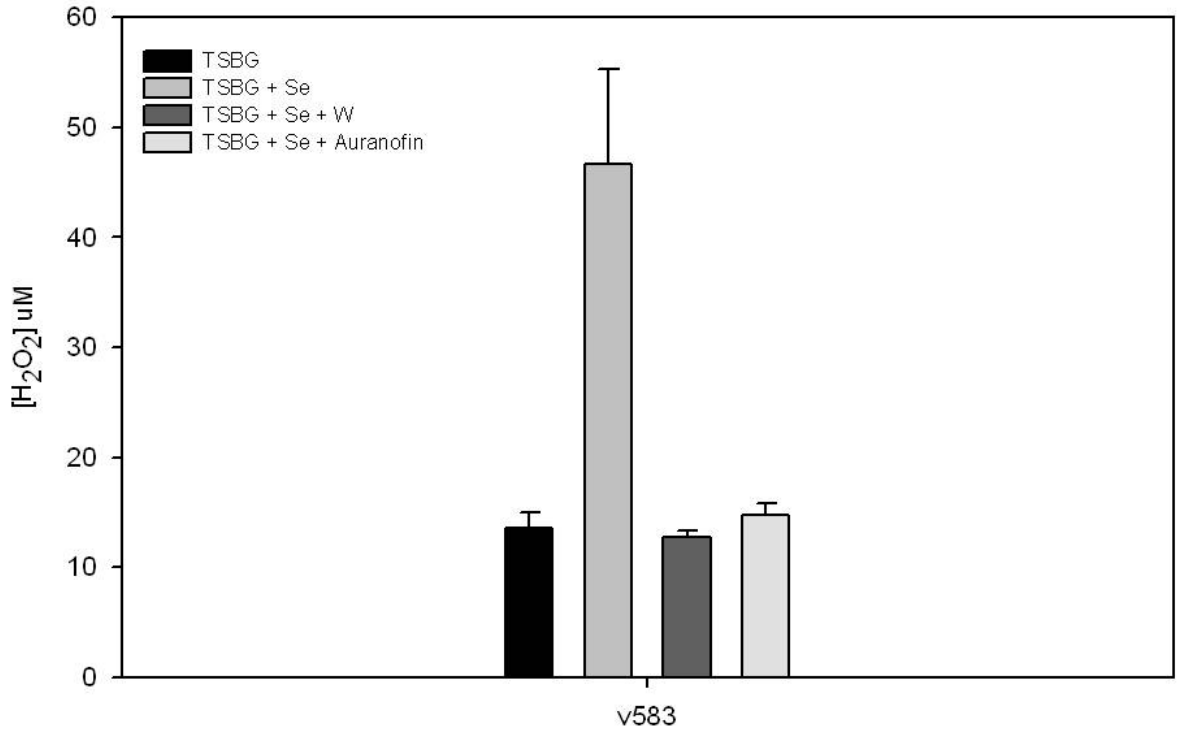

Figure 7: Selenium supplementation leads to enhanced extracellular hydrogen peroxide production

E. faecalis V583 cultures were incubated at 37°C for 48 hours in 6 well dishes of differing conditions of tryptic soy broth + 1% glucose (TSBG). Selenite was added to a final concentration of 50 nM, tungsten 1 μM, and auranofin 1 μM. The concentration of hydrogen peroxide in the media was determined using the Invitrogen Amplex Red Kit as described in the methods.

