Exploring the Physiology of Clostridioides difficile: Selenium-Dependent Catabolism of Host-Derived Nutrients

Michael A. Johnstone
University of Central Florida

Part of the Bacterial Infections and Mycoses Commons

Find similar works at: https://stars.library.ucf.edu/etd2023
University of Central Florida Libraries http://library.ucf.edu

This Doctoral Dissertation (Open Access) is brought to you for free and open access by STARS. It has been accepted for inclusion in Graduate Thesis and Dissertation 2023-2024 by an authorized administrator of STARS. For more information, please contact STARS@ucf.edu.

STARS Citation
https://stars.library.ucf.edu/etd2023/154
EXPLORING THE PHYSIOLOGY OF CLOSTRIDIOIDES DIFFICILE: SELENIUM-DEPENDENT CATABOLISM OF HOST-DERIVED NUTRIENTS

by

MICHAEL ALAN JOHNSTONE
B.S., University of Central Florida, 2017
M.S., University of Central Florida, 2019

A dissertation submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
in the Burnett School of Biomedical Sciences
in the College of Medicine
at the University of Central Florida
Orlando, Florida

Spring Term
2024

Major Professor: William T. Self
ABSTRACT

*Clostridioides difficile* is a bacterial pathogen that causes pseudomembranous colitis and the majority of antibiotic-associated diarrheal cases. Broad-spectrum antibiotic usage disrupts the normal gut microbiota and thereby compromises colonization resistance, the main defense against *C. difficile* infection. Treatment options are limited to vancomycin, fidaxomicin, and the fecal microbiota transplant. Addressing the scarcity of these therapeutics, we documented two explorations in *C. difficile* drug discovery: (i) evaluation of antibacterial and toxin-suppressing activity of (+)-puupehenone and similar derivatives, and (ii) clarification of a discrepancy in the hypothesized mechanism of auranofin against *C. difficile*. A better understanding of how *C. difficile* colonizes and thrives in the gut can greatly benefit therapeutic development. Interestingly, *C. difficile* can scavenge nutrients such as amino acids and possibly even purines during infection. Amino acids including proline and glycine act as substrates for Stickland metabolism, a bioenergetics scheme that partially relies on enzymes containing selenium in the form of selenocysteine (e.g., D-proline reductase and glycine reductase). Purines such as xanthine and uric acid can be degraded by bacterial molybdenum hydroxylases harboring an uncharacterized form of selenium, though the role of these enzymes in *C. difficile* physiology is poorly understood. Selenium likely plays a key role in the scavenging of these nutrients during *C. difficile* infection. Our investigation of these selenium-dependent enzymes revealed two new findings in *C. difficile* biology: (i) a link between proline-dependent growth and D-proline reductase, characterized as an energy “addiction,” and (ii) a previously uncharacterized selenium-dependent pathway involved in the catabolism of xanthine and uric acid. Overall, these physiological analyses of *C. difficile* provide promising candidates for therapeutics and key information regarding the organism’s nutrient preferences.
To my mother, Sheri Johnstone, and my father, Steve Johnstone, who passed away on July 25th, 2022. All glory goes to my heavenly Father, Jesus Christ, YHVH.

“Also I heard the voice of the Lord, saying, Whom shall I send, and who will go for us? Then said I, Here am I; send me.” – Isaiah 6:8
ACKNOWLEDGMENTS

Firstly, I must extend unending gratitude to my mentor, Dr. William Self, whose professionalism and patience have been crucial to my development as a scientist. My success could not have been achieved without Dr. Self’s fantastic mentorship. It has been an awesome journey ever since I joined the lab in 2017 as a master’s student—I remember how I struggled to purify the SclA protein back then! From the bottom of my heart: thank you, Doc. I am genuinely proud to have been your student, and I wish you continued success and multiple publications in the near future. Let me know when you finally find that high-affinity selenium transporter—it’ll be a great Nature story!

I humbly acknowledge my committee members Drs Sean Moore, Kenneth Teter, Mollie Jewett, and Joseph Sorg for their advice and for challenging me at every step of my doctoral journey. I am honored to have been formally grilled by all of you. I extend special thanks to my external committee member Dr. Sorg for granting me the opportunity and the tools to expand my genetic skillsets. To the members of the Sorg lab: these projects were made possible because of the tools you created, so thank you. I’m very proud of the fact that the Self lab can now perform genetic investigations of C. difficile!

