BACTERIAL SELENOPROTEINS: A ROLE IN PATHOGENESIS AND TARGETS FOR ANTIMICROBIAL DEVELOPMENT.

by

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ABSTRACT

Selenoproteins are unique proteins in which selenocysteine is inserted into the polypeptide chain by highly specialized translational machinery. They exist within all three kingdoms of life. The functions of these proteins in biology are still being defined. In particular, the importance of selenoproteins in pathogenic microorganisms has received little attention. We first established that a nosocomial pathogen, Clostridium difficile, utilizes a selenoenzyme dependent pathway for energy metabolism. Following this initial characterization, we demonstrate that this pathway is linked to production of toxins by this organism. Finally, we show that interruption of selenium metabolism is a viable pathway for development of antimicrobials against this, and other selenoprotein dependent pathogens.

We investigated whether Stickland reactions (paired amino acid fermentation) might be at the heart of C. difficile’s bioenergetic pathways. Growth of C. difficile on Stickland pairs yielded large increases in cell density in a limiting basal medium, demonstrating these reactions are tied to ATP production. Selenium supplementation was required for this increase in cell yield. Analysis of genome sequence data reveals genes encoding the protein components of two key selenoenzyme reductases; glycine and D-proline reductase. These selenoenzymes were expressed upon addition of the corresponding Stickland acceptor (glycine, proline or hydroxyproline). Purification of the selenoenzyme D-proline reductase revealed a mixed complex of PrdA and PrdB (SeCys containing) proteins. D-proline reductase utilized only D-proline but not L-hydroxyproline, even in the presence of an expressed and purified proline racemase. The enzyme was found to be independent of divalent cations, and zinc was a potent inhibitor. These results show that Stickland reactions are key to the growth of C. difficile and that the mechanism of D-proline reductase may differ significantly from similar enzymes from non-pathogenic species.
C. difficile pathogenesis is due to the production of toxins, A and B, members of the large clostridial cytotoxin family. Previous studies have shown that toxin production by this organism is influenced by the composition of the growth medium. We examined the impact of Stickland acceptor amino acids (Stickland acceptors; glycine, proline and hydroxyproline) on growth kinetics and yield, protein synthesis, toxin production and gene expression. Although addition of Stickland acceptors moderately increases growth yield and total protein synthesis, there does not appear to be a clear impact on entry into stationary phase. Glycine dramatically increases the amount of toxin released into the growth medium. Conversely, the addition of hydroxyproline suppresses toxin production. We examine possible mechanisms of regulation and demonstrate that CodY, a regulator of toxin gene transcription does not appear to mediate this effect.

Given the importance of selenium dependent Stickland reactions to C. difficile growth and toxin production we aimed to examine the efficacy of blocking such pathways as a means of antimicrobial development. Selenide is the only known substrate for selenophosphate synthetase, the first enzyme involved in the specific incorporation of selenium into selenoproteins. We have identified a stable complex formed upon reaction of auranofin (a gold containing drug) with selenide in vitro. Auranofin potently inhibits the growth of C. difficile but does not similarly affect other clostridia that do not utilize selenoproteins to obtain energy. Moreover, auranofin inhibits the incorporation of radioisotope selenium (\(^{75}\)Se) in selenoproteins in both E. coli, the prokaryotic model for selenoprotein synthesis, and C. difficile without impacting total protein synthesis. Auranofin blocks the uptake of selenium and results in the accumulation of the auranofin-selenide adduct in the culture medium. Addition of selenium in the form of selenite or L-selenocysteine to the growth media significantly reduces the inhibitory action of auranofin on the growth of C. difficile. Based on
these results, we propose that formation of this complex and the subsequent deficiency in available selenium for selenoprotein synthesis is the mechanism by which auranofin inhibits *C. difficile* growth.

The antimicrobial potential of blocking selenium metabolism is further demonstrated in the dental pathogen *Treponema denticola*. We show that auranofin blocks the growth this organism which also participates in Stickland fermentation. In addition, we provide evidence that the antimicrobial action of stannous salts against *T. denticola* is also mediated through inhibition of the metabolism of selenium. These studies clearly show that, at least in a subset of microbes that use selenium for the synthesis of selenoproteins, the need for this metalloid can be a useful target for future antimicrobial development.
For my parents
who taught me to believe in myself
and to persevere in the pursuit of my dreams.
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CHAPTER 1
INTRODUCTION

The role of selenium in human health is well studied in terms of nutritional supplementation, mammalian selenoproteins, redox biochemistry and carcinogenesis. The impact of selenoproteins on microbial pathogenesis, however, is largely unstudied. This work consists of four independent manuscripts. The first is the characterization of a selenoenzyme in a major human pathogen, \textit{Clostridium difficile}. The second indicates that selenoproteins may contribute to the pathogenesis of this organism. The final two chapters demonstrate that specifically targeting selenoproteins and selenium metabolism provides a viable pathway for antimicrobial development.

Selenium was discovered by Jöns Jakob Berzelius in 1817. Its name is derived from the Greek word Selene, meaning moon. It is a chalcogen, sharing many physical properties with sulfur (169). The first indication that it played a role in biology wasn’t until the 1950s when it was recognized as an essential trace element for several organisms (123, 126, 136). The first selenoproteins, proteins in which selenium is covalently bound, were discovered in 1973- glycine reductase from \textit{Clostridium sticklandii}, and the first human selenoprotein, glutathione peroxidase (37, 163). These findings were followed by the recognition that selenocysteine, the twenty first amino acid, is uniquely encoded by the stop codon, UGA (20). Since these early studies, at least twenty-five human selenoproteins have been identified (55). The rationale for the evolution of specialized machinery for the insertion of selenocysteine instead of cysteine into these proteins is unclear. It is likely due to the lower pKa and higher reactivity of SeH which is necessary for specific cellular tasks (169).

Selenoprotein synthesis is a complex process in both prokaryotes and eukaryotes. It consists of many steps and involves a cadre of specialized protein machinery. Prokaryotic selenoprotein
biosynthesis is best understood in *Escherichia coli* (10). A serine is first loaded onto tRNA^Sec^, the gene product of *selC*, which possesses the UCA anticodon (91). Selenophosphate, the reactive selenium donor (51), is produced in an ATP dependent manner by the enzyme selenophosphate synthetase (SPS) which is encoded by *selD*. The substrate for this reaction is the reduced form of selenium, selenide (HSe⁻) (32, 90, 166, 167). Selenocysteine synthase, an enzyme encoded by the *selA* gene, ligates selenophosphate to the serine charged tRNA to form selenocysteine (40). A specialized translation factor, SelB, transports the selenocysteine bound tRNA to the ribosome by binding to the stem-loop structure within the mRNA. This structure is known as the selenocysteine insertion sequence (SECIS) element and is located immediately downstream of the UGA codon in the coding region (39). The process is similar in mammalian cells with conversion of a serine charged tRNA to selenocysteine (89). Like *E. coli*, mammalian selenoprotein biosynthesis requires a SECIS element and recognition by RNA binding proteins (93). Unlike prokaryotes, however, the SECIS element is located within the 3’ untranslated region (UTR) (9).

To date, the role of selenium and selenoproteins in human health is the subject of much study and debate. Selenium is an essential element in the mammalian diet and is required for the production of selenoproteins. Deficiency is associated with a wide variety of human diseases including cancer, cardiovascular disease, male infertility, and immune suppression (130). Selenoproteins are critical players in a variety of essential biological processes. Iodothyronine deiodinases are selenoproteins that are critical for the production of active thyroid hormone (84). Glutathione peroxidase (Gpx) and thioredoxin reductase (TrxR) are two abundant mammalian selenoproteins involved in defense and repair of oxidative damage. Both contain selenocysteine at their active sites (35, 159, 160). Gpx catalyzes the reduction of hydroperoxides and lipid peroxides to their corresponding alcohols and water using glutathione as the electron donor (164). TrxR, which is
required for DNA synthesis, catalyzes the NADPH dependent reduction of thioredoxin (Trx) and other oxidized dithiols (5, 147).

Much of current research regarding selenium and human health has focused on the chemopreventative effect of selenium supplementation against cancer. Recent large scale clinical studies, however, have cast doubt on the efficacy of such activity (131, 156). In addition to being a micronutrient, selenium can also have potent toxic effects. This was underscored recently with the deaths of twenty one polo horses after an overdose of selenium in an injected vitamin supplement (142). The toxic effects of selenium overdose include hair loss, damage to skin and nails, unsteady gait and paralysis (92). High doses of selenium are considered carcinogenic. Ironically, the toxicity and carcinogenicity of selenium is attributed to oxidative stress (94).

Furthermore, few studies have examined the importance of selenoproteins in human pathogens. Several important human pathogens rely upon selenoproteins for their survival. The parasites Trypanosoma brucei and Leishmania major, responsible for African sleeping sickness and Chaga’s disease respectively, both express selenoproteins (98). Recently, selenoproteins in the malaria parasite, Plasmodium falciparum, were suggested as possible drug targets (97). Selenoproteins have also been identified in many bacterial pathogens including Campylobacter jejuni, Escherichia coli, Haemophilus influenza, and Salmonella typhimurium (174). Work in our laboratory has focused on selenoproteins involved in bacterial fermentation of amino acids. This metabolic pathway, known as the Stickland reaction, is unique to anaerobic microorganisms (23, 141, 149, 150). It is found in several species of clostridia, including Clostridium difficile, a major nosocomial pathogen (68). It has also been described in Treponema denticola, a spirochete that is implicated in periodontal disease (134).
The unique reactivity of selenocysteine and the specialized machinery required for selenoprotein synthesis make selenoproteins attractive targets for antimicrobial development.
CHAPTER 2
ANALYSIS OF PROLINE REDUCTION IN THE NOSOCOMIAL PATHOGEN CLOSTRIDIUM DIFFICILE

2.1 Introduction.

Clostridium difficile is the primary causative agent for antibiotic-induced diarrhea (termed C. difficile associated disease or CDAD) and is a rapidly emerging nosocomial pathogen in healthcare facilities around the world (46, 47, 71, 80). An outbreak of a more virulent ‘epidemic’ strain of C. difficile in Quebec, Canada was correlated with the death of more than 100 patients within a six month period in a single hospital (29, 30, 99). A higher level of toxin produced by this toxigenic variant has been implicated in a higher death rate (30, 99, 165), and this strain has emerged not only in Canada but also in the United States (100, 109, 168). As concerning as this data is, it is likely a gross underestimate of the actual infection rate since there was not an active surveillance in place for reporting such infections and death rates due to CDAD during this time period.

Even more concerning is the dearth of information on the metabolic pathways of this emerging pathogen – few studies have addressed the means by which this anaerobe obtains energy. The only studies focused on physiology have determined that C. difficile requires five amino acids when grown in a defined medium - leucine, isoleucine, proline, tryptophan and valine (61, 74, 120). Addition of glycine to this minimal defined medium increases growth significantly (74). Toxin production (the primary means by which this organism causes disease) in C. difficile has been shown to increase upon addition or omission of certain amino acids to culture media (66, 74-76, 170). Although the synthesis of toxins appears to be related to the level of certain amino acids in the culture medium, the molecular mechanism behind this link to fermentation pathways is still
unexplained. Clearly a better understanding of energy and carbon metabolism is needed since it may be tied directly to pathogenesis.

Figure 2-1 Overview of Stickland Reactions.

Based on previous studies in *C. sticklandii*, *E. acidaminophilum* and *C. sporogenes* (3, 21, 141, 149, 152, 153) a schematic overview is presented on the coupled oxidation and reduction of pairs of amino acids (Stickland reactions). In addition to oxidation of amino acids, Stickland reactions may also couple to the oxidation of purines and sugars, based on early work by Barker (6). The thioredoxin (Trx) and thioredoxin reductase (TrxR) system has been suggested by Andreesen to be directly linked to glycine reductase based on the co-localization of genes encoding Trx and TrxR with components of the glycine reductase in several organisms (3). The means by which reducing potential couples to D-proline reductase to produce proton motive force is unknown, although proline-dependent production of ΔpH has been demonstrated in *C. sporogenes* (101, 103).

Using extracts of *C. sporogenes*, Stickland (152, 153) described the coupled fermentation of two amino acids in which one is oxidatively deaminated or decarboxylated (Stickland donor) and another amino acid (Stickland acceptor) is reductively deaminated or reduced (Figure 2-1). This coupled amino acid fermentation has been demonstrated as a primary source of ATP generation in several model (non-toxigenic) organisms including *C. sporogenes*, *C. sticklandii* and *Eubacterium acidaminophilum* (3, 14, 23, 141, 149, 150). Barker, also using cell extracts of *C. sporogenes*, established which amino acid combinations act as efficient Stickland donors and acceptors (6). The most
efficient Stickland donors identified in these studies were leucine, isoleucine and alanine and the most efficient Stickland acceptors were glycine, proline and hydroxyproline. Since these early reports on Stickland pairs, studies using *C. sporogenes* and *C. sticklandii* have demonstrated that both glycine and D-proline are efficient Stickland acceptors (6, 149-152). The two reductases that catalyze the reduction of the Stickland acceptors glycine and D-proline (glycine reductase and D-proline reductase) have been best characterized by Stadtman and her colleagues in *C. sticklandii* (20, 21, 141, 149-151). Studies by Andreesen and his colleagues have also demonstrated that glycine derivatives (betaine, sarcosine) can also act as Stickland acceptors in *E. acidaminophilum* (3, 111), and the enzymes catalyzing the reduction of these amino acids are likely to contain the core subunits from the glycine reductase (3). Andreesen also uncovered a selenoprotein subunit in the D-proline reductase (73). It should be noted that in the initial description of *C. sticklandii*, Stadtman reported that the closest related strain (based on biochemical characteristics) was the poorly understood *C. difficile* (151).

D-proline reductase catalyzes the reductive cleavage of the D-proline ring to yield δ-aminovaleric acid. Although this reaction does not result in production of a molecule with high group-transfer potential (as compared to acetyl-phosphate production by GR), it has been reported that PR is coupled to the generation of proton motive force (PMF, (103)). This PMF generation likely represents the key energy-yielding pathway critical for anaerobes that specialize in Stickland fermentation of amino acids. The molecular mechanism by which PR couples to generate PMF has yet to be investigated. Although these selenoenzymes have been characterized in non-toxigenic strains, their putative role in growth of the proteolytic toxigenic species of clostridia such as *C. difficile* and *C. botulinum* in the host has not yet been evaluated.

In this report we have begun to probe the metabolic pathways for energy metabolism in *C. difficile*. We have determined the nature of Stickland reactions in growth studies, and purified and
characterized both a D-proline reductase and proline racemase to determine whether hydroxyproline could act directly as a Stickland acceptor. These studies are aimed at characterizing the bioenergetics of pathogenic clostridia, and furthering our understanding of the biochemistry of Stickland reactions.

2.2 Materials and Methods.

2.2.1 Growth of C. difficile.

Two strains of C. difficile were used in this study, ATCC 9689 and strain 630. C. difficile 630 was kindly provided by Peter Mullany (Eastman Dental Institute, London). The genome of strain 630 has been completely sequenced and an initial analysis of the genome has been reported (137). Sequences utilized for annotation of genes were derived from data obtained by Pathogen Sequencing Group at the Sanger Institute and can be obtained from the website ftp://ftp.sanger.ac.uk/pub/pathogens/cd. For routine growth and maintenance, cultures were grown in rich medium TYPG (1.0% Tryptone, 0.5% Yeast extract, 10 mM KH$_2$PO$_4$, and 0.3% Glucose) or in Brain Heart Infusion (BHI, Oxoid).