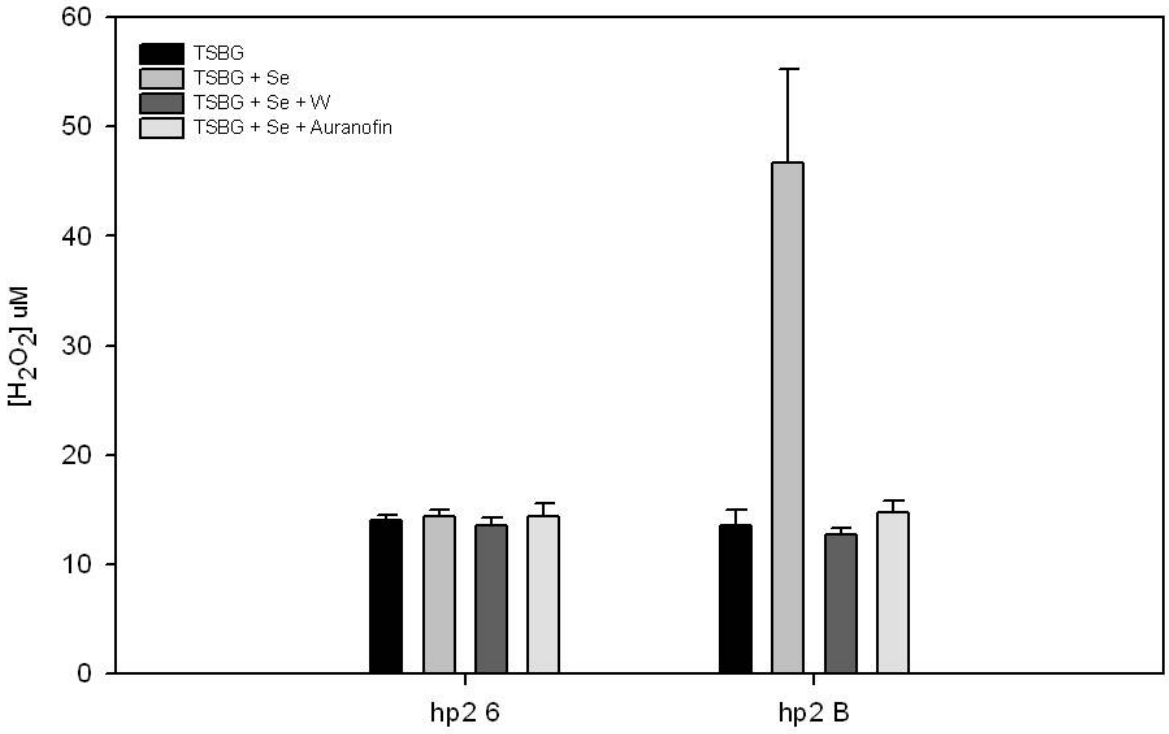

Figure 8: Deletion of the *hp2* gene leads to a significant decrease in extracellular hydrogen peroxide production

Δ*hp2* and wild type revertant cultures were incubated at 37°C for 48 hours in 6 well dishes of differing conditions of tryptic soy broth + 1% glucose (TSBG). Selenite was added to a final concentration of 50 nM, tungsten 1 μM, and auranofin 1 μM. The concentration of hydrogen peroxide in the media was determined using the Invitrogen Amplex Red Kit as described in the methods.

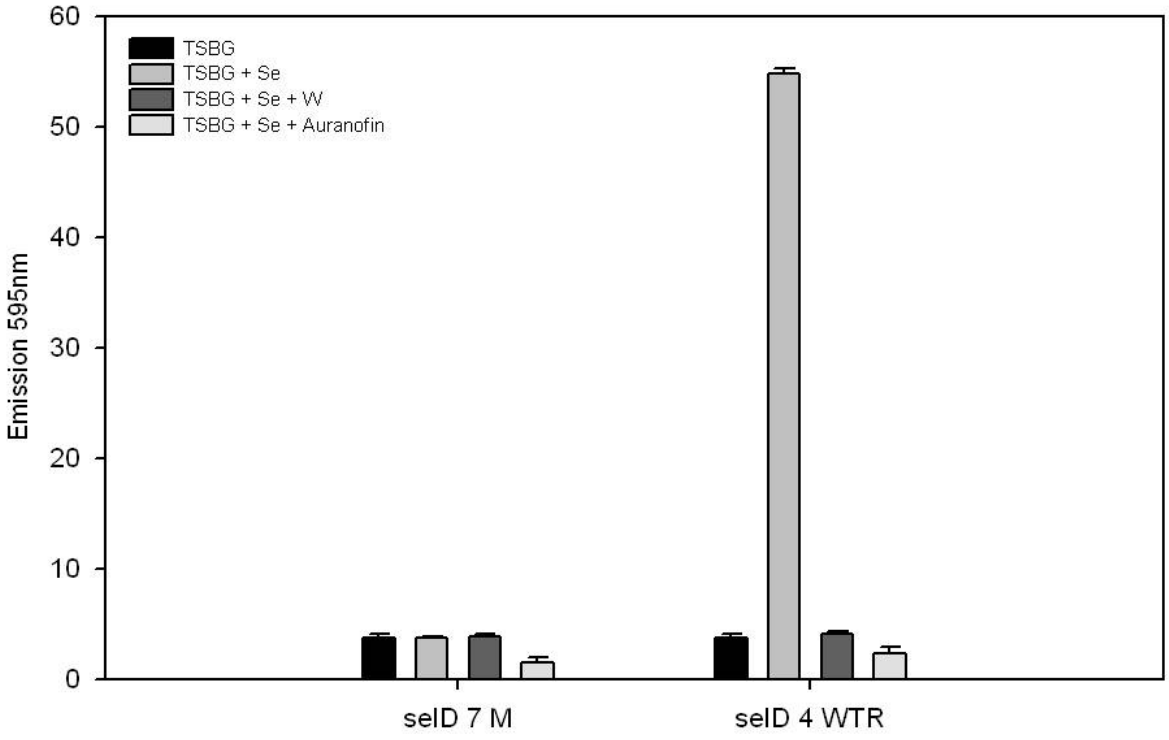

Figure 9: Deletion of the *selD* gene leads to a significant decrease in extracellular hydrogen peroxide production

Δ*selD* and wild type revertant cultures were incubated at 37°C for 48 hours in 6 well dishes of differing conditions of tryptic soy broth + 1% glucose (TSBG). Selenite was added to a final concentration of 50 nM, tungsten 1 μM, and auranofin 1 μM. The concentration of hydrogen peroxide in the media was determined using the Invitrogen Amplex Red Kit as described in the methods.