I acknowledge the esteemed gentlemen in my Dungeons and Dragons group: Dr. Nikhil Bose, Jonhoi Smith, Christian Millot, Kohlton Bendowski, and Andrew Kwiat. Aside from serving as a great way to take a break from the lab, this activity allowed me to channel my creativity and practice my storytelling which, interestingly enough, improved my overall ability to think scientifically. I’m still unsure how we managed to convince a bunch of full-time graduate students to play pretend for 3–4 hours every other Monday night, but it happened! It’s been an absolute
pleasure serving as your Dungeon Master, and I foresee an exciting campaign ahead of us. Adventurers, the realm awaits your return.

I acknowledge the members of my Bible study group, particularly Anthony Gordon who originally came up with the idea. Our study sessions helped me realize that my lack of spirituality had caused me to misplace my priorities in life. Indeed, while I had been certainly enthralled by the call of academia, I eventually realized that I longed for something else besides publications and endless pipetting. I thank this community for helping me with this journey, and I’m excited to get back into the Word with you. God bless and rock on, Spiritual Vagabonds.

I acknowledge the past and present colleagues in the Self lab, especially Deepa Shah, Dominika Dzurny, and Erika Serravalle. I am grateful for your friendship, and I thank you for all the fun memories we made during my academic career. It’s crazy how much the lab’s changed since I joined (there are other grad students in the lab besides me for once!), and I’m excited to see what you all achieve after I’m gone. I wish you all successful and blessed careers in your future.

Lastly, as I approach the last stages of my academic career, I end with a quote, which I found quite provocative for reasons unknown: “There are two men in each one of us: the scientist, he who starts with a clear field and desires to rise to the knowledge of Nature through observations, experimentation and reasoning, and the man of sentiment, the man of belief, the man who mourns his dead children, and who cannot, alas, prove that he will see them again, but who believes that he will, and lives in the hope – the man who will not die like a vibrio, but who feels that the force that is within him cannot die. The two domains are distinct, and woe to him who tries to let them trespass on each other in the so imperfect state of human knowledge.” – Louis Pasteur
# TABLE OF CONTENTS

LIST OF FIGURES ............................................................................................................. xi

LIST OF TABLES .................................................................................................................. xiii

CHAPTER ONE: INTRODUCTION ......................................................................................... 1

CHAPTER TWO: EVALUATION OF DERIVATIVES OF (+)-PUUPEHENONE AGAINST CLOSTRIDIOIDES DIFFICILE AND OTHER GRAM-POSITIVE BACTERIA ........................................ 7

Preamble ............................................................................................................................. 7

Introduction ......................................................................................................................... 7

Results and Discussion ...................................................................................................... 10

Methods .............................................................................................................................. 17

  Bacterial Growth Conditions ........................................................................................ 17

  MIC Determination ....................................................................................................... 17

  Toxin Analysis ............................................................................................................... 18

CHAPTER THREE: INHIBITION OF SELENOPROTEIN SYNTHESIS IS NOT THE MECHANISM BY WHICH AURANOFIN INHIBITS GROWTH OF CLOSTRIDIOIDES DIFFICILE ........................................................................ 21

Preamble ............................................................................................................................. 21

Introduction ......................................................................................................................... 21

Results ................................................................................................................................ 24

  Wild-type C. difficile and mutants lacking selenoproteins exhibit similar sensitivity to auranofin ................................................................................................................... 24

  Selenite supplementation neutralizes auranofin’s activity against C. difficile even in the absence of selenoproteins ............................................................................................ 29

Discussion .......................................................................................................................... 32

Materials and Methods ................................................................................................... 34
Bacterial strains and growth maintenance .......................................................... 34
Broth microdilution assay .................................................................................. 34
Selenite sensitivity assay .................................................................................... 35

CHAPTER FOUR: D-PROLINE REDUCTASE UNDERLIES PROLINE-DEPENDENT GROWTH OF CLOSTRIDIOIDES DIFFICILE .................................................. 36

Preamble ............................................................................................................. 36
Introduction ......................................................................................................... 36

Results ............................................................................................................... 40
PrdB is present throughout extended culture in various growth media .......... 40
Proline and hydroxyproline enhance growth yields in rich media, but the latter does not require proline reductase ......................................................... 45
In the absence of proline reductase, proline is no longer required for growth in a defined minimal medium ................................................................. 49
Proline and hydroxyproline addition leads to a decrease in toxin production that is diminished in a ΔselD mutant strain .............................................. 52