In order to determine the need for selenium for growth of C. difficile on Stickland pairs, a basal medium was formulated to minimize contaminating selenium present in standard yeast extract. Torula yeast extract (Candida utilis, ICN) has been used in previous studies to establish selenium requirements for the production of the selenoenzyme nicotinic acid hydroxylase in C. barkeri (50), as well as in the formulation of a mammalian diet demonstrated to cause selenium deficiency (15, 129). An autolysate of Torula yeast (ICN) was generated by incubating yeast (250 g/L in dH$_2$O) at 50°C
overnight followed by clarification by centrifugation at 20,000 x g for 1 hour at 4°C. The clarified autolysate was sterilized by autoclaving and stored at 4°C.

A basal medium formulated to minimize selenium contamination (TTYP) includes the following: 2 gm/L Tryptone (Fisher Scientific), 0.1% Torula Yeast autolysate, 10 mM KH$_2$PO$_4$, and metal salts (10 µM CaCl$_2$, 100 µM MgCl$_2$, 100 µM FeSO$_4$, 5 µM CoCl$_2$, 5 µM NiCl$_2$, 8 µM MnCl$_2$, 3 µM Na$_2$MoO$_4$). Selenium was added as selenite at a final concentration of 1 µM. After autoclaving, culture tubes (12 x 75 mm) were transferred to an anaerobic chamber with a 95% nitrogen, 5% hydrogen atmosphere (Coy Laboratories, Grass Lake, Michigan) and Na$_2$S was added to a final concentration of 0.03% to pre-reduce the medium. A 1% inoculum from a culture grown in the basal medium (TTYP) was used in all experiments to minimize carry over selenium from rich medium (Brain Heart Infusion) stock cultures. Cultures were incubated in the anaerobic chamber in a Model 2002 incubator (Coy Labs) at 37°C. Optical density measurements of cultures at 600 nm were determined using a Hewlett Packard 8453 diode array UV-Visible spectrophotometer at the times indicated for each experiment.

Growth in defined medium was carried out as described by Karasawa et al (74). L-4-hydroxyproline was substituted for L-proline. In addition, phosphate (10 mM) was substituted for carbonate in the medium to maintain pH of the culture, as CO$_2$ is not a component of the gas mixture in the anaerobic chamber used in these studies.

2.2.2 $^{75}$Se labeling studies.

C. difficile strain 630 or ATCC 9689 was cultivated either in Brain-Heart Infusion (BHI, Oxoid LTD, Basingstoke, England) or TYPG medium. For identification of selenoproteins 10 µCi
of $^{75}$Se (University of Missouri, Columbia, Missouri), in the form of sodium selenite (50 nM), was added to each 9 mL culture (12 x 75 mm test tubes). Cells were harvested by centrifugation for 5 min at 5000 x g, washed once with buffer A (0.1 M tricine, pH 7.5, 0.1 mM PMSF, 0.5 mM EDTA), and resuspended in buffer A. Cells were lysed by sonication using a sonic dismembranator model 100 (Fisher Scientific) for 10 seconds at a power output of 12 watts, and the resultant crude cell extracts were clarified by centrifugation at 13,500 x g for 10 min at 4° C. Protein concentration was determined by Bradford assay (13) using bovine serum albumin (Pierce) as a standard. Selenoproteins were identified by separating cell extracts using 15% polyacrylamide gel, and radioisotope-labeled proteins were detected by phosphorimage analysis (Molecular Dynamics).

### 2.2.3 Immunoblot analysis for glycine reductase selenoprotein A.

Polyclonal sheep antibodies to native selenoprotein A from *C. sticklandii* were a generous gift from Dr. Thressa Stadtman (NHLBI, NIH, Bethesda, Maryland). Cell extracts were separated by SDS-PAGE (15%), subsequently transferred to polyvinylidene difluoride (PVDF) membrane, and blocked with Tris-buffered saline-Tween (TBS-Tween) containing 2% BSA for 1 hour at 25° C. Membranes were incubated with primary antibody at 25° C for 4 hours at a dilution of 1:500 in blocking buffer. After washing with TBS-Tween, the membrane was incubated with secondary antibody (Rabbit anti-sheep) conjugated with alkaline phosphatase (Sigma-Aldrich) at 25° C for 1 hour. The blot was developed using BCIP/NBT as substrates for alkaline phosphatase in 100 mM Tris buffer, pH 9.0, 100 mM NaCl and 5 mM MgCl$_2$. 
2.2.4 Purification of D-proline reductase.

Ten liters of *C. difficile* 630 were cultivated in a rich growth medium containing: 20 g/L tryptone, 10 g/L yeast extract, 1.75 g/L K$_2$HPO$_4$, 40 mM L-proline, 40 mM alanine, 1 μM selenite and 0.05% Na$_2$S to reduce the medium. The optimal concentrations of Stickland donor (alanine) and acceptor (proline) were determined experimentally and chosen based on cell yield determined by optical density at 24-36 hours after inoculation (data not shown). Cells (harvested by centrifugation at 5000 x g for 30 min) from individual 1 liter cultures (10 x 1 liter) grown under identical conditions were pooled after resuspension in 25 mL of buffer A (50 mM Tris, pH 8.4, 1 mM EDTA, 1 mM dithiothreitol) which contained 100 μM benzamidine to inhibit proteolysis. Cells were lysed by sonication at a power setting of 10 Watts using a Fisher Scientific Model 100 sonifier in 30 second bursts until cell lysis was apparent. This crude cell lysate was clarified by centrifugation at 21,000 x g for 30 min at 4° C. Stepwise ammonium sulfate fractionation (25, 40 and 60 and 85% saturation) was used as a first step to separate D-proline reductase containing fractions from other cell components. The majority of the PR activity was obtained in the 40-60% ammonium sulfate pellet, and this fraction was subsequently applied to phenyl sepharose (20 x 2.5 cm) column equilibrated in buffer A containing 2M ammonium sulfate. Proteins were eluted using a 400 mL linear gradient of decreasing ammonium sulfate (2.0 M – 0.0 M) in buffer A. Active PR fractions (see assay below) were pooled and applied to EAH sepharose (1.25 x 8 cm). After washing the column in buffer A containing 0.1M KCl to remove loosely bound proteins, PR was eluted by a 100 mL linear gradient of KCl (0.1-0.7M) in buffer A. PR containing fractions were then concentrated in an Amicon ultrafiltration membrane apparatus (YM-3 membrane) to a volume of 3.5 mL and applied to an S-200 gel filtration column (90 x 2.5 cm) equilibrated in buffer B (50 mM Tris, pH 8.4, 1 mM DTT, 250 mM KCl). PR containing fractions eluted as a single sharp peak and these fractions were
 aliquoted and quick-frozen in liquid nitrogen. This preparation was judged to be approximately 95% pure based on analysis by SDS-PAGE.

$^{75}$Se-labeled PR was purified following essentially the same procedure for purification as described above, with minor variations that did not affect the final protein preparation. The radiolabeled preparation of PR from $^{75}$Se-labeled cells was used for N-terminal sequence analysis and to confirm the presence of selenium within specific protein subunits of the purified enzyme complex.

2.2.5 *Size Exclusion Chromatography.*

Molecular weight standards used to calibrate the gel filtration column (Sephacryl S-200) were apoferritin (480 kDa), gamma globulin (160 kDa), bovine albumin (67 kDa), chymotrypsinogen (24 kDa) and cytochrome C (13 kDa). Dextran blue was utilized to calculate the void volume of the column. From these data, a calibration curve was obtained to calculate the native molecular weight of the PR complex.

2.2.6 *Fluorometric assay for D-proline reductase activity.*

D-proline reductase activity was assayed by following the DTT and D-proline-dependent production of $\delta$-aminovaleric acid, based on the method of Seto (140). The complete assay contained 50 mM Tricine, pH 8.4, 10 mM dithiothreitol, 1 $\mu$g of PR, and 1 mM D-proline. The detection was as previously described (140) with the only exception being the use of 96-well microplates for detection of the fluorometric product of reaction of $\delta$-aminovalerate with O-phthalaldehyde in a final volume of 200 $\mu$L. Initial rates were calculated based on production of $\delta$-
aminovaleric acid per min at several time points (e.g., 2, 4, 6, 8 min after addition of enzyme). PR activities are reported as nanomoles per min per milligram of cell protein.

### 2.2.7 Expression and purification of Proline Racemase.

The gene encoding a putative proline racemase (*prdF*) was amplified by polymerase chain reaction from genomic DNA (strain 630) isolated using a Wizard Genomic DNA kit (Promega, Madison, WI). A high fidelity DNA polymerase enzyme mixture was utilized (Dynazyme EXT, Finnzymes, Finland) for PCR. The oligonucleotides utilized for amplification were as follows: 5’-GAATTTCATATGAAATTTAGCAGA-3’ and 5’-ATTGGATCCTTATTTAAGAATAAA-3’.

The resulting PCR product was directionally cloned, following digestion with *NdeI* and *BamHI* restriction enzymes, into these corresponding sites in the expression vector pET15b (Novagen). The resultant plasmid, termed pSJ1, fuses the full length PrdF protein with an N-terminal 6-His tag to allow efficient purification by affinity chromatography.

For purification of the racemase, plasmid pSJ1 was transformed into Rosetta(DE3) and fresh transformants were used to inoculate 1 liter of modified Luria Broth (10 g tryptone, 5 g yeast extract, 5 g NaCl) cultivated at 37°C in Fernbach flasks shaking at 200 rpm. When the culture reached an optical density of 0.7 (600 nm) IPTG was added to 1 mM and the culture was incubated for an additional 16 hours at 37°C before harvesting cells by centrifugation at 5000 x g for 15 min. Cells were subsequently washed with buffer C (50 mM Tricine, pH 7.5, 150 mM NaCl) and resuspended in 10 mL of buffer C and lysed by sonication (Fisher Scientific Sonifier Model 100) at a power of 4 watts in 30 seconds cycles until lysis was apparent. This crude cell lysate was clarified by centrifugation at 19,500 x g for 30 min at 4°C. The lysate was filtered through a 0.8 μM filter (Whatman) and NaCl was added to give a final concentration of 2 M. Lysate was applied to a 1 mL
Hi-trap chelating column (Amersham Pharmacia) and subsequently washed with buffer D (50 mM Tricine, pH 7.5, 2 M NaCl). Proline racemase was eluted by washing the column with buffer C containing 300 mM imidazole. This protein fraction was found to be at least 95% pure (based on SDS-PAGE analysis followed by commassie blue staining) and was subsequently concentrated ten-fold and stored at -80° C.

2.3 Results and Discussion

2.3.1 Growth of C. difficile on Stickland amino acid pairs.

Both glycine and L-proline are required for optimal growth of C. difficile in defined media (61, 74, 120). Since the Stickland amino acid reductases (GR and PR) are selenoenzymes in non-toxigenic Clostridium sp., the metabolism of glycine and D-proline in the pathogenic C. difficile may also require selenium. To test this, we determined the growth of C. difficile in the presence of Stickland pairs in a basal culture medium with and without added selenium.
Figure 2-2 Growth of *C. difficile* requires selenium when cultured with Stickland pairs.

Optical densities of cultures were measured at 22 hours after inoculation (using a 1% inoculum), which was determined to be the peak density in growth curve studies (data not shown). Cultures were incubated at 37°C overnight under a 95% nitrogen, 5% hydrogen atmosphere in a Coy anaerobic chamber. Selenium, when added (hatched bars), was in the form of selenite at a concentration of 1 µM. Torula yeast extract was used in the basal medium to reduce trace selenium normally present in TYPG medium (see methods section for details on TTYP medium composition). P = D-proline; G = glycine; A = alanine. The mean optical density from at least three individual cultures is plotted with the standard deviation plotted as error, and the experimental was reproduced at least three times.

Complete removal of selenium from either defined or rich medium is made difficult by its presence as a trace contaminant in inorganic salts, yeast extract and protein hydrolysates. The basal medium with Torula yeast extract, termed TTYP, supported only a low level of growth of the type strain *C. difficile* (ATCC 9689, Figure 2-2). Addition of the Stickland donor alanine had no significant effect on growth. However, addition of Stickland acceptor D-proline increased growth significantly, especially when selenium was added (Figure 2-2). Growth with glycine was only increased when selenium was also present in the medium. In the absence of selenium, glycine actually inhibited growth. This growth inhibition by glycine in the absence of selenium was partially alleviated by the addition of D-proline or by the Stickland donor alanine (Figure 2-2). When the Stickland donor and both acceptors (D-proline and glycine) were added to the culture medium, overall growth increased.
in the presence of selenium. The growth yields (optical density) were obtained after 22 hours as this was determined to be the peak of optical density for growth in these culture media (data not shown). These results demonstrate that the growth of *C. difficile* in the presence of Stickland acceptors is dependent on selenium.

Since several carbohydrates can be utilized as carbon sources by *C. difficile* (116), we determined the effect of added glucose on the stimulatory effect of Stickland amino acids and selenium on growth of *C. difficile*. The addition of glucose to the culture medium only slightly increased the cell yield without changing the stimulation by Stickland acceptors and selenium (data not shown). This clearly demonstrates that although *C. difficile* can utilize glucose as a carbon source, the availability of amino acids as electron acceptors (Stickland fermentation) is a key limiting factor in growth yields of this pathogen.

We also tested growth of a different strain, *C. difficile* 630, to confirm that increases in cell yield by addition of Stickland pairs of amino acids are consistent among two *C. difficile* isolates. In the presence of equimolar (30 mM) alanine and glycine, *C. difficile* 630 cell yield was increased by about 10-fold by the addition of selenium, reaching optical densities (measured at 600 nm) higher than 1.3 units in the TTYP basal medium. Growth on equimolar concentrations (30 mM) of alanine and D-proline was also enhanced by addition of selenium, but only by about 2-fold (data not shown). Increasing the concentration of Stickland amino acid pairs to 100 mM did not increase the cell yield any higher than the values with the 30 mM amino acid additions, likely due to limitations by other components in the basal growth medium. At all concentrations of amino acids tested, selenium was an integral requirement for higher growth rate and cell yield. These results show that for *C. difficile* high cell yields can be obtained with either Stickland pair, but only in the presence of selenium. Furthermore, these growth studies suggest that *C. difficile* likely uses the GR and PR
selenoenzymes as a primary means of generating ATP, and this is reflected in the higher growth yields in selenium containing media.

2.3.2 Hydroxyproline can substitute for proline in defined medium.

Hydroxyproline is a modified amino acid that is found in the host as a post-translational modification of proline residues in collagen. To determine if L-4-hydroxyproline can be utilized as a substitute for proline (as an electron acceptor) to support higher growth yield of *C. difficile*, we included L-4-hydroxyproline in a defined medium and omitted L-proline. Peak optical densities (22 hours) obtained in the presence of L-4-hydroxyproline were similar (0.97 ± 0.03 OD) to readings for cultures with L-proline in the culture medium (1.1 ± 0.10 OD). In the absence of either amino acid no significant growth was observed, as expected (74). These results not only show that an amino acid derived solely from collagen (L-4-hydroxyproline) can serve to replace L-proline in defined medium, but also demonstrate that the role of proline may not be for protein synthesis, but as a needed electron acceptor, since hydroxyproline has been shown to be a Stickland acceptor in previous studies (6).
2.3.3 Analysis of genes encoding GR and PR

Figure 2-3 Identification of putative genes encoding glycine reductase and D-proline reductase in *C. difficile* 630.