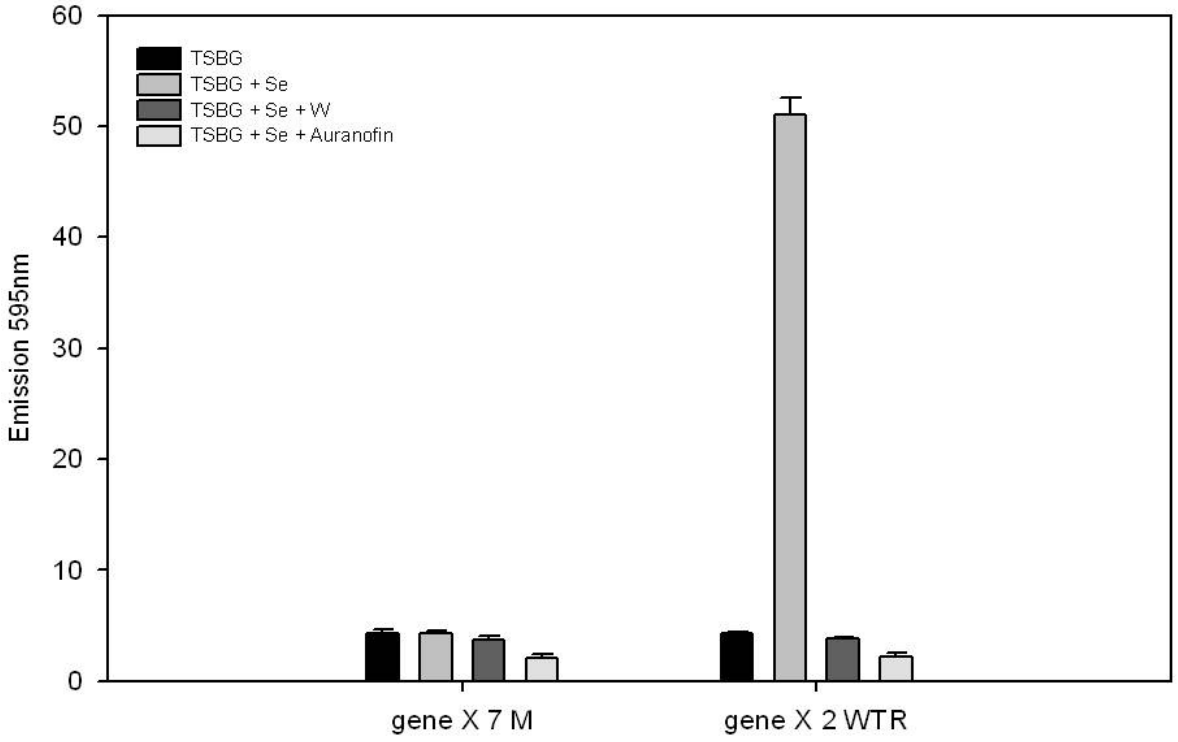

Figure 10: Deletion of *gene X* leads to a significant decrease in extracellular hydrogen peroxide production

Δ*gene X* and wild type revertant cultures were incubated at 37°C for 48 hours in 6 well dishes of differing conditions of tryptic soy broth + 1% glucose (TSBG). Selenite was added to a final concentration of 50 nM, tungsten 1 μM, and auranofin 1 μM. The concentration of hydrogen peroxide in the media was determined using the Invitrogen Amplex Red Kit as described in the methods.