Discussion ......................................................................................................... 55
The strict physiological dependence on Prd and evidence for a hierarchy of electron acceptors ................................................................. 55
The complex role of glycine and the effects of Grd .......................................... 61
The varying physiological effects of hydroxyproline in different strains ........ 63
Toxin production and the impact of selenoproteins on its regulation .............. 65

Materials and Methods ..................................................................................... 69
Bacterial strains and culture media ................................................................. 69
Growth studies and analysis ............................................................................ 70
Radiolabeling studies with $^{75}$Se .................................................................. 70
Analysis of TcdA production ......................................................................... 71
CHAPTER FIVE: CLOSTRIDOIDES DIFFICILE EXPLOITS XANTHINE AND URIC ACID AS NUTRIENTS BY UTILIZING A SELENIUM-DEPENDENT CATABOLIC PATHWAY ... 72

Preamble ............................................................................................................................. 72

Introduction ....................................................................................................................... 72

Results ............................................................................................................................... 77

C. difficile contains gene clusters that putatively encode molybdenum hydroxylases ....... 77

Hypoxanthine, xanthine, and uric acid enhance C. difficile growth in a minimal medium lacking glycine and threonine ......................................................................................... 78

Selenophosphate synthetase plays a major role in growth with xanthine and uric acid but not hypoxanthine ......................................................................................................... 81

smhA and smhB are putative genetic determinants for the maturation of the labile Se cofactor .......................................................................................................................... 83

smhA plays a substantial role in growth on xanthine and uric acid but not hypoxanthine ... 85

Discussion .......................................................................................................................... 88

Materials and Methods .................................................................................................... 92

Bacterial strains, culture media, and growth conditions ..................................................... 92

Growth studies and analysis ............................................................................................... 95

Plasmid construction ........................................................................................................ 95

Conjugation into C. difficile ............................................................................................... 99

CRISPR induction .............................................................................................................. 100

CHAPTER SIX: CONCLUSION ......................................................................................... 102

APPENDIX A: COPYRIGHT INFORMATION FOR CHAPTER TWO ............................... 107

APPENDIX B: COPYRIGHT INFORMATION FOR CHAPTER THREE ............................... 109

APPENDIX C: CHAPTER THREE SUPPLEMENTAL INFORMATION ................................ 111

APPENDIX D: COPYRIGHT INFORMATION FOR CHAPTER FOUR ................................. 115

APPENDIX E: CHAPTER FOUR SUPPLEMENTAL INFORMATION ..................................... 118
LIST OF FIGURES

Figure 1: Synthetic meroterpenoid library................................................................. 12
Figure 2: (+)-Puupehenone and several derivatives reduce toxin production in C. difficile NAP1................................................................. 16
Figure 3: A C. difficile ΔselD mutant has the same sensitivity to auranofin as wild type..... 27
Figure 4: Mutations in selenophosphate synthetase, proline reductase, or glycine reductase do not confer resistance to auranofin................................................................. 28
Figure 5: Selenite supplementation decreases auranofin sensitivity even in the absence of selenoproteins................................................................. 30
Figure 6: Selenite supplementation decreases auranofin sensitivity in a manner independent of selenophosphate synthetase, proline reductase, or glycine reductase....... 31
Figure 7: D-Proline reductase persists throughout the entirety of in vitro culture........... 43
Figure 8: Proline and hydroxyproline increase growth yield in BHIS, but the latter does not rely on D-proline reductase................................................................. 47
Figure 9: Growth yield stimulation from proline and hydroxyproline is enhanced in TY. . 48
Figure 10: Proline-dependent growth of C. difficile requires the presence of D-proline reductase................................................................. 51
Figure 11: Proline and hydroxyproline suppress toxin production in a SelD-dependent manner in BHIS. ................................................................. 54
Figure 12: An overview of electron donors and acceptors in C. difficile........................ 60
Figure 13: A model depicting C. difficile infection as a series of decisions based on nutrient environment................................................................. 68
Figure 14: Biological pathways for specific incorporation of selenium into macromolecules. ................................................................. 74
Figure 15: Hypoxanthine, xanthine, and uric acid induce rapid growth of C. difficile in a minimal medium devoid of glycine and threonine................................................................. 80
Figure 16: Selenophosphate synthetase is required for rapid growth with xanthine and urate but not hypoxanthine in the absence of glycine and threonine................................. 82
Figure 17: smhA and smhB are located within gene clusters associated with pyrimidine and purine metabolism in C. difficile................................................................. 84
Figure 18: The product of *smhA* but not *smhB* is necessary for rapid growth with uric acid or xanthine in the absence of glycine and threonine................................................................. 87