The sequence data used for annotation were produced by the Pathogen Sequencing Group at the Wellcome Sanger Centre and was obtained from ftp://ftp.sanger.ac.uk/pub/pathogens/cd. Schematic representation of the genes encoded within two separate regions of the genomic DNA of *Clostridium difficile* 630, comparing directly with similar operons in *C. sticklandii*. Numbers indicate the location of the DNA coding regions within the linear genomic DNA sequence (Sanger Institute). A. Alignment of the glycine reductase region from *C. difficile* 630 and *C. sticklandii*. Primary putative assignment of genes in *C. difficile* was made by comparison with genes encoded by *C. sticklandii* (Accession # GI:11065682). Assignment of *olgP* (oligopeptidase) and *proP* (Xaa-Pro aminopeptidase) genes were made based on their homology to similar genes in *C. perfringens* (GI:18145988) and *C. tetani* (NP:782200), respectively. B. Alignment of the proline reductase region from *C. difficile* 630 and *C. sticklandii*. Primary putative assignment of genes in *C. difficile* was made by comparison with genes encoded by *C. sticklandii* (Accession GI:6899992).

The genome sequence of *C. difficile* 630 has recently been reported (137) and the sequence file is available from the Wellcome Trust Sanger Centre. Using established gene and protein sequences for glycine reductase and D-proline reductase from *Clostridium sticklandii* (73), we have compared the genes encoding these enzymes in *C. difficile* 630. Figure 2-3 is a schematic of the organization of the genes that putatively encode the subunits of PR and GR on the chromosome of *C. difficile* 630. Genes encoding subunits of glycine reductase (denoted *grd*) are clustered together in
an apparent operon, based on close linkage of the open reading frames in both *C. difficile* and *C. sticklandii*. Two of the genes, *grdA* and *grdB*, contain an in-frame UGA codon, suggesting that these two genes encode the two previously identified selenocysteine-containing subunits of GR (52). The locations of the SeCys residues in the *C. difficile* 630 GR amino acid sequences are conserved with respect to these residues in GRs from *C. sticklandii* and *E. acidaminophilum*. The gene order from *grdX* to *grdC* is also identical to that from *C. sticklandii* (52). It should be noted that a gene predicted to encode proline aminopeptidase (*proP*) is located at the end of the *grd* genes (Figure 2-3A). This protein has been used as a biochemical confirmation for positive clinical isolates of *C. difficile* (36, 44), and its close linkage to the *grd* genes in *C. difficile* suggests a metabolic link of proline and glycine metabolism in *C. difficile*.

The genes predicted to encode subunits of the PR, including one gene containing an in-frame UGA codon, are located within an apparent operon at a distant location from the genes for GR (Figure 2-3B). The organization of this operon is also identical to that found in *C. sticklandii* with an exception of a duplication of *prdE* in *C. difficile* (73). Located downstream, and potentially within the same operon of the *prd* genes, is an ORF predicted to encode the proline racemase (*prdf*). Proline racemase is critical for converting available L-proline to D-proline to generate the substrate for the D-proline reductase. In all of our growth studies L-proline was interchangeable with D-proline, demonstrating the presence of an active proline racemase in the cell to convert L-proline to D-proline in *C. difficile*. 
Figure 2-4 Radiolabeling (\(^{75}\)Se) \textit{C. difficile} reveals increased Proline Reductase and Glycine Reductase upon addition of glycine, D-proline or L-4-hydroxyproline.

A. Cultures were grown in TYPG medium (see text) with \(^{75}\)Se (10 µCi) for 24 hours, harvested, lysed by sonication and separated by 12% SDS-PAGE. Location of molecular weight markers are indicated at left (in kDa). Arrows indicate the selenoprotein present in cell extracts grown under the following conditions: Lanes 1 and 5, TYPG alone [C]; lanes 2 and 6, TYGP + D-proline (10 mM) [P]; lanes 3 and 7, TYGP + glycine (10 mM) [G]; lanes 4 and 8, TYGP + D-proline and glycine (10 mM each) [PG]. B. Immunoblot to detect presence of glycine reductase selenoprotein A (small subunit). The same extracts from part A, after SDS-PAGE, were transferred to PVDF and probed with polyclonal antibodies raised against GR selenoprotein A from \textit{C. sticklandii} (kindly provided by T. C. Stadtman, NHLBI, NIH). C. L-proline and L-4-hydroxyproline also induce production of a selenoprotein in \textit{C. difficile} (strain 9689). Lane 1, TYPG medium (no addition) [C]; Lane 2, TYPG plus glycine (10 mM) [G]; Lane 3, TYPG plus L-proline (10 mM) [P]; Lane 4, TYPG plus L-4-hydroxyproline (10 mM) [L-Hyp]. Predicted selenoproteins are indicated by arrows based on molecular weights from genes annotated in figure 2-3.
2.3.4 Selenium incorporation into Glycine reductase and Proline reductase.

Given the efficient growth of *C. difficile* strains on Stickland pairs of amino acids (including L-4-hydroxyproline) and the requirement for selenium, we sought to confirm the production of the predicted selenoproteins of (GR and PR) by labeling the cells using $^{75}$Se. Cells were cultured in the presence of 50 nM selenite ($10 \mu$Ci $^{75}$Se) for 48 hours in TYPG. The selenium-labeled proteins were identified by autoradiography of protein bands after separation on reducing, denaturing, 12% PAGE (Figure 2-4A).

In both *C. difficile* 630 and type strain ATCC 9689 three major selenoproteins were identified upon labeling with $^{75}$Se (Figure 2-4A). All three selenoproteins were expressed in the rich medium, with no additional amino acids, demonstrating Stickland reactions are utilized during growth in protein hydrolysates. Addition of D-proline increased the $^{75}$Se-labeled protein band at 27 kDa, and addition of glycine increased the level of both a 17 kDa and 47 kDa protein. These sizes correspond to the selenoprotein subunits predicted by genes encoding subunits of PR and GR (Figure 2-3). Similar results were obtained when using BHI medium (data not shown). Additional minor radioactive bands may be attributed to other unknown selenoproteins or degradation products of selenoproteins.

The 17 kDa selenoprotein is GR Selenoprotein A. Polyclonal antibodies raised against the selenoprotein A from *C. sticklandii* were used to probe whether the small 17 kDa radiolabeled protein corresponds to GR selenoprotein A. These results are shown in Figure 2-4B. An immunoreactive protein was detected at the same location as the radiolabeled protein (based on calibrated protein marker). In addition, the intensity of the immunoreactive protein agrees with the level of the radiolabeled protein band from Figure 2-4A. This confirms the identification of selenoprotein A, and indicates that when glycine is present at higher concentrations in the culture
medium, the use of glycine reductase is preferred and synthesis of the selenoprotein subunits of D-proline reductase is apparently repressed, since equal protein amounts from cell extracts were present on the membrane.

2.3.5 Hydroxyproline induces a selenoprotein similar in size to PrdB.

Barker originally reported the use of L-hydroxyproline as a Stickland acceptor in studies using cell extracts and reduced redox-active dyes (or hydrogen) as electron donors (6). Little is known about the use of hydroxyproline as a Stickland acceptor. Upon addition of L-4-hydroxyproline to the culture medium, a selenoprotein similar in molecular weight to one produced when D-proline was added to the medium was produced at higher levels (Figure 2-4C). This suggests that a similar enzyme to the previously studied PR from *C. sticklandii* (73, 149) is produced. Addition of either D-proline or hydroxyproline to the culture medium resulted in a substantial decrease in the level of glycine reductase selenoproteins A and B. This raised the question of whether the D-proline reductase from *C. difficile* reduces both D-proline and L-4-hydroxyproline (or a downstream metabolite of L-4-hydroxyproline) as substrates. Thus, we purified the radiolabeled selenoprotein induced by D-proline to determine the substrate specificity of this enzyme from *C. difficile* strain 630.

2.3.6 Purification and initial biochemical characterization of D-proline reductase.

Several preparations of PR were obtained in our studies, either by following radioisotope labeling (^75Se) or by D-proline dependent production of δ-aminovalerate in the presence of dithiothreitol as the electron donor as previously described (140). A purification scheme for PR that utilized enzyme activity for protein purification (as opposed to following radioisotope labeled
protein) is presented in Figure 2-5 and Table 2-1. As described for PR from *C. sticklandii* (141), the enzyme complex did bind to EAH sepharose, but some loss of activity was observed at this purification step (Table 2-1). A similar preparation (based on specific activity and SDS-PAGE analysis) was obtained by following $^{75}$Se labeled fractions using the same chromatographic steps (data not shown), but with a slightly higher specific activity (875 units).

![Figure 2-5 Purification and substrate specificity of *C. difficile* D-proline reductase.](image)

A. Fractions from sequential steps of purification of D-proline reductase are separated by SDS-PAGE (15%), after staining with commassie blue. Lane 1, Molecular weight marker (size in KDa indicated at left); Lane 2, Crude cell extract; Lane 3, 60% ammonium sulfate fraction; Lane 4, Phenyl sepharose; Lane 5, EAH sepharose; Lane 6, Sephacryl S-200. 5 μg of protein was loaded in each lane. Identification of protein subunits was accomplished by Edman degradation, see text for details. PrdA* is a proteolytically cleaved product of the precursor subunit PrdA (73).

B. Substrate specificity of D-proline reductase. Proline racemase (PrdF, figure 3) was purified by affinity chromatography (see methods) and used to elucidate the substrate specificity of D-proline reductase from *C. difficile*. Specific activity was measured by assaying for δ-aminovaleric acid production as previously described (140). Proline racemase (1 µg) was pre-incubated with proline substrate for 30 min (30 ºC) prior to addition of the D-proline reductase. Each proline substrate was present in the reaction at a concentration of 1 mM. ND indicates no detectable activity. Activity is expressed as nmol min$^{-1}$ mg$^{-1}$ protein.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>PR</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-proline</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>524</td>
</tr>
<tr>
<td>D-proline</td>
<td>-</td>
<td>517</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>603</td>
</tr>
<tr>
<td>L-4-OH-proline</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>D-4-OH-proline</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>ND</td>
</tr>
</tbody>
</table>
Table 2-1 Purification of *C. difficile* D-proline reductase

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein (mg)</th>
<th>Specific activity (nmol min(^{-1}) mg(^{-1}))</th>
<th>% yield</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Extract</td>
<td>742</td>
<td>89.5</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>60% (NH(_4))(_2)SO(_4)</td>
<td>125</td>
<td>173</td>
<td>33</td>
<td>1.9</td>
</tr>
<tr>
<td>Phenyl Sepharose</td>
<td>24.9</td>
<td>314</td>
<td>11</td>
<td>3.5</td>
</tr>
<tr>
<td>EAH sepharose</td>
<td>5.3</td>
<td>203</td>
<td>1.7</td>
<td>2.3</td>
</tr>
<tr>
<td>Sephacryl S-200</td>
<td>1.4</td>
<td>513.6</td>
<td>1.1</td>
<td>5.7</td>
</tr>
</tbody>
</table>

Three separate protein subunits were observed in the purified preparation (Figure 2-5). Edman degradation of these subunits confirmed that the largest subunit was indeed PrdA (SITLEAQ, initial methionine not present) and that the 27 kDa subunit was PrdB (SLTTVQGL). Excision of the protein bands from SDS-PAGE revealed \(^{75}\)Se was present only in the PrdB subunit. The smallest subunit was initially blocked at the N-terminus. Treatment of the protein with 0.1 N trifluoroacetic acid (which resulted in cleavage into smaller peptides) and subsequent analysis by Edman degradation revealed an internal sequence of PrdA (VRIM). Since no other gene within the *prd* or *grd* operons encodes a protein containing this amino acid sequence it strongly suggests this is a processed PrdA subunit (PrdA\(^*\)). The proteolysis of PrdA has been described in previous studies that demonstrate post-translational modification of this protein subunit is required for activation of the enzyme complex (3, 73, 141).

2.3.7 D-proline reductase does not utilize either L- or D-Hydroxyproline as a substrate.

Since it appears that PrdB selenoprotein is induced in radiolabeled cultures either by addition of D-proline (L-proline) or L-4-hydroxyproline, we determined the substrate specificity of our preparation of PR. For this analysis we also expressed and purified by affinity chromatography the
proline racemase enzyme (see Materials and Methods section for details). D-proline reductase utilized D-proline as a substrate, but in the presence of L-proline no δ-aminovaleric acid was produced (Figure 2-5B). Addition of proline racemase and L-proline did result in comparable activity (Figure 2-5B), confirming the activity of the expressed and purified proline racemase. We analyzed the kinetics of PR (higher activity preparation) with D-proline and found the apparent $K_m$ to be 200 μM with a $V_{max}$ of 1000 nmol min$^{-1}$ mg$^{-1}$. We also determined the optimum pH - which increased steadily upon increasing pH above pH 7.0 up to pH 8.6. We chose to use pH 8.4 for our assays since increasing pH at or above 8.4 did not significantly increase PR activity. We also determined the native molecular weight of the enzyme complex by chromatography on Sephacryl S-200. The estimated native molecular weight was approximately 280 kDa, indicating several subunits of PrdA and PrdB are present in the purified enzyme complex, although the composition cannot yet be determined. This is made difficult by the presence of a significant amount of unprocessed PrdA protein in the preparation, as this likely cannot contribute to enzyme activity (73, 141). To our knowledge this is the first determination of the kinetic properties of PR from any organism.

Although the presence of proline racemase supported conversion of L-proline to D-proline, neither L-4-hydroxyproline nor D-4-hydroxyproline were utilized as a substrate by the purified PR complex, even when present in concentrations up to 5 mM. Addition of proline racemase also had no effect on the enzyme activity in the presence of these substrates (Figure 2-5B). This would indicate that the D-proline reductase, in conjunction with the proline racemase, cannot directly convert hydroxyproline derived from collagen for this enzyme complex. However, the possibility remains that the product of the reduction of hydroxyproline to a hydroxyl derivative of δ-aminovaleric acid is not detected with O-phthalaldehyde (see methods for details).
We also cultivated *C. difficile* 630 in the presence of $^{75}$Se-selenite and L-4-hydroxyproline as the Stickland acceptor and partially purified the radiolabeled protein induced under these growth conditions. This partially purified preparation also did not utilize L-4-hydroxyproline or D-4-hydroxyproline as substrates in a DTT-dependent manner, but did have good activity in the presence of D-proline. These results indicate that the selenoprotein induced upon addition of L-4-hydroxyproline is in fact PR.