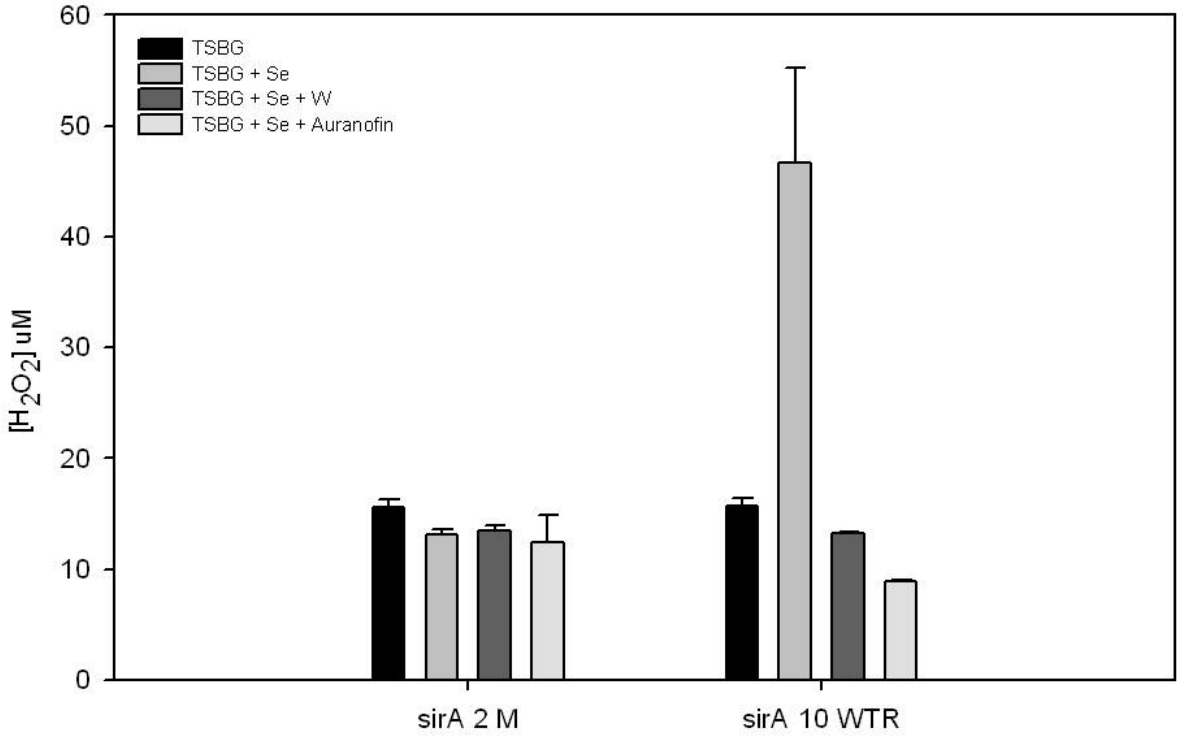

Figure 11: Deletion of the *sirA* gene leads to a significant decrease in extracellular hydrogen peroxide production

Δ*sirA* and wild type revertant cultures were incubated at 37°C for 48 hours in 6 well dishes of differing conditions of tryptic soy broth + 1% glucose (TSBG). Selenite was added to a final concentration of 50 nM, tungsten 1 μM, and auranofin 1 μM. The concentration of hydrogen peroxide in the media was determined using the Invitrogen Amplex Red Kit as described in the methods.