Figure 19: Fidaxomicin and vancomycin activity against R20291 and JIR8094. ..................112

Figure 20: The R20291 strains are not sensitive to selenite up to 100 µM. .........................113

Figure 21: The JIR8094 strains are not sensitive to selenite up to 100 µM. .......................114

Figure 22: The Grd subunits disappear in the absence of glycine........................................119

Figure 23: Confirmed selenoprotein profiles of R20291, KNM6, and KNM9. ..................... 120

Figure 24: Proline and hydroxyproline enhance maximum growth yields in rich media. 121

Figure 25: *C. difficile* contains five gene clusters putatively encoding molybdenum hydroxylases. ............................................................................................................................... 125

Figure 26: Addition of hypoxanthine, xanthine, or uric acid does not affect growth of R20291 and JIR8094 in BHIS and CDMM................................................................. 126

Figure 27: In-frame deletions of *smhA* and *smhB* were successfully constructed in *C.
difficile* R20291. .................................................................................................................. 127

Figure 28: Deletion of *smhA* and *smhB* does not affect growth of *C. difficile* in BHIS and CDMM................................................................. 128

Figure 29: A wild-type copy of *smhA* provided *in trans* fully complements the Δ*smhA* mutant and partially complements the Δ*smhA* Δ*smhB* mutant........................................ 129
**LIST OF TABLES**

Table 1: MICs of compounds against Gram-positive bacteria ....................................................... 13
Table 2. Bacterial strains used in Chapter Three ............................................................................. 26
Table 3. Bacterial strains used in Chapter Four .................................................................................. 44
Table 4. Bacterial strains used in Chapter Five ................................................................................... 94
Table 5. Plasmids used in this study .................................................................................................. 98
Table 6. Doubling times in BHIS supplemented with proline, glycine, and hydroxyproline. .................. 122
Table 7. Doubling times in TY supplemented with proline, glycine, and hydroxyproline. 123
Table 8. Genome IDs and locations for genes encoding putative selenium-dependent molybdenum hydroxylases in *C. difficile* 630 and R20291 ................................................................. 130
Table 9. Oligonucleotides used in this study. ..................................................................................... 131
CHAPTER ONE: INTRODUCTION

The Gram-positive bacterium *Clostridiodioses difficile* (formerly *Clostridium*) is a nosocomial pathogen that causes severe intestinal inflammation, often resulting in diarrhea and pseudomembranous colitis (PMC) (1), which is characterized by the appearance of yellow-white plaques completely covering the colonic mucosa (2). Originally isolated from the gut flora of healthy newborns in 1935 (3), *C. difficile* rose to prominence as a significant antibiotic-induced pathogen in the 1970s as reports of clindamycin-associated diarrhea and PMC began to surface (2, 4). Indeed, since 1978, the majority of PMC cases have since been caused by *C. difficile* infections (CDIs) (4). Generally considered to be the leading cause of antibiotic-associated diarrhea (5), *C. difficile* is classified as an urgent threat by the United States Centers for Disease Control and Prevention (CDC) (6). According to the CDC, CDIs approximated 223,900 cases and resulted in 12,800 deaths in 2017 (6). Exposure to antibiotics such as clindamycin and fluoroquinolones increases the risk for CDI as these broad-spectrum drugs greatly disrupt the composition of the normal gut microbiota (1, 7, 8). Once colonization resistance is compromised, *C. difficile* can now exploit the newly dysbiotic state of the large intestine, ultimately colonizing and causing disease (8).