2.3.8 *Divalent cations are not required for D-proline reductase activity.*

All previous characterization of D-proline reductases, whether in cell extracts or using purified preparations, have reported an absolute requirement for divalent cations such as Mg$^{2+}$. Our analysis of the required components for PR from *C. difficile* by omission of individual components of the enzyme assay revealed no requirement for any divalent cation (data not shown). Addition of EDTA or EGTA also did not significantly affect the activity of the enzyme. This is in contrast to previous reports (73, 149, 150). To further study this apparent discrepancy, we determined the activity of PR in the presence of varying concentrations of magnesium, manganese, calcium and zinc and these results are summarized in Figure 2-6.
Figure 2-6 Analysis of the requirement for divalent cations for D-proline reductase activity.

The requirement for divalent cations for D-proline reductase activity was tested in the presence of several cations (A, Mg$^{2+}$; B, Mn$^{2+}$; C, Ca$^{2+}$; D, Zn$^{2+}$). Specific activity is plotted versus cation concentration. The mean of these activities is plotted with the standard deviation shown as error. At least three independent enzyme assays are represented. Activity was determined by following production of δ-aminovalerate as described in experimental procedures section.

The activity of PR increases slightly with magnesium added to 250 μM, however this increase is not statistically significant. Addition of higher concentrations of magnesium reduced activity, as much as 30% when magnesium was present at 5 mM. It should be noted that this is the concentration typically used in previous reports of PR enzyme assays (73, 149, 150). No significant change in activity occurred in the presence of manganese, except for a decrease again at 5 mM. In contrast, addition of calcium at 500 μM significantly increased PR activity. Nonetheless this increase
was not consistent when calcium was increased to millimolar levels. Addition of zinc dramatically reduced PR activity. This likely indicates the binding of zinc to the active site selenocysteine and/or cysteine residues, which prevents catalytic reduction of the proline ring by the selenoprotein subunit.

Biochemical analysis of D-proline reductase revealed that hydroxyproline derivatives could not be used directly as an electron acceptor. This suggests that a novel pathway may exist in *C. difficile* to interconvert hydroxyproline to proline so that it can be used to drive PMF via Stickland fermentations, since growth studies clearly show that hydroxyproline can be used as a Stickland acceptor. The lack of a requirement for divalent cations for PR catalysis, and resistance to chelating agents was unexpected. This suggests that the PR from *C. difficile* may reduce the proline ring by a slightly different mechanism than PR isolated from other Stickland fermenters. The need for divalent cations is not well understood since the reaction mechanism for this enzyme has yet to be explored. The inhibition by zinc may indeed prove useful to probe this mechanism in future studies.

Based on the available nutrients in the host, it is tantalizing to suggest that collagen could serve as a primary source of these amino acids to act as electron acceptors. The structural domain of collagen is Gly-X-Y, where x is often proline and y often hydroxyproline (128). It has been reported that large clostridial toxin B from *C. difficile* can induce the production of matrix metalloproteinases (82, 115). MMP-2 could generate the needed oligopeptides for growth of *C. difficile* (glycine and proline rich) and gives a rationale to the type of non-invasive infection that is characteristic of this pathogen. This would also shed light on the apparent lack of collagenase activity exhibited by clinical isolates and explain a linkage between fermentation pathways and toxin regulation (127). Once small peptides are released by MMP-2, the oligopeptidase and proline aminopeptidase encoded by genes located adjacent to the *grd* operon (Figure 2-3) would generate free proline, glycine and hydroxyproline for use as Stickland acceptors. These free amino acids
would then be used as Stickland acceptors for synthesis of ATP by substrate-level phosphorylation (GR) as well as production of PMF (PR). This potential linkage both to toxin regulation and the use of collagen as a source of Stickland acceptors will be the focus of future studies.
CHAPTER 3
STICKLAND ACCEPTORS ALTER CLOSTRIDIUM DIFFICILE TOXIN PRODUCTION

3.1 Introduction

*Clostridium difficile* has recently garnered much attention as the causative agent of antibiotic induced diarrhea, leading to severe colitis and even death. An outbreak of a more virulent strain (NAP1/O27) in Quebec, Canada in 2003 and the spread of this strain throughout North America and Europe, together with an increasing trend of community acquired illness firmly established this organism as a significant public health threat (1, 7, 31, 100, 109, 168). In the United States, the incidence has more than doubled since the year 2000 and it is estimated that 15000 to 20000 people die each year from *C. difficile* associated disease (135).

*Clostridium difficile* associated disease generally occurs when antimicrobial therapy disrupts the natural flora of the gastrointestinal tract. The organism colonizes the newly available ecological niche and begins to produce two large molecular weight toxins, toxin A and toxin B. These toxins inactivate GTPases of the Rho/Rac family through glucosyltransferase activity ultimately resulting in depolymerization of the actin cytoskeleton, mucosal damage and inflammation (72). Symptoms typically range from mild to severe diarrhea. Complications include pseudomembranous colitis, toxic megacolon and death (80).

Regulation of toxin transcription is not well understood. Production of toxin varies between strains and based on environmental conditions (75, 119, 170). In addition, an increase in the level of toxin produced by the epidemic NAP1/O27 strain has been suggested to play a role in the higher mortality rates observed with this strain (30, 99, 165). The toxin genes (*tcd*A and *tcd*B) are encoded within the pathogenicity locus (PaLoc) along with two other genes that are thought to be involved in
regulation of toxin production. \textit{tcdC} encodes a negative regulator of toxin production that is highly transcribed during exponential growth, but down regulated during stationary phase, the peak of toxin production (65, 108). Polymorphisms in \textit{tcdC} are associated with increased toxin production (145). \textit{tcdD} encodes a sigma factor that is positively associated with toxin production (27, 28, 107, 112). Another protein encoded outside of the PaLoc, CodY, is implicated in environmental regulation of toxin production (26). \textit{C. difficile} has a highly variable genome with the core components consisting of only 16% of the encoded genes (69, 137). This creates difficulties in defining the mechanisms of toxin gene regulation and pathogenesis. Additionally, studies of toxin regulation are limited by the resistance of \textit{C. difficile} to genetic manipulation.

A diverse selection of factors influences toxin production including environmental stress, temperature, biotin limitation and purine biosynthesis (77, 105, 119, 171). Of particular interest is the impact of the amino acid composition of the growth medium. Previous studies have shown that regulation of toxin production in \textit{C. difficile} is altered upon addition or omission of certain amino acids to culture media (66, 74-76, 170). The recent analysis of this regulation showed the addition of L-proline or L-cysteine to the culture medium significantly reduced intracellular toxin levels in a concentration-dependent manner (78). The molecular mechanism behind this linkage to amino acid metabolism is unexplained.

Previous work in our laboratory described Stickland reactions in \textit{C. difficile} (68). Stickland reactions are described as the fermentation of two amino acids in which one is oxidatively deaminated or decarboxylated (Stickland donor) and another amino acid (Stickland acceptor) is reductively deaminated or reduced (152). This coupled amino acid fermentation has been demonstrated as a primary source of ATP generation in several organisms including \textit{C. spongines}, \textit{C. sticklandii} and \textit{E. acidaminophilum} (3, 14, 23, 141, 149, 150). The enzymes responsible for amino acid
reduction in *C. difficile* are D-proline reductase and glycine reductase. Proline reductase catalyzes the reduction of proline to δ-amino-valeric acid which is secreted into the growth medium (149, 150). It is suggested that this generates proton motive force (102). Glycine reductase, using reducing potential from thioredoxin, deaminates glycine and activates it to acetyl phosphate which can be utilized for substrate level phosphorylation or generation of biomass through production of acetyl-CoA (2, 3). Interestingly, both D-PR and GR are highly conserved across strains and are considered to be part of the core genome of *C. difficile* (69). Recent proteomic analysis indicates that these enzymes are present within the *C. difficile* spore (88). In addition, glycine has been shown to be a potent spore germinant (144). These data suggest that amino acid fermentation may play a critical role in transmission of the disease. In this study we examine the impact of Stickland fermentation on toxin production in *C. difficile*.

3.2 Materials and Methods

3.2.1 Growth of *C. difficile*.

A high toxin producing strain, VPI 10463, (purchased from ATCC) was used in this study. The CodY deficient strain (JIR8094::pSD21) and the parent strain (JIR8094) are previously described (9). They were kindly provided by Abraham L. Sonenshein (Tufts University, Boston, MA). For routine growth and maintenance cultures were grown in rich medium, Brain Heart Infusion (BHI, Oxoid). For all experiments, *C. difficile* was cultivated in a rich medium (2% tryptone, 0.5% yeast extract, 0.3% glucose, 1µM sodium selenite). After autoclaving, the culture medium was transferred to an anaerobic chamber with an atmosphere containing 95% nitrogen and 5% hydrogen (Coy Laboratories, Grass Lake, Michigan) and Na₂S was added to a final concentration of 0.03% to
pre-reduce the medium. Thiamphenicol (10 µg/ml) was added to the growth media of JIR8094::pSD21 in order to maintain the integration of the plasmid. Where indicated, growth medium was supplemented with 16.7mM L-proline, glycine, or L-4-hydroxyproline. A 1% inoculum from an overnight culture grown in the basal medium was used in all experiments. Cultures were incubated in an anaerobic chamber at 37°C (Model 2002 incubator, Coy Labs, Great Lakes, MI).

3.2.2 Growth curve and extracellular protein analysis.

Optical density measurements of cultures at 600 nm were determined using a Molecular Devices SpectraMax 190 96-well plate reader at the times indicated for each experiment. Cultures were centrifuged at 16,100 x g for five minutes. The resulting cell free supernatants were assayed for protein as described by Bradford (13) using bovine serum albumin (Thermo Scientific, Rockford, IL) as a standard.

For analysis of extracellular toxins, 50 µL aliquots of cell free supernatant from each *C. difficile* culture across a 72 hour growth study was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5% polyacrylamide). The separated proteins were stained overnight (GelCode Blue, Thermo Scientific).

3.2.3 Real time RT-PCR analysis of toxin gene expression.

*C. difficile* was cultivated as described with the addition of Stickland acceptors (L-proline, L-4-hydroxyproline or glycine) at a final concentration of 16.7 mM. 72 hours following inoculation cells were harvested by centrifugation (5000 x g). Cells were subsequently washed with diethylpyrocarbonate (DEPC) treated phosphate buffered saline (PBS). Total RNA was isolated utilizing the ChargeSwitch Total RNA Cell kit (Invitrogen, Carlsbad, CA) and quantified by UV-
visible spectrophotometry at 260 nm. cDNA was generated with 1µg of purified RNA utilizing the iScript cDNA synthesis kit (BioRad, Hercules, CA). Real-time PCR amplification was performed utilizing the BioRad i-Cycler. Primer pairs specific to tcdA and tcdB have been described previously (18). Amplification of the transcripts for 16S rRNA were used as an internal standard and this primer pair has also been described for C. difficile (19). Primer pairs for tcdC, tcdD, and tcdE were designed with the Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) utilizing the gene sequences from strain 630 and are listed in Table 3-1. BioRad iQ SYBR green supermix was utilized for real-time PCR amplification with primers at a concentration of 200 nM each. cDNA was diluted in sterile H₂O before addition to the reaction mix. Reaction conditions consisted of a single cycle at 95.0°C for 3 min; subsequent 40 cycles of 95.0°C for 10 s, 55.0°C for 45 s. Melt curve analysis and agarose gel electrophoresis were performed to confirm the presence of a single product of expected size. Efficiency of amplification for each target gene was calculated utilizing a 10-fold dilution series of control cDNA. Relative expression was calculated according to the method described previously (20).
Table 3-1 Primers for Real Time PCR

<table>
<thead>
<tr>
<th>Gene of interest</th>
<th>Direction</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>tcdA</td>
<td>forward</td>
<td>tctaccactgaagcattac</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>taggtactgtaggtttattg</td>
</tr>
<tr>
<td>tcdB</td>
<td>forward</td>
<td>atacagagaactgagag</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>tagcatattcagagaatattgt</td>
</tr>
<tr>
<td>tcdC</td>
<td>forward</td>
<td>ecagttgcagatcagaca</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>tgaagacatgaggaggagca</td>
</tr>
<tr>
<td>tcdD</td>
<td>forward</td>
<td>aactcatagatggatttgaga</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>tcttttcctctctcataatgt</td>
</tr>
<tr>
<td>tcdE</td>
<td>forward</td>
<td>ttgtcctatgattaccag</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>ttcatctatctctcttccate</td>
</tr>
<tr>
<td>16S</td>
<td>forward</td>
<td>tgagcatttaactctgtaaga</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>ecacgctgtaactggetacct</td>
</tr>
</tbody>
</table>

\(^a\) described previously (4)
\(^b\) described previously (32)

3.2.4 ELISA for toxins A and B

Culture media was collected by centrifugation (1 min, 16,100 x g) after 72 hours of growth. Cell free supernatants were filtered to remove any remaining cell debris (0.2 µm filter). Quantification of extracellular toxins was performed utilizing the C. difficile TOX A/B II ELISA (TechLab, Blacksburg, VA). Samples were diluted in the diluent provided to obtain readings (O.D.\(_{450nm}\)) within the acceptable range specified by the kit.

3.3 Results and Discussion

3.3.1 Growth with Stickland acceptors increases toxin production.

The impact of additional Stickland acceptors in rich medium on C. difficile growth and protein production was evaluated over a period of seventy-two hours (Figure 3-1). The additional amino acids increased growth approximately 20-30% over the control at twelve hours post-
inoculation. The largest increase in growth occurred with L-hydroxyproline (Figure 3-1A). Similar results were obtained for total protein (Figure 3-1B). In contrast, extracellular protein levels within stationary phase (24 to 72 hours) were significantly higher in cultures supplemented with glycine than the control and those supplemented with L-proline or L-hydroxyproline (Figure 3-1C).

Media supernatants were analyzed by SDS-PAGE over the course of the 72 hour growth period. Staining with GelCode Blue revealed the presence of a protein band larger than 225 kDa that appeared after the cells entered into stationary phase (Figure 3-2). This corresponded with the migration pattern of the purified toxin A control. The limitations of the 7.5% polyacrylamide gel prevented resolution between toxin A and toxin B. Media supernatants from glycine and L-proline treated cells exhibited higher toxin levels than the control. Glycine treated cells also produced toxin approximately 24 hours earlier. Strikingly no band corresponding to toxin was visible in media supernatants from cells treated with L-hydroxyproline despite the fact that these cultures exhibited comparable growth to glycine and proline treated cells. This data corresponds with the results obtained from cell culture based cytotoxicity assays and ELISA (data not shown).
Figure 3-1 The addition of glycine increases the production of extracellular protein by *C. difficile*.