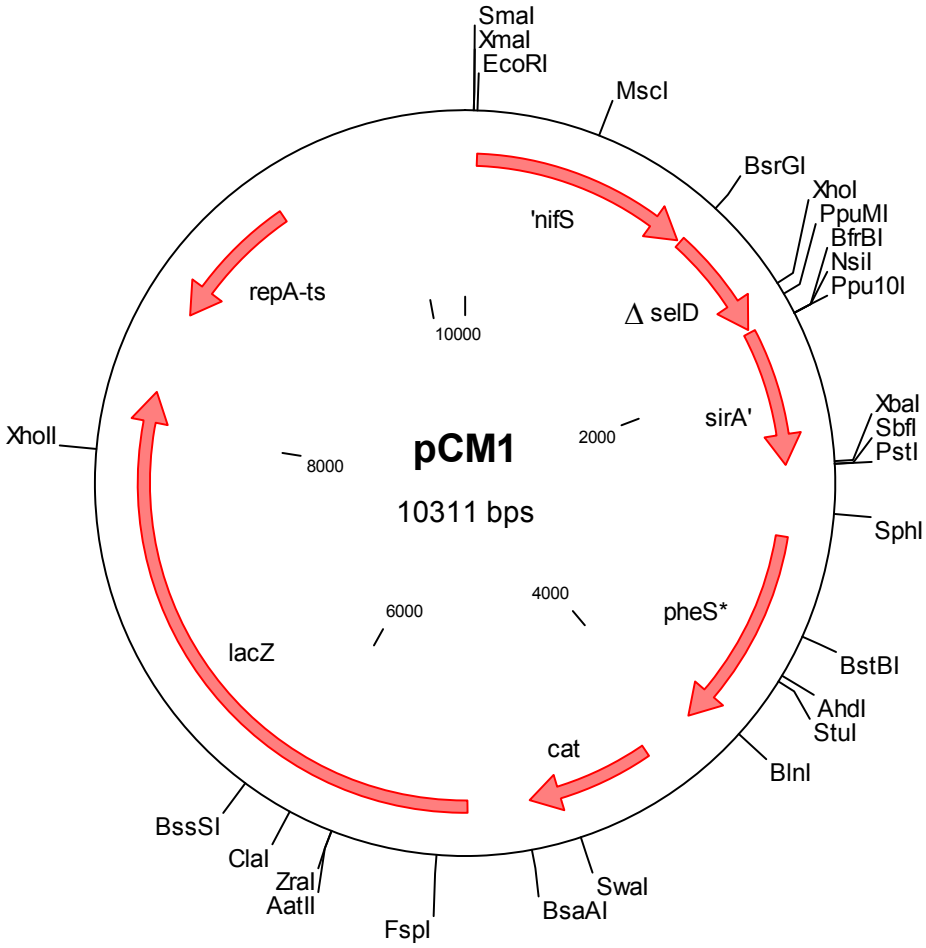

Figure 12: Construction of suicide vector to obtain Δ*selD* mutation

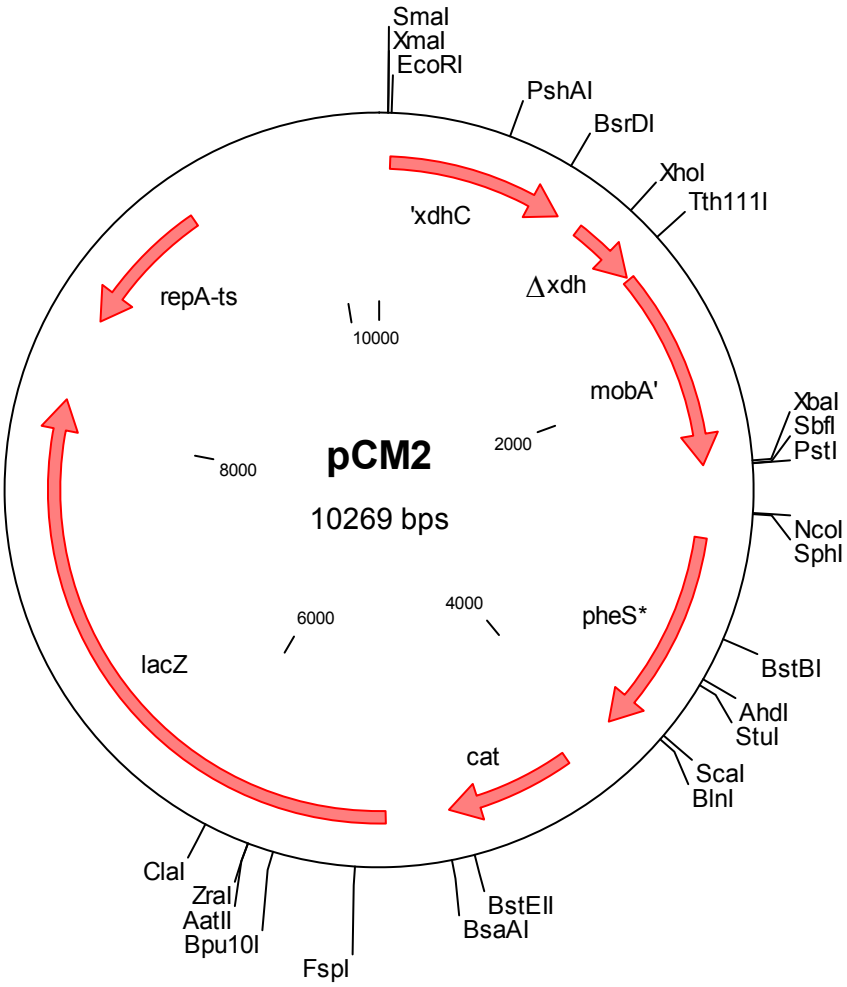

Figure 13: Construction of suicide vector to obtain Δ*xdh* mutation