*C. difficile* must be transmitted through the fecal-oral route in order to infect the host (1); however, because it is a strict anaerobe, it is not possible for vegetative cells to survive outside of the intestines in the presence of oxygen for extended periods of time (9). To overcome this issue, *C. difficile* will form spores, which are refractory to almost all types of environmental challenge (e.g., ethanol, hydrogen peroxide, and heat) (10). *C. difficile* spores travel through the human gastrointestinal tract until they germinate in response to nutrients such as taurocholate and glycine.
found in the small intestine (11). Following germination, vegetative cells subsequently colonize the large intestine and can cause disease by producing two exotoxins known as TcdA (toxin A) and TcdB (toxin B), which are glucosyltransferases that target small GTPases (e.g., Rho, Rac, Cdc42) in host cells (12, 13). Once internalized by the gut epithelium via receptor-mediated endocytosis, TcdA and TcdB inactivate Rho-family GTPases via glucosylation of a critical active-site threonine residue using host UDP-glucose, eventually resulting in cell rounding, apoptosis, and necrosis (12, 13).

Treatment of CDI typically involves the administration of the standard-of-care antibiotics vancomycin and fidaxomicin (14, 15). Unfortunately, recurrence of the disease significantly increases after each antibiotic treatment (16, 17), and both drugs have even been associated with increased rates of recurrence (18, 19). This phenomenon has been especially observed with the former first-line CDI therapeutic metronidazole which fell into disuse due to its association with high rates of disease recurrence (20). Since recurrence results from the continued disruption of the gut microbiota by repeated broad-spectrum treatments (16), there exists a need for new antibiotics with narrow-spectrum activity against \textit{C. difficile}. Fidaxomicin serves as a great example of a narrow-spectrum antibiotic exhibiting potent activity against \textit{C. difficile} while also suppressing the pathogen’s ability to produce toxins via inhibition of RNA polymerase (21, 22). Despite the limited repertoire of CDI therapeutics and increased recurrence rates associated with both drugs, the U.S. Food and Drug Administration (FDA) has not approved any other antibiotic for CDI since fidaxomicin in 2011. A more detailed description of \textit{C. difficile} drug discovery is given in the Introduction sections of Chapters Two and Three. Our lab has shown interest in searching for lead compounds with high potency against \textit{C. difficile} in order to address this issue. This dissertation documents two different explorations into \textit{C. difficile} drug discovery: (i) evaluation of the
antimicrobial activity of chemically synthesized derivatives of a marine natural compound called (+)-puupehenone against *C. difficile* (Chapter Two), and (ii) clarification of the proposed mechanism of the antirheumatic drug auranozin against *C. difficile* using modern genetics (Chapter Three). These results may provide the foundation for further development of both compounds as potential CDI therapeutics.

An alternative to antibiotics exists in the form of fecal microbiota transplants (FMTs) (23), which have been approved very recently by the FDA likely due to their high efficacy in resolving CDI recurrence (24). While the mechanism behind this treatment is unknown, it is assumed that this type of bacteriotherapy reestablishes colonization resistance by repopulating the dysbiotic gut with a microbial population derived from the stool of healthy donors (25). However, since the microbes essential in establishing colonization resistance against *C. difficile* have not been identified, it is hard to predict whether certain microbes in donor samples will improve or worsen outcomes. Indeed, Girinathan et al. (26) observed differential survival outcomes of germ-free mice co-colonized with *C. difficile* and either *Paraclostridium bifermentans* or *Clostridium sardiniense*; specifically, CDI severity was reduced with *P. bifermentans* and worsened with *C. sardiniense*. Interestingly, various nutrients were differentially enriched depending on the co-colonization status of the mice (26), suggesting that nutrient status of the gut plays a significant role in *C. difficile* colonization. In support of this, Aguirre et al. (27) reported that colonization resistance against *C. difficile* appears to result from microbial competition for certain nutrients such as proline and glycine, which are preferred substrates for *C. difficile* energy metabolism (28, 29). These findings highly suggest that colonization resistance against *C. difficile* relies on competition for its preferred resources. Therefore, in order to identify which organisms are needed to outcompete *C. difficile*, the metabolic preferences of the pathogen must be clearly outlined.
In order to develop targeted therapies for *C. difficile*, it is important to understand how *C. difficile* thrives in the gut during infection. This idea can be understood philosophically by posing a simple question: why does *C. difficile* make toxin? To our knowledge, TcdA and TcdB appear to play no role in directly influencing bacterial metabolism and physiology. Instead, the main function of both toxins is solely to induce severe host inflammation (30), which seems irrational since this potentially threatens elimination of the pathogen via the host immune response. A simple explanation for this risky strategy would be that toxin-dependent disease (e.g., diarrhea) merely facilitates dissemination of the pathogen. However, the decision to cause disease may be more complex than originally thought as it is now known that many pathogens such as *Escherichia coli* and *Salmonella enterica* leverage virulence strategies in order to establish a metabolic niche within the host (31-34). For example, respiratory electron acceptors such as nitrate and tetrathionate are generated during gut inflammation from *E. coli* and *S. enterica* infections, respectively (35, 36).