*C. difficile* (VPI10463) was cultivated in rich medium with the addition of Stickland acceptor amino acids- proline, glycine or hydroxyproline (16.7mM). Optical density (A) was followed for 72 hours after inoculation. Total protein (B) and extracellular protein (C) were quantified in cell free supernatants according to the method described by Bradford (13).
3.3.2 Stickland acceptors enhance transcription of toxin genes

Real time rt-PCR was used to determine if the observed changes in toxin levels in the extracellular growth medium are due to transcriptional regulation of the toxin genes, \( tcdA \) and \( tcdB \) (Figure 3-3). mRNA was harvested after 72 hours of growth. Relative to control cells, those grown with additional L-proline exhibited approximately three times as much transcript for \( tcdA \) and \( tcdB \). Those treated with glycine produced nearly nine times as much toxin mRNA. Cells grown with L-hydroxyproline exhibited at least a 75% reduction in \( tcdA \) and \( tcdB \) transcription. These results confirm that the variations in the cytotoxicity of the growth medium and the amounts of toxin visible by SDS-PAGE analysis are due to transcriptional regulation rather than a post-translational effect. Similar results were obtained with the NAP1/O27 epidemic strain (data not shown).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3-2.png}
\caption{Toxin production occurs earlier and is increased with the addition of L-proline or glycine to the culture media of \textit{C. difficile}, but is diminished with L-hydroxyproline. \textit{C. difficile} (VPI10463) culture media was collected by centrifugation at the indicated time points. Cell free supernatants were filtered (0.2\textmu m filter) and equal volumes (50\textmu L) were separated by SDS-PAGE and stained overnight with GelCode Blue. Gel migration was compared with that of purified toxin A (List Biologicals).}
\end{figure}
Figure 3-3 Expression of \textit{C. difficile} toxin mRNA is consistent with the observed changes in extracellular toxin production.

\textit{C. difficile} (VPI 10463) mRNA was harvested after 72 hours of growth in rich media supplemented with proline, hydroxyproline and glycine. Expression of \textit{tcdA} (A) and \textit{tcdB} (B) was analyzed using real time RT-PCR.

### 3.3.3 The remaining genes within the pathogenicity locus are co-regulated with toxins

Two genes within the PaLoc are reported to play important roles in toxin regulation- \textit{tcdC}, encoding a putative negative regulator, and \textit{tcdD}, encoding a sigma factor associated with toxin production. We utilized real time RT-PCR to determine the level of mRNA transcribed from these
genes, and \textit{tcdE}, which is also location within the PaLoc, in response to the addition of Stickland acceptors to the growth medium. Transcription of \textit{tcdC} is known to exhibit an inverse relationship to toxin transcript levels \cite{65}. If the observed transcriptional regulation of \textit{tcdA} and \textit{tcdB} in response to additional amino acids were mediated by the product of \textit{tcdC}, it would be expected that \textit{tcdC} transcription itself would be decreased in cells treated with L-proline and glycine. Although there was some increase in \textit{tcdC} transcription in cells grown with glycine, transcription of \textit{tcdC} did not vary significantly. The transcriptional regulation of \textit{tcdA} and \textit{tcdB} in response to Stickland acceptors appears to occurs independent of \textit{tcdC} \cite{34A}. The patterns of \textit{tcdD} and \textit{tcdE} transcription mirrored that of \textit{tcdA} and \textit{tcdB} \cite{34B and C}. This may be attributed to the polycistronic nature of \textit{tcdABDE} transcription \cite{59, 65}.
Figure 3-4 Expression of the remaining genes within the PaLoc following addition of Stickland acceptor amino acids is consistent with co-transcription with \textit{tcdA} and \textit{tcdB}.

Analysis was performed as in Figure 3-3.
3.3.4 The role of CodY in the response to Stickland acceptors

Recent work by Dineen et al (26) demonstrated that CodY, a protein known to regulate sporulation in *Bacillus subtilis*, is a regulator of toxin production in *C. difficile*. In this study CodY was shown to bind to the promoter of tcdD, the sigma factor responsible for toxin gene transcription, in the presence of GTP and branched chain amino acids (BCAAs). It was also shown to bind weakly to the promoters of the toxin genes. This binding resulted in repression of toxin production. Interruption of the gene encoding CodY resulted in a strain with up regulated toxin transcription and an increase of toxin in the growth medium.

CodY regulates genes involved in BCAA biosynthesis in several organisms (162). As such, promoter binding is activated by directly binding to isoleucine and GTP (60, 95). This allows organisms to quickly respond to BCAA availability. While Stickland fermentation of amino acids involves the reduction of acceptor amino acids (*i.e.* glycine and proline), it is coupled to the oxidation of donors such as alanine, leucine and isoleucine. It is conceivable that the addition of an excess of acceptors to the growth media may deplete the pool of BCAAs for biosynthesis reactions and thus result in derepression of the CodY regulon.
A previously described CodY deficient (JIR8094::pSD21) strain of *C. difficile* (26) was cultivated in the presence of Stickland acceptors. (A) mRNA was extracted after 72 hours of growth in batch culture. Expression of tcdA was quantified by real time rt-PCR. (B) Filtered culture media supernatants were assayed for toxins A and B utilizing the *C. difficile* TOX A/B II ELISA (TechLab).

To address this possibility we obtained the previously described CodY deficient strain (JIR8094::pSD21). We examined toxin production in response to Stickland acceptors in these strains. Real time PCR analysis (Figure 3-5) demonstrated that JIR8094::pSD21 responded to the addition of glycine in the growth medium in a manner similar to VPI10463 and NAP1/O27 with an increase in toxin transcription. These results were confirmed by ELISA analysis of cell free culture supernatants, in which the levels of toxins were nearly 100 fold higher than the controls. These
results indicate that the response to glycine is not due to a decrease in CodY repression of toxin genes, but rather occurs by a separate unknown mechanism. It should be noted that JIR8094::pSD21 produces amounts of toxin comparable to both VPI10463 and NAP1/O27; however the isogenic wild type (JIR8094) strain produces substantially less toxin than and the response of this strain to Stickland acceptors is less pronounced (data not shown).

### 3.3.5 Discussion

The addition of Stickland acceptors to the growth media of *C. difficile* has a clear impact on toxin production. Both proline and glycine increase toxin production, whereas hydroxyproline appears to decrease the amount of toxin released into the media. The molecular mechanism of this response remains unclear. Our research indicates that the response to Stickland acceptors is not dependent on *tcdC* transcription or the presence of CodY. Further studies are required to understand the mechanisms of toxin regulation in this organism.

Much work remains to define *C. difficile* pathogenesis. Most studies of this organism are focused on epidemiology and inter-strain variation. Our current knowledge has been frustratingly limited by the lack of available genetic tools. Recent advances in this area open up many avenues of study (26, 62, 104). Our results, in light of current research, further demonstrate the importance of Stickland fermentation in the ecology of *C. difficile* disease. In particular, recent studies have suggested a role for this metabolic pathway in germination, suggesting that amino acid fermentation may provide the energy required for the cell to exit the dormant state (88). In addition, glycine is a potent germinant (144). How the organism senses the presence of specific amino acids to exert such pronounced phenotypic responses is a topic of further study. Without a doubt, further characterization of Stickland fermentation in *C. difficile* as a whole is necessary.
CHAPTER 4
AURANOFIN DISRUPTS SELENIUM METABOLISM IN CLOSTRIDIUM DIFFICILE BY FORMING A STABLE AU–SE ADDUCT

4.1 Introduction

Auranofin [2,3,4,6-tetra-o-acetyl-1-thio-β-D-glucopyranosato-S-(triethyl-phosphine) gold] is a Au(I) complex containing a Au-S bond stabilized by a triethyl phosphine group (79). It is used clinically to treat rheumatoid arthritis (110). It is a potent inhibitor of the mammalian selenoenzyme thioredoxin reductase (TrxR) and it is proposed that the mechanism of action in arthritis treatment is, in part, due to its activity against this and other selenoenzymes (8, 54). Recently, auranofin has been shown to inhibit growth of the parasite Trypanosoma brucei (98). It has also exhibited activity against Schistosoma mansoni in a mammalian host (85). In both reports the proposed mechanism of action was the inhibition of selenoenzymes critical for survival. It is hypothesized that auranofin inhibits selenoenzymes through interactions with the reduced selenocysteine residues at the active sites (54).

Recently we found that auranofin blocks the incorporation of selenium into selenoproteins in mammalian cells in culture (158), however the mechanism of this inhibition has not yet been determined. Selenoprotein synthesis is well defined in both prokaryotic and eukaryotic systems. It should be noted, however, that transport and metabolism of selenium upstream of the specific selenoprotein synthesis machinery is not well understood. In the first step of selenoprotein synthesis, the highly reactive, reduced form of selenium, hydrogen selenide (HSe⁻), serves as the substrate for selenophosphate synthetase (SPS) (32, 51, 90, 166, 167). SPS produces selenophosphate in an ATP dependent manner. Selenocysteine synthase subsequently catalyzes the reaction of selenophosphate with a serine charged tRNA to form selenocysteine (38, 40). Specialized
translation factors and a stem-loop structure within the mRNA (selenocysteine insertion sequence or SECIS) then direct the insertion of selenocysteine into the polypeptide chain (39). Given its reactivity with active site selenols, the possibility exists that auranofin could interact with reactive selenium metabolites upstream of SPS, such as HSe\(^{-}\), thus blocking selenoprotein synthesis entirely.

The role of selenium and selenoproteins in human health has been studied extensively. At least 25 human selenoproteins, have been identified (121). In recent years studies have focused on the role of human selenoproteins as catalytic antioxidants and the impact of selenium supplementation on cancer incidence. In addition to humans, several pathogens, including, but not limited to, Clostridium difficile, Treponema denticola and Plasmodium falciparum also produce selenoproteins (68, 97, 134). The importance of these selenoproteins and how they impact pathogenesis has yet to be fully elucidated. These unique enzymes and their specialized assembly machinery present an intriguing target for antimicrobial development.

*Clostridium difficile* is a gram positive, anaerobic, spore forming bacillus that has emerged as a significant nosocomial pathogen. Pathogenesis is mediated by two large clostridial cytotoxins, toxins A and B, and symptoms typically range from mild to severe diarrhea. In more severe infections, patients develop pseudomembranous colitis (80). *C. difficile* associated disease (CDAD) contributes an estimated $1 billion in excess health care costs annually (86). Recently the emergence of an epidemic strain (NAP1/O27) that exhibits increased virulence, and increasing mortality rates in the United States has been of particular concern (109, 132). In addition, isolates of this strain have exhibited a wide array of antibiotic resistance (11, 109). Analysis of data collected before and after the emergence of NAP1/O27 indicated a reduction in the effectiveness of vancomycin over metronidazole in treating *C. difficile* infection (124). A recent update of the literature regarding this epidemic has suggested that *C. difficile* now rivals methicillin resistant *Staphylococcus aureus* (MRSA) as a
significant clinical pathogen (48). Given the increased incidence in the clinic and emerging resistant strains, new approaches to target this pathogen are certainly justified.

In this report we demonstrate that auranofin reacts with HSe⁻ to form a stable complex. Subsequently, we show that auranofin blocks selenium utilization by both *Escherichia coli*, a model organism for prokaryotic selenoprotein synthesis, and *C. difficile*, a significant human pathogen. In addition, auranofin exhibits antimicrobial activity against *C. difficile*. We propose that the molecular mechanism of this growth inhibition is the formation of the complex with HSe⁻ which prevents uptake and nutritional utilization of selenium by *C. difficile*.

4.2 **Materials and Methods**

4.2.1 **Reaction of Hydrogen Selenide with Auranofin.**

Hydrogen selenide (HSe⁻) was synthesized by reaction of elemental selenium with sodium borohydride as previously described (81). For experiments examining the oxidation of hydrogen selenide to elemental selenium, equal volumes (50µL) of varying concentrations of auranofin (in DMSO) and hydrogen selenide were added to the wells of a 96 well plate, mixed thoroughly by pipetting and incubated for 30 minutes under anaerobic conditions. The reactions were then exposed to ambient atmospheric conditions for 30 minutes followed by visual examination. Under these conditions hydrogen selenide oxidizes to Se⁰ and forms a red precipitate. Similarly, equal volumes of varying concentrations of auranofin and hydrogen selenide were reacted anaerobically before analysis by mass spectrometry or high performance liquid chromatography (HPLC).

HPLC-MS data were collected on an Agilent 1100 HPLC and Agilent 1969A time of flight mass spectrometer. The capillary voltage was 5,000 V, and the fragmentor voltage was 100 V. The
nitrogen gas temperature was 300° C. The range scanned was from 145 to 2000 amu at 10,000 transients/scan in positive ion mode. The HPLC column was a Vydac C_{18} (218TP5105) with flow at 20 µL/min and temperature held at 30° C. Solvent A was 0.05% trifluoroacetic acid and solvent B was 0.05% trifluoroacetic acid in acetonitrile. The gradient ran from 0 to 95% B at 2%/min. The 0.05% TFA is added to the HPLC solvents to improve chromatographic separation and to increase the solubility of eluted compounds in acetonitrile (106). However, TFA can severely suppress ionization of proteins, so 20 µL/min neat acetic acid is mixed in a tee placed between the HPLC UV detector and the electrospray needle. The resulting 50% acetic acid displaces TFA from the protein (4). Some spectra were obtained by direct injection of the sample into the mass spectrometer, without HPLC separation, as shown in Figure 2. The mass of the Auranofin-selenide adduct was the same whether identified by direct injection or after separation by LC.

HPLC analysis (UV-visible detection) was performed using a Hewlett Packard 1050 system (diode array detection). 20 µL samples were loaded onto a C_{18} column at a flow rate of 0.5 mL per minute. The starting solvent (used for injection) was 0.05% trifluoroacetic (TFA) acid in H₂O. A linear gradient (50 minutes) was developed to 100% acetonitrile, 0.05% TFA. Eluting compounds were monitored spectrophotometrically at 254 nm.

4.2.2 Growth of Escherichia coli.

Wild type *E. coli* (MC4100) and a selenoprotein deficient strain (WL400, ΔselD) were cultured in modified Luria broth (10 gm/L tryptone, 5 gm/L torula yeast extract, 5 gm/L NaCl, 1% dextrose) at 37°C in a Coy anaerobic chamber. A 1% inoculum of an overnight culture was used in each experiment. Optical density measurements were taken 24 hours after inoculation and hydrogen production was assessed by Durham tubes and/or bubbling of cultures upon vigorous shaking. For
experiments utilizing *E. coli*, auranofin was dissolved in ethanol rather than DMSO because the latter inhibits gas production in this organism.

### 4.2.3 Growth of Clostridium difficile, C. perfringens and C. tetani.

Four strains of *C. difficile* were used in this study, ATCC 9689, VPI 10463, NAP1/O27 and strain 630. *C. difficile* 630 was kindly provided by Peter Mullany (Eastman Dental Institute, London, United Kingdom), and NAPI/O27 was provided by Dr. Michel Warny (Acambis, Inc., Cambridge, MA). Two pathogenic clostridia that do not produce selenoproteins, *C. perfringens* (ATCC 19406) and *C. tetani* (ATCC 10543), were used as experimental controls. Cultures were grown in brain heart infusion (BHI; Oxoid) supplemented with 0.5 g/L L-cysteine to pre-reduce the culture media. All cultures were grown at 37°C in a Coy Laboratories anaerobic chamber under an atmosphere of 98% nitrogen, 2% hydrogen. A 1% inoculum of an overnight culture was used in each experiment. Auranofin (Alexis Biochemicals, San Diego, CA) was diluted in DMSO before addition to the culture media. Equal volumes of the resulting auranofin dilutions, or DMSO as a vehicle control, were added to each culture. To examine the role of selenium metabolism in the toxicity of auranofin the growth medium was supplemented with selenium (sodium selenite or L-selenocysteine) as indicated. Optical density measurements of cultures at 600 nm were determined using a Spectramax multiwell plate reader (Molecular Devices, Sunnyvale, CA) from 200 µL of culture after 24 hours of growth in each experiment.