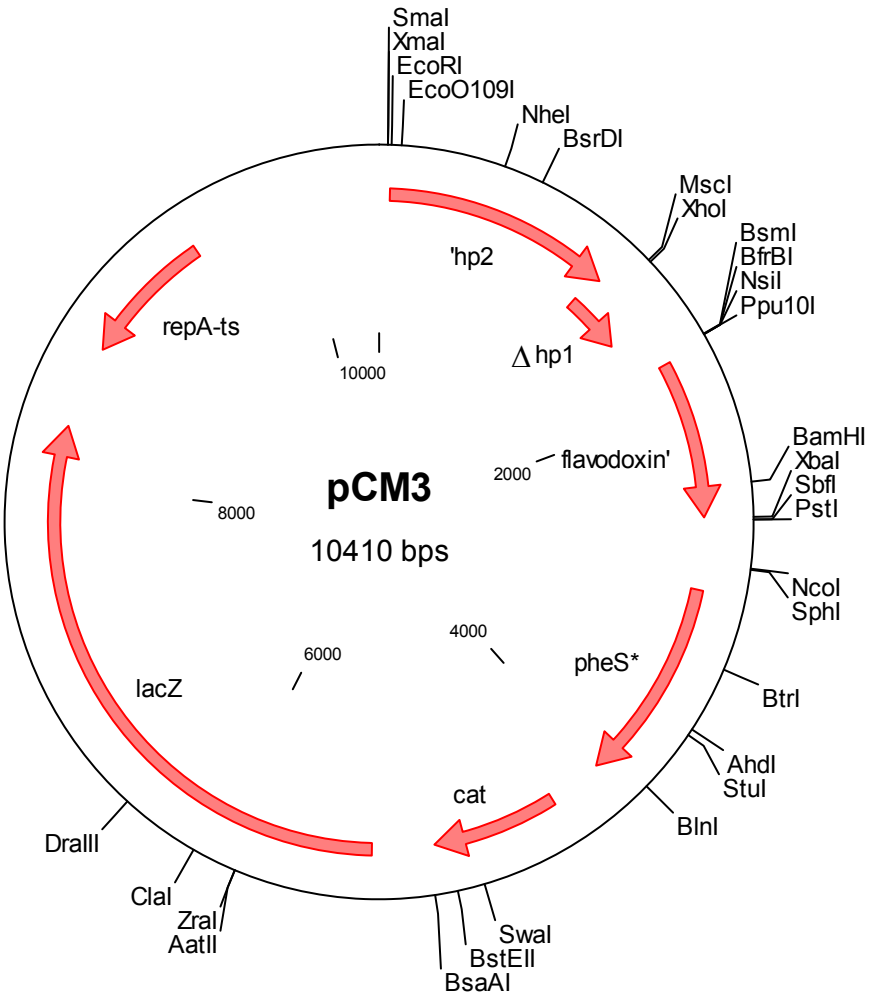

Figure 14: Construction of suicide vector to obtain Δ*hp1* mutation

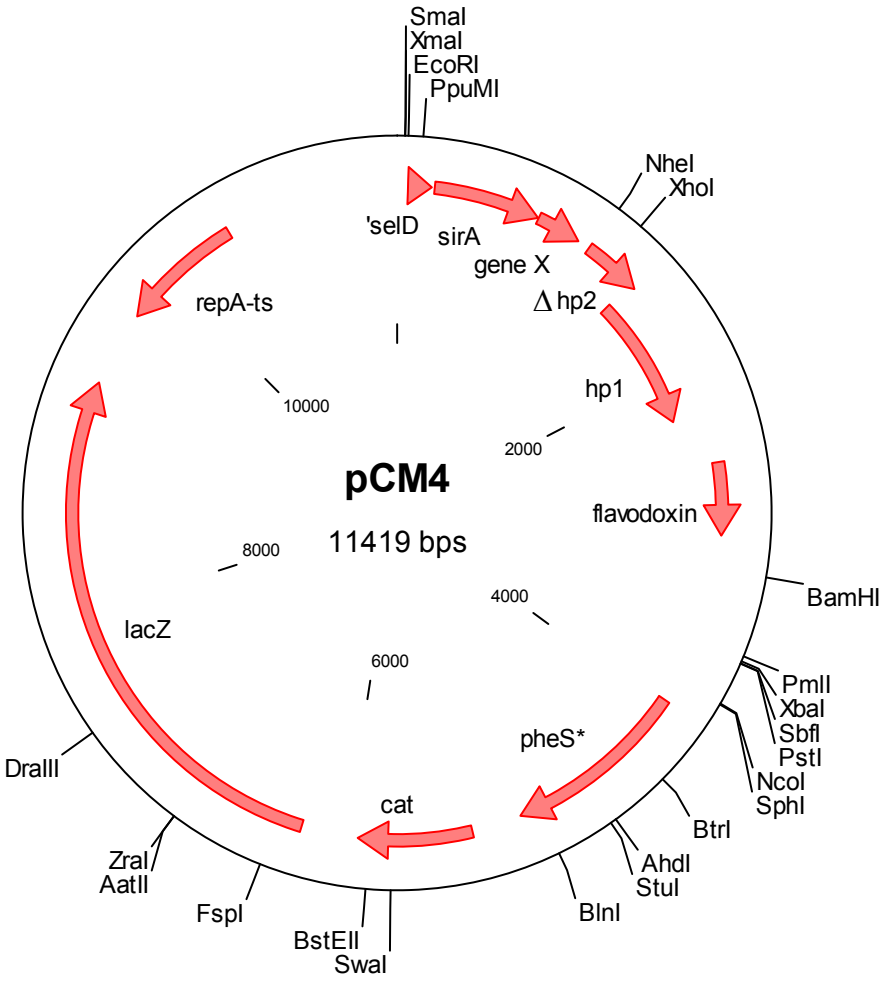

Figure 15: Construction of suicide vector to obtain Δ*hp2* mutation