### 4.2.4 75Se Incorporation into selenoproteins.

One mL cultures of *E. coli* (MC4100) were prepared in modified Luria both as described above in the presence of increasing concentrations of auranofin (5, 10, and 50 µM). For the
identification of selenoproteins, 6 µCi of $^{75}$Se (University of Missouri, Columbia), in the form of sodium selenite (100 nM), was added to each culture. To examine total protein synthesis, 20 µCi of $^{35}$S (methionine/cysteine mixture) was added to replicate cultures. After 24 hours of growth under anaerobic conditions, cells were harvested by centrifugation for 5 min at 5,000 x $g$ and resuspended in lysis buffer (25 mM Tris, pH 8.8, 1 mM DTT, 0.5 mM EDTA, 0.1 mM benzamidine). Cells were lysed by sonication using a sonic dismembranator, model 100 (Fisher Scientific), for 10 seconds at a power output of 12 W, and the resultant crude cell extracts were clarified by centrifugation at 13,500 x $g$ for 10 minutes. Protein concentration was determined by the Bradford assay using bovine serum albumin (Pierce) as a standard (13). Selenoproteins and total protein synthesis were analyzed by separating 25 µg of cell extracts using a 12% SDS-polyacrylamide gel, and radioisotope-labeled proteins were detected by PhosphorImager analysis (Molecular Dynamics).

The impact of auranofin on selenoprotein synthesis in C. difficile (NAP1/O27) was determined in 1 mL mid-logarithmic phase cultures. A 20% inoculum of an overnight culture was used in each experiment. C. difficile was cultivated in BHI + cysteine for 4 hours before addition of auranofin and 6 µCi of $^{75}$Se in the form of sodium selenite (100 nM) or 20 µCi of $^{35}$S (methionine/cysteine mixture). The cultures were incubated for an additional 4 hours before harvesting. Cell extracts were prepared and analyzed as described for E. coli.

4.2.5 $^{75}$Se Uptake studies.

The impact of auranofin on selenite uptake in E. coli and C. difficile (NAP1/O27) was determined in 1 mL mid-logarithmic phase cultures. A 20% inoculum of an overnight culture was used in each experiment. Both organisms were cultivated in BHI + cysteine for 2 hours before the addition of chloramphenicol (30 µg/mL) to inhibit protein synthesis during uptake analysis in
response to drug treatment. Various concentrations of auranofin (0 to 10 µM) were added to the cultures immediately followed by 4 µCi of $^{75}$Se in the form of sodium selenite (2 nM selenium). Cultures were incubated for 20 minutes at 37ºC. Cells were harvested by centrifugation (1 minute at 16200 x g). Culture media supernatants were placed on ice for further analysis by HPLC. Cells were washed once in 500 µL PBS and harvested by centrifugation (1 minute at 16,200 x g). The supernatant was discarded and the cells were resuspended in 500 µL PBS before measuring the total uptake of $^{75}$Se using a Model 1470 Gamma counter (Perkin–Elmer, Wellesley, ME).

4.2.6 HPLC analysis of $^{75}$Se in C. difficile growth media.

20 µL samples of reserved growth medium from the $^{75}$Se uptake study were separated by HPLC as described above for the mixtures of auranofin and selenide (above). Fractions were collected every minute and the distribution of $^{75}$Se (counts per minute) was measured using a gamma counter (Perkin-Elmer).

4.3 Results and Discussion

4.3.1 Auranofin reacts with HSe– to form an adduct.

HSe– is highly reactive and exquisitely sensitive to oxidation under aerobic conditions. When it is exposed to oxygen it is rapidly oxidized to elemental selenium forming a red precipitate. We took advantage of this simple oxidation reaction to determine if HSe– interacts directly with auranofin. Reactions of HSe– and auranofin were prepared anaerobically and subsequently exposed to ambient conditions. HSe– alone formed the expected red precipitate; however, reaction with auranofin protected it from oxidation to elemental selenium, suggesting formation of an oxygen-resistant complex (data not shown).
Auranofin (10 mM in DMSO) was mixed anaerobically with equal volumes of H₂O (a) or 20 mM hydrogen selenide (b). Reactions were incubated for 20 minutes before injection. 20 µL samples were loaded onto a C₁₈ column at a flow rate of 0.5 mL per minute. The starting solvent (used for injection) was 0.05% trifluoroacetic (TFA) acid in H₂O. A linear gradient (50 minutes) was developed to 100% acetonitrile, 0.05% TFA. Products were identified at 254 nm using a diode-array detector.
To isolate and identify this apparently stable complex, mixtures of auranofin and selenide were separated by HPLC using reverse phase chromatography. Auranofin alone elutes as a single peak (denoted as peak 1 in Figure 4-1). When HSe\(^-\) is added to auranofin, this results in the disappearance of peak 1 and coincides with the appearance of an earlier selenium-dependent peak (Peak 2, Figure 4-1). Similar reactions containing sodium selenite or selenocysteine did not alter the elution profile of auranofin, indicating that these forms of selenium are not reactive (data not shown). Reaction of auranofin with \(^{75}\)Se labeled selenide (formed by reaction of \(^{75}\)Se labeled selenite with DTT) confirmed that the earlier peak (peak 2) contained selenium (data not shown). Mass spectrometry revealed that the major product of an equal molar reaction of auranofin and HSe\(^-\) exhibits a selenium isotope signature and has a mass of 1025.10 atomic mass units (amu) (Figure 4-2). Based upon the mass obtained, we hypothesize that hydrogen selenide displaces the sulfur atoms in auranofin (Figure 4-3a) to form a stable complex (Figure 4-3b). Reactions of auranofin and selenide were further analyzed by X-ray absorption spectroscopy and the structure proposed by mass spectrometry was confirmed (67).
Figure 4-2 Mass spectrometry reveals major reaction products of auranofin and hydrogen selenide.

The predominant product from direct injection of an equimolar reaction of auranofin and hydrogen selenide has a mass of 1025.10 atomic mass units and exhibits a selenium isotope signature (22). The inset plot shows all of the products obtained across the entire spectrum. The predominant compound was also obtained after liquid chromatography as described in the methods section, and its mass was confirmed (data not shown) (Mass spectrometry data is courtesy of Dr. Rodney Levine, NIH, NHLBI).

Figure 4-3 Structure of auranofin (A) and the putative structure of the stable product formed upon reaction of auranofin and selenide (B), based on mass spectrometry.
4.3.2  Auranofin impacts the growth of anaerobically grown E. coli.

Given that auranofin reacts with HSe\textsuperscript{−} \textit{in vitro}, we next examined whether formation of this adduct impacts microbial growth in culture. Although \textit{E. coli} does not require selenium under normal laboratory conditions, selenoproteins play an important role in mixed acid fermentation during anaerobic growth. The most predominant selenoprotein in anaerobically grown \textit{E. coli} is formate dehydrogenase (FDH\textsubscript{h}). FDH\textsubscript{h} production is required for activity of the formate hydrogen lyase complex (FHL) and the production of hydrogen gas (49). Thus, \textit{E. coli} can be used to study dynamically the impact of auranofin on prokaryotic selenoprotein synthesis.

![Figure 4-4 Auranofin reduces growth yield of wild type \textit{E. coli}, but not a \textit{selD} mutant.](image)

Both wild type (MC4100) and a \textit{selD} mutant (WL400), which does not produce selenoproteins, were cultured in modified Luria broth under anaerobic conditions at 37°C. Auranofin (dissolved in 95% ethanol) was added to the culture medium before inoculation (1, 5, 10, 25, 50 µM). Optical density of the cultures was measured after 24 hours of growth. Data shown is from at least three independent cultures. Error bars indicate standard deviation.
We tested the effect of auranofin on growth and gas production in wild type *E. coli* (MC4100). Auranofin reduced the growth yield of MC4100 at 24 hours when present at concentrations of 25 and 50 µM (Figure 4-4). The growth inhibition observed under these conditions was similar to that seen with the deletion of *selD* and may be attributed to build-up of formic acid in the growth medium. In addition, growth of the isogenic *selD* deletion mutant (WL400) was not affected by auranofin. Further, gas production was significantly reduced in MC4100 cultures containing 10 and 20 µM auranofin (as assessed by Durham tubes) and was completely absent in those grown with 50 µM auranofin (data not shown). The effect of auranofin on MC4100 could be due interruption of selenoprotein synthesis, or possibly due to direct inhibition of FDH₁⁻

**4.3.3 Auranofin inhibits growth of *C. difficile*.**

Recent work in our laboratory demonstrated the central role of Stickland reactions in the growth of *C. difficile* (68). Stickland reactions are described as the coupled fermentation of amino acids in which one, the Stickland donor, is oxidized and the other, the Stickland acceptor, is reduced (153, 155). Glycine reduction results in production of acetyl phosphate, and thus ATP synthesis via substrate-level phosphorylation (143, 146). Reduction of proline has been tied to membrane gradients (103). The enzymes that catalyze these reactions in *C. difficile* are glycine reductase and D-proline reductase respectively. Both are selenoproteins (2, 68).
Figure 4-5 Auranofin inhibits growth of \textit{C. difficile}.

\textit{C. difficile} cultures (NAPI/O27, VPI10463, 630, and ATCC 9689) were grown anaerobically in BHI + cysteine. Auranofin in DMSO (0.25, 0.5, 0.75, 0.8, 0.85, 0.9, 0.95, 1.0, 1.5, and 2.0 µM) was added to the growth medium prior to inoculation. Growth was measured as optical density at 600 nm after 24 hours at 37°C. Percent growth (growth yield of inhibited cultures versus control) is plotted at the indicated concentrations of auranofin. Data shown is from at least three independent cultures. Error bars indicate standard deviation.

Based upon the knowledge that \textit{C. difficile} utilizes Stickland reactions for energy metabolism and the enzymes that catalyze these reactions are selenoproteins, we decided to determine the impact of auranofin on the growth of the organism. To determine the antimicrobial activity of auranofin, it was tested against four strains of \textit{C. difficile}. As with \textit{E. coli}, variable concentrations of auranofin (0.25 to 2 µM) were added to rich culture medium (BHI) before inoculation. The turbidity
of *C. difficile* cultures was measured spectrophotometrically (at 600 nm) following 24 hours of anaerobic growth. Growth of *C. difficile* is potently inhibited by auranofin and this growth inhibition is consistent among all four strains (Figure 4-5). At 2 µM auranofin, no appreciable growth was observed. A sharp decrease in growth occurred between 750 nM and 1 µM auranofin in all strains tested. The estimated IC<sub>50</sub> values are as follows: NAP1/O27, 775 nM; VPI 10463, 1000 nM; 630, 800 nM; ATCC 9689, 750 nM. Thus all strains were significantly inhibited by concentrations of auranofin in the high nanomolar range. In order to insure that vegetative growth rather than spore germination was examined, a 1% inoculum of mid-exponential phase cells was used in these experiments. Moreover we followed the inhibition of growth of one strain for the entire batch growth period and found concentration dependent inhibition of growth at each time point (Figure 4-6).

![Figure 4-6 Growth of *C. difficile* over a 24 hour time period in the presence of auranofin.](image)

*C. difficile* (NAP1/O27) cultures were grown anaerobically at 37 °C in BHI + cysteine in 96-well polystyrene plates. Auranofin in DMSO (0.25, 0.5, 0.75, 1.0, 1.5, and 2.0 µM) was added to the growth medium prior addition of a 1% inoculum from an overnight culture. Growth was measured as optical density at 600 nm at each time point. Data shown is from at least three independent cultures. Error bars indicate standard deviation.
**Figure 4-7 Inhibition of growth of C. difficile, C. perfringens and C. tetani by auranofin.**

*C. perfringens* (ATCC 19406) and *C. tetani* (ATCC 10543) were grown anaerobically in BHI + cysteine. Auranofin in DMSO (1.0, 5.0 and 10.0 µM) was added to the growth medium prior to inoculation. Growth was measured as optical density at 600 nm after 24 hours at 37°C. Percent growth (growth yield of inhibited cultures versus control) is plotted at the indicated concentrations of auranofin. Data shown is from at least three independent cultures. Error bars indicate standard deviation.

*C. tetani* and *C. perfringens* are human pathogens that are classified with *C. difficile* in a group of organisms known as the toxigenic clostridia. We therefore tested the growth of these organisms in the presence of auranofin to determine the relative specificity within this class. No significant inhibition of growth was observed at concentrations of auranofin up to 10 µM for *C. tetani* or *C. perfringens* (Figure 4-7). A similar pattern of sensitivity of *C. difficile* relative to other toxigenic species was observed in cultures grown in reinforced clostridial medium (data not shown). Based on genomic DNA sequence, neither *C. tetani* nor *C. perfringens* carry genes encoding glycine or D-proline reductase. There is also no evidence presented in the literature that these strains can catalyze Stickland reactions. In addition, neither strain carries genes encoding the needed components for selenoprotein synthesis (*selA, selB, selC* or *selD*) (148). We experimentally confirmed that no specific selenoproteins were expressed in *C. perfringens* using $^{75}$Se radiolabeling (data not shown).
inhibitory action of auranofin in *C. difficile* can be attributed to the organism’s reliance upon selenium and selenoproteins for growth.

4.3.4 *Auranofin inhibits selenoprotein synthesis in E. coli model and C. difficile.*

Previous work in our laboratory has demonstrated that auranofin prevents the incorporation of selenium into selenoproteins in mammalian cells. To determine the impact of auranofin on overall selenoprotein synthesis in *E. coli*, we cultivated MC4100 in the presence of several concentrations of auranofin with the addition of radiolabeled selenium (**75**Se) in the form of selenite. Cell extracts were prepared after 24 hours of anaerobic growth. The results clearly demonstrate a concentration dependent decrease of selenium incorporation into FDH$_H$ (Figure 4-8a). Total protein synthesis was not impaired by auranofin as indicated by **35**S labeling (Figure 4-8b).

Because auranofin potently inhibits the growth of *C. difficile* we treated exponentially growing cells with several concentrations of auranofin plus **75**Se and prepared cell extracts after four hours of anaerobic growth. We have previously used these same techniques to describe the presence and expression of both glycine and D-proline reductase in *C. difficile* (68). This allowed us to examine if auranofin also interferes with the incorporation of selenium into selenoproteins in this organism. As was the case with *E. coli*, a clear decrease in selenoprotein synthesis was observed (Figure 4-8c), but there was no impact on total protein synthesis (Figure 4-8c). A slight increase in the selenoprotein component of D-proline reductase (PrdB, Figure 4-8c) is also observed. The cause for this increase is not yet known. Nonetheless, these results clearly demonstrate that auranofin prevents selenoprotein synthesis in both organisms. The mechanism of *C. difficile* growth inhibition is not likely due to direct inhibition of glycine or D-proline reductase. Rather, auranofin does not allow the production of these critical selenoenzymes.
Figure 4-8 Auranofin prevents incorporation of $^{75}\text{Se}$ in anaerobically grown *E. coli* and *C. difficile*.