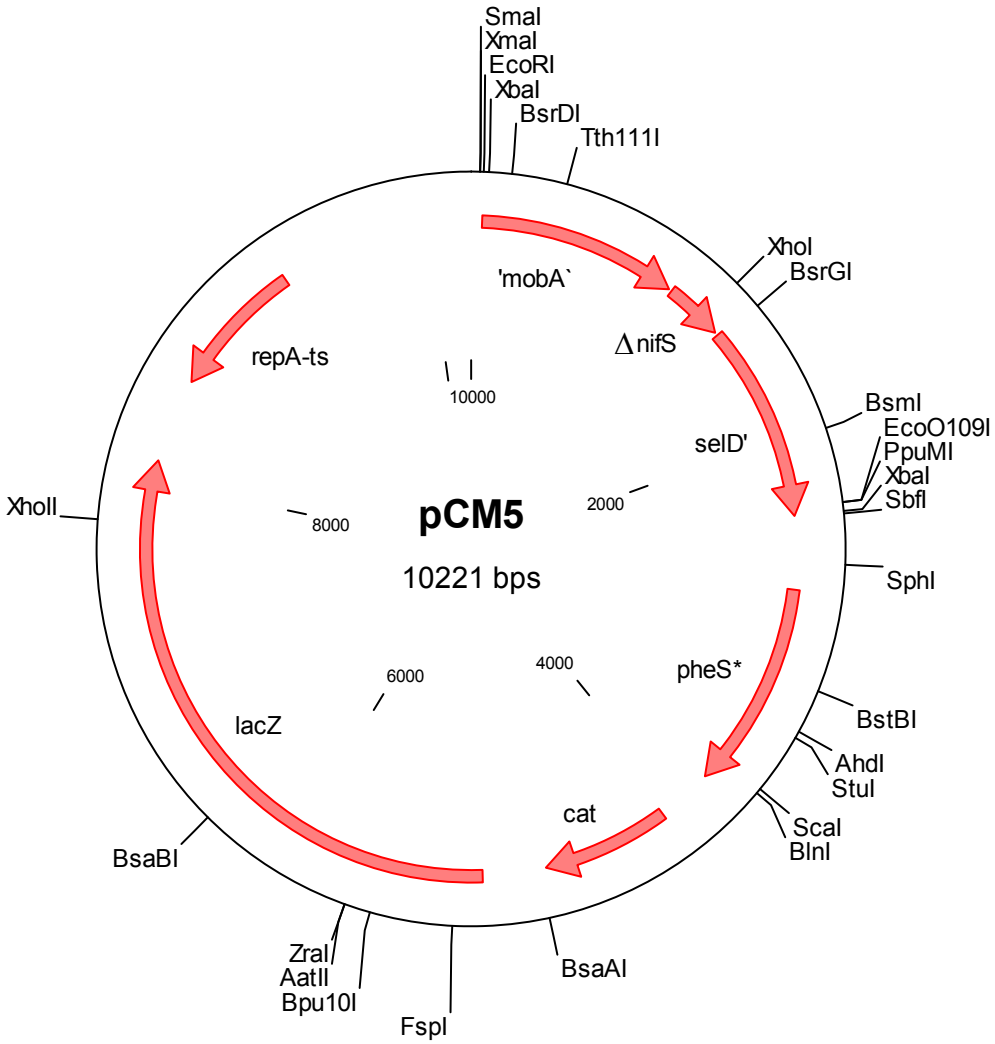

Figure 16: Construction of suicide vector to obtain Δ*nifS* mutation

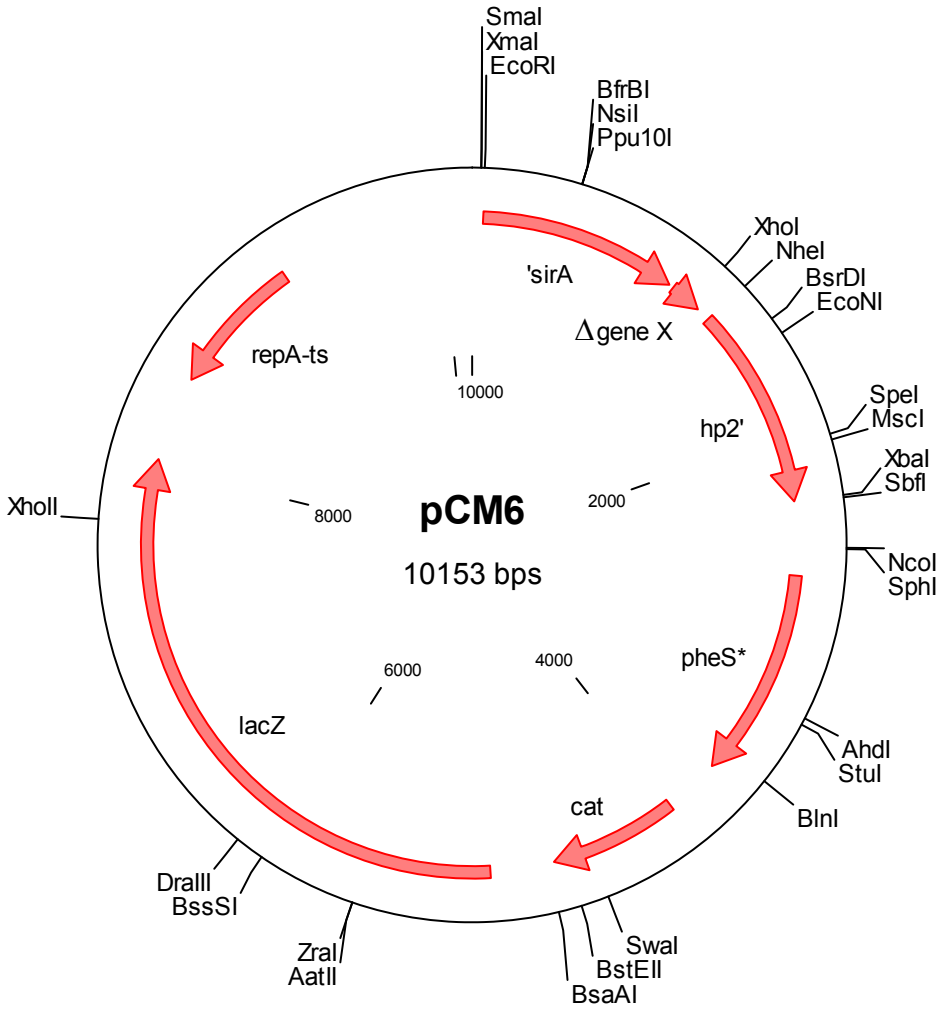