Soluble extracts of MC4100 treated with varying concentrations of auranofin (0, 5, 10, 50 µM lanes 1-4 respectively) were prepared following anaerobic growth overnight in the presence of 6 µCi of $^{75}\text{Se}$ (0.1 µM Na$_2$SeO$_3$) (a) or 20 µCi of $^{35}\text{S}$ (b). Twenty-five micrograms of protein from crude cell extracts were separated by a 12% SDS-polyacrylamide gel, and the radioactive bands were visualized using a phosphoimager. (c). Soluble extracts of *C. difficile* (NAPI/O27 strain) were prepared after treatment mid-logarithmic phase with auranofin (0, 5, 10 µM lanes 1-3 respectively) in the presence of 6 µCi of $^{75}\text{Se}$ (0.1 µM Na$_2$SeO$_3$) (a) or 20 µCi of $^{35}\text{S}$ (d). Twenty-five micrograms of protein from crude cell extracts were separated by a 12% SDS-polyacrylamide gel, and the radioactive bands were visualized using a phosphoimager. The predicted selenoproteins are indicated by arrows based on the molecular weights of the genes as described previously (68).
4.3.5 \textit{Auranofin prevents the uptake of selenium by E. coli and C. difficile.}

The mechanisms of selenium uptake in both prokaryotes and eukaryotes are undefined. Thus far we have shown that auranofin forms a complex with HSe\textsuperscript{−} \textit{in vitro} and auranofin inhibits selenoprotein synthesis in both \textit{E. coli} and \textit{C. difficile}. We examined whether the formation of the auranofin-selenide adduct prevents the uptake of selenium from the growth medium. Uptake of radiolabeled selenite (\textsuperscript{75}Se) occurs rapidly in mid-logarithmic cultures of both organisms in rich media (BHI) and is easily quantified using a gamma counter. For this study, actively growing cultures were treated with varying concentrations of auranofin immediately followed by the addition of \textsuperscript{75}Se (2 \mu Ci). The uptake of selenium was followed kinetically for a period of 60 minutes and was found to be essentially linear over this period (data not shown). Using a fixed time point (20 minutes) we observed a clear inhibition in the amount of radiolabel transported into the cells (Figure 4-9) when auranofin was added. This inhibition was also concentration dependent. For \textit{C. difficile}, 500 nM auranofin reduced the uptake of \textsuperscript{75}Se by approximately 50\%. The slight variation in the effect of auranofin on \textsuperscript{75}Se uptake between \textit{E. coli} and \textit{C. difficile} may be attributed to differences in the cell membranes of these organisms (\textit{E. coli} is gram negative whereas \textit{C. difficile} is gram positive).
Figure 4-9 Auranofin prevents uptake of $^{75}$Se by *E. coli* and *C. difficile*.

Actively growing cultures of *E. coli* (A) and *C. difficile* (B) were treated with auranofin (0 to 25 µM as indicated) as well as $^{75}$Se. Cultures were incubated for 20 minutes at 37ºC. Cells were harvested by centrifugation and washed with PBS. Total uptake of $^{75}$Se was analyzed using a Model 1470 Gamma counter (Perkin–Elmer, Wellesley, ME) and is reported as counts per minute (CPM). Data shown is from at least three independent cultures. Error bars indicate standard deviation.
Figure 4-10 Auranofin forms a complex with radiolabeled selenium in *C. difficile* growth medium.

Growth media from the uptake experiments (0 and 10µM auranofin, *a* and *b* respectively) were separated by HPLC as described for the analysis of the mixtures of auranofin and hydrogen selenide. Fractions were collected every minute and analyzed using the gamma counter.

4.3.6 *Auranofin forms a complex with selenium in C. difficile growth media.*

Media supernatants from the uptake study with *C. difficile* were fractionated by HPLC as described for the analysis of the mixtures of auranofin and selenide above. In media treated with DMSO alone (vehicle control) a small peak of radioactivity was observed at the beginning of the trace with no other distinguishable peaks (Figure 4-10a). In contrast, media treated with 10 µM
auranofin exhibited a clearly defined peak of $^{75}$Se at approximately 22 minutes (Figure 4-10b). These data indicate that the earlier identified auranofin-selenium complex is formed in the growth media preventing uptake and nutritional utilization of selenium by *C. difficile*.

**4.3.7 Selenium supplementation prevents auranofin dependent growth inhibition.**

*C. difficile* expresses at least three major selenoproteins that could be directly inhibited by auranofin (glycine reductase, D-proline reductase, and selenophosphate synthetase). If the formation of the auranofin-selenide adduct is indeed blocking the metabolic use of selenium in bacterial cultures, then supplementation would alleviate this inhibition. Conversely, if auranofin is directly inhibiting one or more selenoproteins, then supplementation would be unlikely to affect the action of the drug. Thus, we evaluated the impact of selenium supplementation on growth of the NAP1/O27 strain in the presence of inhibitory concentrations of auranofin.
Figure 4-11 Selenium supplementation prevents auranofin-dependent growth inhibition of *C. difficile*.

*C. difficile* (NAPI/O27) was cultivated in BHI + cysteine. The culture medium was supplemented with either sodium selenite (0.5, 1.0, 5.0 µM) (a) or L-selenocysteine (0.5, 1.0, 5.0 µM) (b). Percent growth (growth yield of inhibited cultures versus control) is plotted at the indicated concentrations of auranofin after 24 hours at 37ºC. Data shown is from at least three independent cultures. Error bars indicate standard deviation.

The addition of 5 µM sodium selenite to the growth medium significantly reduces the impact of auranofin on *C. difficile*, with lower concentrations of selenite also exhibiting a protective effect (Figure 4-11a). Selenocysteine was not as potent, but the addition of 5 µM selenocysteine to the growth medium was also protective (Figure 4-11b). This disparity may be due to differences in the ability of the organism to utilize selenium from selenite versus selenocysteine. It should be noted...
that supplementation of BHI with selenite or selenocysteine did not significantly increase growth yield alone.

4.3.8 Discussion

Recently, targeting selenoproteins has become an interesting avenue for the development of anticancer therapies (172, 173). These strategies provide a new angle to combating an immensely complex human health problem. In addition to their role in mammalian cells, selenoproteins are necessary for the growth of several significant human pathogens. It is becoming clear that the potential of selenoenzyme inhibition and interruption of selenoprotein synthesis as a means for antimicrobial development cannot be overlooked. The unique enzymatic characteristics of selenoproteins and their complex assembly machinery provide several prospective antimicrobial targets.

*Clostridium difficile* continues to be a major cause of hospital acquired infection that warrants further attention. With the mortality rates from *C. difficile* increasing by 35% per year from 1999 to 2004 (133), and an increasingly poor response to metronidazole, the preferred treatment for CDAD, (114) new tactics for combating this disease must be developed. In this study we capitalized on *C. difficile*’s unique reliance on selenoenzymes for energy metabolism. We demonstrated that auranofin diminishes the growth of *C. difficile* at nanomolar concentrations.

Based upon the data that we have gathered so far, we cannot eliminate the possibility that auranofin may directly inhibit one or more of the selenoenzymes in *C. difficile*. Preliminary experiments did not indicate that D-proline reductase was inhibited by auranofin (data not shown), but *C. difficile* expresses at least two other selenoproteins, glycine reductase and selenophosphate synthetase. The observed effect of selenium supplementation on auranofin toxicity may be due to
relief of enzyme inhibition through competitive binding to free hydrogen selenide. Figures 4-12 and 4-13, however, clearly show a reduction in the incorporation of radiolabeled selenium into selenoproteins with the addition of auranofin to the growth medium. In this context it appears that potential direct inhibition of glycine reductase, or D-proline reductase, is irrelevant.

Previous studies suggest that Au(I) containing compounds, such as auranofin, inhibit selenoenzymes by binding to the reduced selenocysteine at the active site. Although there is substantial literature examining the chemical interactions between Au and Se, little research has focused on the biological implications. The alteration of mammalian selenium metabolism by Au(I) containing drugs has been demonstrated suggesting that covalent reactions between Au(I) and the nucleophilic metabolites of selenium could limit the nutritional availability of selenium for the production of selenoproteins (53). In this report we have clearly shown that auranofin reacts with \( \text{HSe}^- \) in vitro to form a stable complex. This is consistent with other chemical studies utilizing organic selenolate compounds to demonstrate similar Au-Se interactions (33). If auranofin reacts with hydrogen selenide in vivo then the pool of available selenium for selenoprotein synthesis would be reduced. We have shown that this complex occurs in bacterial culture to prevent the uptake and nutritional utilization of selenium by both E. coli and C. difficile. The implications of these results for mammalian systems must be further studied.

Recently there have been several studies that have demonstrated the activity of auranofin against significant eukaryotic human pathogens (85, 98). They examined inhibition of selenoenzymes in these organisms, but did not consider the possibility that auranofin could inhibit overall selenoprotein synthesis. In light of our results, this warrants further examination.

The mechanisms of selenium transport and reduction to hydrogen selenide remain enigmatic. Our results provide some insight into the metabolism of selenium upstream of
selenophosphate synthetase, suggesting that the reduction of selenite to HSe\(^-\) occurs before it is taken up by the bacterial cell. In addition to its potential implications for human health, auranofin may be used as a tool to study selenium metabolism. We can take advantage of the ability of auranofin to form a complex with hydrogen selenide to further elucidate prokaryotic selenium metabolism upstream of selenophosphate synthetase.

Finally, given that auranofin appears to block the metabolic use of an essential nutrient, rather than acting upon a single enzyme, the development of resistant strains is improbable. Studies are underway to determine whether strains can be isolated that are resistant to auranofin. Given its importance in energy metabolism, and the fact that multiple enzymes require selenium, resistance is unlikely to occur by point mutation.
CHAPTER 5
INHIBITION OF SELENIUM METABOLISM IN THE ORAL PATHOGEN TREPONEMA DENTICOLA

5.1 Introduction

The biological use of selenium as a catalyst, incorporated into proteins as selenocysteine, is broad. It plays an essential role in energy metabolism, redox balance, and reproduction in a variety of organisms from bacterial pathogens, to eukaryotic parasites, to humans. Several epidemiological studies indicate that higher levels of selenium in the mammalian diet can have a negative effect on dental health (12, 56-58, 161). Although the impact of selenium is attributed to its influence on the physical properties of the enamel surface (25), the role of selenium in supporting the oral microbial community has not been studied.

The oral cavity is a highly complex microbiome with a large proportion of its residents uncharacterized due to their fastidious nature and resistance to traditional culture methods (34). Analysis of whole saliva indicates that bacterial metabolism influences the amino acid composition and indicates a role for amino acid fermentation (157). Curtis et al demonstrated the occurrence of Stickland reactions in dental plaque (24). These reactions were first described in clostridia (153-155). They involve the coupled fermentation of amino acids in which one amino acid is oxidized (Stickland donor) and another (Stickland acceptor) is reduced (118). Treponema denticola, an established resident of the oral cavity, performs Stickland reactions via the selenoprotein glycine reductase (134). Glycine reductase is composed of a multi-protein complex that contains two separate selenoproteins, termed selenoprotein A and selenoprotein B (3, 20, 21, 45, 52). This complex of proteins converts glycine to acetyl-phosphate using inorganic phosphate and reducing potential from thioredoxin. For the organisms that use this complex, this is a vital source of ATP.
Thus far, the requirement for selenocysteine at the active site of this enzyme complex is universally conserved, even though all other selenoproteins that have been identified using computational techniques have a putative cysteine homologue (83).

*T. denticola* is considered one of the primary pathogens responsible for periodontitis, a chronic inflammatory disease that is the major cause of adult tooth loss (34, 113, 138). It is the best studied oral spirochete, commonly found with other spirochetes within the periodontal pocket. It expresses a variety of virulence factors and is capable of adhering to and penetrating endothelial cell monolayers (125). Its health impact may reach beyond the oral cavity. A recent study linked periodontitis with peripheral arterial disease and detected *T. denticola* along with other periodontal pathogens in athlerosclerotic plaque (16). Sequence analysis indicates the presence of several selenoproteins within the genome of *T. denticola* in addition to glycine reductase (83). This organism exhibits a strict growth requirement for selenium (134).

A significant literature exists clearly demonstrating the antimicrobial activity of fluoride compounds against microorganisms associated with dental decay and periodontitis. Both sodium fluoride and stannous fluoride inhibit growth of *T. denticola*, as well as stannous ions alone (64). The inhibitory effect of stannous salts on *T. denticola* growth is unexplained. It should be noted that toothpastes containing stannous fluoride are more effective in reducing gingivitis and plaque (117, 122).

Tin, as well as several other trace elements, modulates the effects of acute selenium toxicity (63). Conversely, selenium affects the activity of tin in animal models (17-19). In this study we examine the possibility that stannous ions interfere with selenium metabolism in *T. denticola*. 
5.2 Materials and Methods

5.2.1 Growth of Treponema denticola

ATCC strain 33520 was cultivated in rich medium (ATCC 1357: NOS Spirochete medium). Cultures were prepared inside the anaerobic chamber in 96-well polystyrene plates and incubated at 37°C in multi-plate Bio-Bag Environmental Chambers (Type A-Becton Dickinson) which produce CO₂ to supplement growth. Optical density measurements were obtained after 48 hours of growth using a Spectramax 190 UV-visible spectrophotometer (Molecular Devices). Varying concentrations of tin fluoride, tin chloride, sodium fluoride (1mg/mL in H₂O) and auranofin (in DMSO) were added prior to inoculation as indicated. For supplementation studies, selenium was added to the growth medium in the form of sodium selenite or L-selenocysteine before inoculation.

5.2.2 Selenoprotein synthesis

The impact of the metal salts and auranofin on selenoprotein synthesis in T. denticola was determined in 1mL mid-logarithmic phase cultures. A 48 hour batch culture was used in each experiment and distributed into 1mL aliquots before addition of the metal salts or auranofin. Radioisotope, 3µCi of ⁷⁵Se in the form of sodium selenite (10 µM) or 44 µCi of ³⁵S (methionine/cysteine mixture), was subsequently added to each culture. The cultures were incubated for 4 hours before harvesting. Cells were harvested by centrifugation for 5 min at 5,000 x g and resuspended in lysis buffer (50 mM Tris, pH 7, 1 mM DTT, 0.5 mM EDTA, 0.1 mM benzamidine). Cells were lysed by sonication using a sonic dismembranator, model 100 (Fisher Scientific), for 10 seconds at a power output of 12 W, and the resultant crude cell extracts were clarified by centrifugation at 13,500 g for 10 minutes. Protein concentration was determined by the Bradford assay using bovine serum albumin (Pierce) as a standard (5). Selenoproteins and total protein
synthesis were analyzed by separating 20 µg of cell extracts using a 12% SDS-polyacrylamide gel, and radioisotope-labeled proteins were detected by PhosphorImager analysis (Molecular Dynamics). Relative incorporation of 75Se or 35S into proteins was determined by quantitative phosphorimage analysis (ImageQuant, Molecular Dynamics).

5.2.3 75Se uptake

The impact of tin ions on selenite uptake was determined in 1 mL aliquots of an actively growing batch culture (48 hours after inoculation). Chloramphenicol (30 µg/mL) was added to inhibit protein synthesis during uptake analysis in response to drug treatment. Various concentrations of tin chloride (5, 10, 25, 50 mg/mL) were added to the cultures immediately followed by 4 µCi of 75Se in the form of sodium selenite (2 nM selenium). Cultures were incubated for 20 minutes at 37°C. Cells were harvested by centrifugation (1 minute at 16200 x g). Cells were washed once in 500 µL PBS and harvested by centrifugation (1 minute at 16,200 x g). The supernatant was discarded and the cells were resuspended in 500 µL PBS before measuring the total uptake of 75Se using a Model 1470 Gamma counter (Perkin–Elmer, Wellesley, ME).

5.3 Results and Discussion

5.3.1 T. denticola growth is inhibited by stannous salts and auranofin.

We first aimed to define the inhibitory concentrations of sodium fluoride, stannous fluoride and stannous chloride against T. denticola (ATCC strain 33520) in NOS spirochete medium (ATCC 1357). We cultivated cells in 96-well plates and incubated at 37°C (Figure 5-1A). Optical density measurements were obtained after 48 hours of growth. Under these conditions we found that cultures grew steadily over a period of 48-72 hours, and thus 48 hours represents an appropriate
point in the batch growth to assess changes in growth yield. A fifty percent reduction in growth yield was observed in the presence of 10 µg/mL stannous fluoride, whereas those treated with sodium fluoride did not show a similar reduction until a dose of 50 µg/mL. This difference in inhibitory concentrations could be attributed to the concentration of fluoride ions. Cultures treated with stannous chloride, however, exhibited growth inhibition similar to those treated with stannous fluoride, indicating that stannous ions, independent of fluoride, inhibit growth of *T. denticola*. This is consistent with a previous study on the inhibition of growth by stannous salts (64).
Figure 5-1 *T. denticola* growth inhibition by stannous fluoride, stannous chloride, sodium fluoride and auranofin.

Cultures (200µL) were incubated anaerobically at 37°C in NOS Spirochete medium (ATCC 1357) in 96 well polystyrene plates. Optical density was measured after 48 hours of growth at a wavelength of 600 nm. (A) Stannous salts, sodium fluoride (0, 2.5, 5, 10, 25, 50, 75, 100 µg/mL, final) and (B) auranofin (0.068, 0.34, 0.68, 3.4, 6.8 µg/mL, final) were added to the culture media prior to inoculation. Percent growth (growth yield of inhibited cultures versus control) is plotted. Data shown is from at least three independent cultures. Error bars indicate standard deviation.

Recent work in our laboratory demonstrated that auranofin [2,3,4,6-tetra-o-acetyl-1-thio-β-D-glycopyranosato-S-(triethyl-phosphine) gold], a known inhibitor of selenocysteine containing enzymes, can interact with the reduced form of selenium, selenide (Se^3^-), to form a stable complex.
and thus prevent its nutritional utilization and incorporation into selenoproteins (67). Given that tin is known to interact with selenium in biological systems, we chose to also examine the impact of auranofin on the growth of *T. denticola* (Figure 5-1B). As with the stannous salts, cultures were incubated in the presence of auranofin for 48 hours before analyzing growth yield. Auranofin potently inhibits the growth of *T. denticola* at concentrations less than 5 µg/mL.
Figure 5-2 Supplementation with sodium selenite counteracts the antimicrobial nature of stannous ions, but not fluoride.

Cultures were grown and optical densities recorded as described in Figure 1 in the presence of (A) stannous fluoride, (B) sodium fluoride, and (C) stannous chloride. Sodium selenite was added prior to inoculation. Data shown is from at least three independent cultures. Error bars indicate standard deviation.
Figure 5-3 Supplementation with selenocysteine also alleviates stannous ion dependent growth inhibition.

Cultures were grown and optical densities recorded as described in Figure 1 in the presence of (A) stannous fluoride, (B) sodium fluoride, and (C) stannous chloride. Selenocysteine was added prior to inoculation. Data shown is from at least three independent cultures. Error bars indicate standard deviation.
5.3.2  Selenium supplementation modulates the effects of stannous salts, but not sodium fluoride.

If stannous ions present in the growth medium are interacting with available selenium, then selenium supplementation should alleviate the observed growth inhibition. Selenium was added to the growth medium in the form of sodium selenite (Figure 5-2) or L-selenocysteine (Figure 5-3) before inoculation. This addition reduced, but did not eliminate the effect of stannous fluoride on T. denticola growth. Similar results were obtained for stannous chloride. It should be noted that the lowest concentration of selenite tested had the most pronounced affect on growth either when added to cells alongside stannous fluoride or stannous chloride, suggesting that a simple titration of selenium and tin cannot fully explain the changes in growth observed. It is possible that an insoluble complex of tin and selenium is formed and this complex is more toxic at higher concentrations.

Both selenite and selenocysteine can be utilized for selenoprotein synthesis (87). In either case, the selenium atom must be reduced to Se\(^2\) which is highly reactive and serves as the substrate for selenophosphate synthetase (166). Se\(^2\) is the most likely candidate for interaction with stannous ions in T. denticola. For both stannous chloride and stannous fluoride, selenocysteine had a more pronounced effect on toxicity of tin than sodium selenite. Moreover there was clearly not an additional toxicity when selenocysteine was present in increasing levels as was the case with addition of selenite. Without a further understanding of how T. denticola processes and utilizes selenium, this difference cannot yet be explained.

In contrast to the results obtained with the stannous salts, the toxicity of sodium fluoride was unaffected by the addition of selenium to the growth medium (Figures 5-2B and 5-3B). The antimicrobial property of the fluoride ion alone does not appear to be related to the T. denticola’s dependence on selenoproteins for growth. Taken together, these results suggest an intersection exists between selenium metabolism and stannous salt toxicity. We used auranofin to further
demonstrate this point. The addition of both selenite and selenocysteine in the presence of auranofin induced a protective effect similar to that observed with the stannous salts (Figure 5-4). Likewise, selenocysteine was more potent in reducing the toxicity.

Figure 5-4 Selenium supplementation in the presence of auranofin yields a similar effect to that observed with stannous salts.

Cultures were grown and optical densities recorded as described in Figure 5-1. Cultures were treated with varying concentrations of auranofin (0.068, 0.34, 0.68, 3.4, 6.8 µg/mL, final). Selenium in the form of (A) sodium selenite or (B) L-selenocysteine were added prior to inoculation. Percent growth (growth yield of inhibited cultures versus control) is plotted. Data shown is from at least three independent cultures. Error bars indicate standard deviation.
Figure 5-5 Stannous chloride and auranofin inhibit $^{75}$Se incorporation into *T. denticola*.

Actively growing cultures were treated with stannous chloride (12.5 and 50 µg/mL) or auranofin (1.7, 3.4, 6.8 µg/mL) followed by the addition of (A) $^{75}$Se (sodium selenite) or (B) $^{35}$S (methionine/cysteine mixture). Cultures were harvested after 4 hours at 37°C and crude cell extracts were analyzed by SDS-PAGE. Radiolabeled proteins were visualized by phosphorimager (Molecular Dynamics). Identification of glycine reductase subunits (GrdA and GrdB, 18 and 45 kDa respectively) is based upon predicted molecular weights (134).
Table 5-1 Quantification of selenium incorporation into selenoproteins

<table>
<thead>
<tr>
<th>Condition</th>
<th>Concentration (µg/mL)</th>
<th>^75^Se/^35^S Ratio(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>9.7</td>
</tr>
<tr>
<td>SnCl</td>
<td>12.5</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>2.9</td>
</tr>
<tr>
<td>Auranofin</td>
<td>1.7</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>3.4</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>6.8</td>
<td>0.75</td>
</tr>
</tbody>
</table>

\(^a\) Relative incorporation of ^75^Se or ^35^S into proteins was determined by quantitative phosphoimage analysis (ImageQuant, Molecular Dynamics).

5.3.3  Stannous salts and auranofin inhibit selenoprotein synthesis.

Radiolabeling studies were performed to further examine the relationship between stannous salts and selenium metabolism. Inhibitory concentrations of stannous chloride and auranofin were used to treat actively growing cultures of *T. denticola* (48 hours after inoculation) followed by the addition of the radiolabel, ^75^Se, in the form of sodium selenite (10 nM). Cultures were incubated for 4 hours to allow for de novo protein synthesis. Cells were harvested by centrifugation and lysed by sonication. Selenoproteins and total protein synthesis were analyzed by separating 20 µg of cell extracts by SDS-PAGE. Both stannous chloride and auranofin inhibited ^75^Se incorporation (Figure 5-5A). Interestingly, the most profound effect was to reduce the presence of small molecular weight selenium compounds.
Replicate cultures were labeled with $^{35}$S to evaluate total protein synthesis. Both stannous chloride and auranofin induced a slight reduction in $^{35}$S labeling (Figure 5-5B). Relative incorporation of $^{75}$Se or $^{35}$S was determined by quantitative phosphorimage analysis (ImageQuant, Molecular Dynamics). To control for the differences in total protein synthesis, the ratio of $^{75}$Se band intensity to that of the major bands labeled with $^{35}$S was calculated (Table 5-1). Despite the decrease in total protein, both stannous chloride and auranofin specifically reduce selenoprotein synthesis as compared to the control. While this reduction was expected with auranofin based on our recently published results studying selenium metabolism in *C. difficile* (67), the effect of stannous chloride provides additional evidence that stannous ions interfere with selenium metabolism in this organism.

### 5.3.4 Stannous chloride prevents uptake of selenium.

The reduction in the small molecule selenium compounds and selenoprotein synthesis observed by radiolabel indicates that stannous ions interfere with the ability of *T. denticola* to utilize nutritionally available selenium from the growth medium. It is unclear at which point in selenium metabolism this interference occurs. The mechanisms of uptake and processing of nutritional sources of selenium by either prokaryotic or eukaryotic cells are poorly understood. However, we have recently shown that hydrogen selenide is likely the form of selenium taken up by mammalian cells (43) or *Clostridium difficile* (67). We can determine if stannous salts inhibit selenium uptake by *T. denticola* by following the assimilation of $^{75}$Se in whole cells. The impact of stannous ions on selenium uptake was determined in actively growing cells. Chloramphenicol was added to inhibit protein synthesis. Stannous chloride was added to the cultures immediately followed by $^{75}$Se, selenite. Stannous chloride potently inhibits uptake of selenium (Figure 5-6).
Figure 5-6 Stannous chloride prevents uptake of $^{75}$Se by *T. denticola*.

Actively growing cultures were treated with stannous chloride (5, 10, 25, 50 µg/mL) followed by the addition of $^{75}$Se (sodium selenite). Cultures were incubated for 20 minutes at 37°C. Cells were harvested by centrifugation and washed with PBS. Total uptake of $^{75}$Se was analyzed using a Model 1470 Gamma counter (Perkin–Elmer, Wellesley, ME) and is reported as percent uptake (as compared to control). Data shown is from at least three independent cultures. Error bars indicate standard deviation.

Although we have added selenium in the form of selenite for our labeling study (only form available), it is clear from many chemical and biochemical studies that selenite will react with any available thiols present in the culture medium to form selenotrisulfides and hydrogen selenide (41, 42, 96, 139). Tin selenides are commonly studied in materials science, especially in the formation of solid thin films (70), yet no literature exists on solution complexes or adducts of SnSe or SnSe$_2$ in a biological context. In a recent work we describe an adduct of auranofin that is a stable Au-Se complex formed upon reaction of the gold drug with hydrogen selenide (67). Since hydrogen selenide is unstable under aerobic conditions (quickly oxidizes and forms a red precipitate) we found that reaction of auranofin and selenide led to formation of a stable adduct that did not yield a red precipitate (67). Likewise, we have also mixed tin chloride and tin fluoride with hydrogen selenide and found that a red precipitate was not formed, but that a colored precipitate did form (data not
shown). This reactivity suggests that we are forming SnSe and SnSe₂ insoluble salts, and the chemistry of this reaction will be the subject of ongoing investigation emanating from this work. It should be noted that although we did see a reduction in selenium transport, we are still not sure that any chemical reaction that occurs between tin and selenium is limited to the extracellular milieu.

The impact of stannous salts on the growth of *T. denticola* was previously established without deriving the mechanism of action. Here we demonstrate that stannous salts impair selenium metabolism in this organism. Given that selenium is required for the synthesis of glycine reductase and, consequently, acetyl phosphate for ATP synthesis, we proposed that is the root of growth inhibition. Stannous fluoride is widely used in toothpastes and other oral treatments. Understanding the implications of these results requires a further understanding of the role of selenium metabolism and amino acid fermentation in the oral bacterial community.
CHAPTER 6
GENERAL DISCUSSION

The dawn of antibiotics in the first half of the twentieth century revolutionized health care. By the 1960s many scientists believed that infectious disease would soon become obsolete. Clearly this has not been the case. The emergence of “superbugs” such as methicillin resistant *Staphylococcus aureus* and vancomycin resistant *Enterococcus faecalis* has demonstrated that we cannot let down our guard against our microbial foes. While it seems we have reached the limits of traditional antimicrobial development, in this post genomic era we have the opportunity to tackle the problem from a new angle. The wide array of information found within bioinformatic databases and the vast number of tools that are now available allow us to explore and find new drug targets through inquiry at the molecular level. This begins by developing an understanding of the mechanisms of pathogenesis and the underlying processes that drive the growth of microorganisms. Building upon the knowledge gained, we can carefully tailor our approaches to combating infectious disease.

Our study began with the recognition that the genome of *C. difficile* encodes the genes required for Stickland fermentation. We set out to define the relevance of a unique means of energy generation in the context of a significant human pathogen. The breadth of our work ranges from the study of this specialized metabolic pathway to the proof of principle that blocking selenium metabolism can prevent the growth of this, and other organisms that impact human health. We demonstrated that Stickland fermentation plays a central role in *C. difficile* energy metabolism. We showed that this has important consequences for the pathogenesis *C. difficile* in terms of toxin production. Initially, we attempted to use auranofin to specifically inhibit the enzymes responsible for Stickland fermentation. Our results indicate that it blocks selenium metabolism in the organism.
as a whole. What began with the study of a very narrow topic became significant with regard to many human pathogens that rely upon selenoproteins for growth.

The pathway for selenoprotein synthesis is a multistep process that is dependent on many different protein partners. The formation of a complex between auranofin and selenide prevents C. difficile and T. denticola from acquiring selenium from the growth medium. This effectively blocks the entire pathway. Additional work is required to determine if this effect occurs in vivo. As with any antimicrobial strategy, we must also examine the potential for the development of resistance. Although we feel it is unlikely, further studies are required to address the possibility.

We feel that this work has broad implications; however, it is clear that there is much that remains to be elucidated. For example, are there specific inhibitors of D-proline reductase or glycine reductase that can inhibit growth of C. difficile? Since this organism utilizes both enzymes for Stickland reactions, it is unlikely that an inhibitor that blocks just one of them would be effective in preventing growth. Also, what role does Stickland fermentation play in the overall ecology of C. difficile disease? Although we demonstrated that C. difficile responds to the presence of Stickland acceptor amino acids in the growth medium to alter toxin production, the mechanism by which this occurs is unknown. Does the organism specifically sense these amino acids to regulate gene expression or is the observed phenotypic response due to downstream effects of inducing Stickland fermentation? What is the influence of Stickland fermentation on sporulation and germination? Generation of strains deficient in D-proline reductase and glycine reductase enzyme would be useful in answering these questions. Unfortunately, genetic manipulation of C. difficile has proven to be quite difficult. There have been recent advances in this area and we hope that we may soon be able
to overcome this challenge. As is often the case with scientific inquiry, we are left with more questions than we have answers.
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