Figure 17: Construction of suicide vector to obtain Δ*gene X* mutation

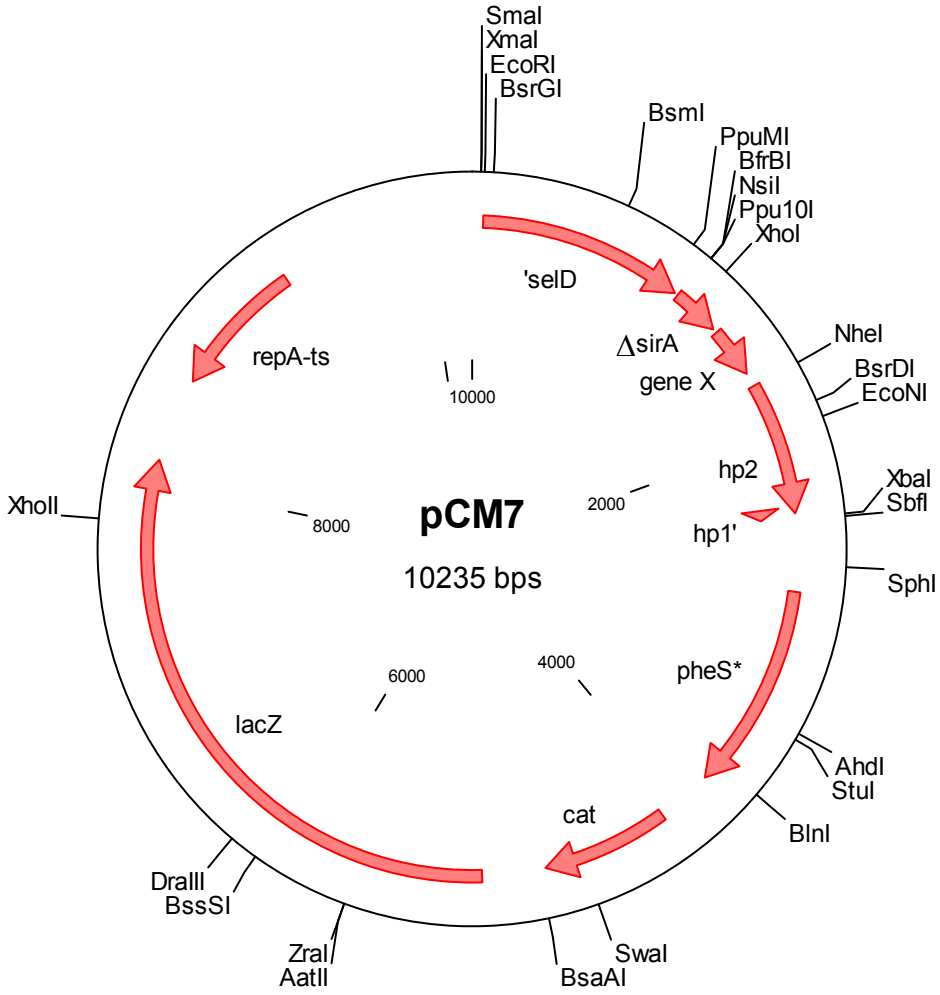

Figure 18: Construction of suicide vector to obtain Δ*sirA* mutation

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