Likewise, metabolism and virulence are intimately linked in *C. difficile* (37), and it is now clear that toxin-dependent inflammation plays a role in scavenging nutrients during infection. For example, Pruss and Sonnenburg (38) reported that *C. difficile* toxin-dependent inflammation results in the generation of sorbitol by aldose reductase in murine gut immune cells. Similarly, Fletcher et al. (39) observed another interesting consequence of toxin-mediated inflammation: the degradation of host collagen, which is a structural extracellular matrix protein mainly comprised of glycine, proline, and hydroxyproline. In that study, amino acid catabolic pathways in *C. difficile* were upregulated during infection (39), suggesting that collagen was being degraded in order to scavenge amino acids. Our lab first proposed that collagen was a metabolic target during CDI since its amino acid constituents glycine and proline coincidentally act as electron acceptors in a clostridial bioenergetics scheme called Stickland metabolism (28), which describes redox half-
reactions catalyzed between pairs of amino acids (40, 41). A thorough review of Stickland reactions and their potential relationship with collagen degradation is given in the Introduction of Chapter Four. In this metabolism, proline and glycine are substrates for D-proline reductase (Prd) and glycine reductase (Grd) (28, 29), which are unique enzymes that harbor selenium in the form of selenocysteine in their active sites (28, 29). The presence of selenium in these core enzymes suggests an important function in *C. difficile* energy metabolism, though the role of this element in infection is still unclear.

Selenium is an important redox-active micronutrient found in all domains of life. Sharing many chemical similarities with sulfur, selenium is specifically incorporated into certain types of biological molecules such as proteins (42), tRNAs (43), and small molecules (44). Specific incorporation of selenium into these molecules requires the activated selenium donor known as selenophosphate (45, 46), which is generated from the transmutation of selenide via selenophosphate synthetase (SelD) (47). Recent studies have shown that SelD plays a role in *C. difficile* physiology (48, 49), but the entire molecular repertoire of selenophosphate-dependent macromolecules (i.e., besides Prd and Grd) lacks adequate characterization. Our lab has shown great interest in two types of selenoenzymes in *C. difficile*: selenocysteine-containing enzymes (e.g., Prd and Grd) and selenium-dependent molybdenum hydroxylases (SDMHs). In the former class of selenoenzymes, incorporation of the 21st amino acid selenocysteine is performed cotranslationally by recoding a UGA stop codon using a specific elongation factor (SelB), selenocysteine-specific tRNA (selC), and downstream stem-loop structure (SECIS element) (50, 51). In the latter example of selenoenzymes, SDMHs contain selenium in the form of a labile, non-selenocysteine cofactor in their active sites (52-56), though it is unclear how this selenium is integrated. SDMHs catalyze the water-dependent hydroxylation of carbon substrates such as
purines (57). The role of these molybdoenzymes in *C. difficile* metabolism has not been described, though there is data that suggests *C. difficile* can scavenge host-derived purines during infection (26). An extensive review of the biological utilization and presence of selenium in both types of selenoenzymes is provided in the Introduction sections of Chapters Three, Four, and Five. This dissertation contains two independent studies that delineate poorly understood aspects of selenium biology while also highlighting the role of selenium in *C. difficile* physiology: (i) the elucidation of the complex relationship between proline-dependent growth and Prd as a form of energy “addiction” (Chapter Four), and (ii) the first genetic characterization of a selenium-dependent purinolytic pathway in *C. difficile* (Chapter Five). These results add nuance to the selenium-dependent metabolic pathways in *C. difficile* and may help researchers understand the pathogen’s nutrient preferences when designing targeted microbial-based therapies for CDI.
CHAPTER TWO:
EVALUATION OF DERIVATIVES OF (+)-PUUPEHENONE AGAINST
CLOSTRIDIODES DIFFICILE AND OTHER GRAM-POSITIVE
BACTERIA

Preamble

Management of CDI is hindered by the limited number of FDA-approved therapeutics available in the clinic, namely vancomycin and fidaxomicin (14, 15, 58, 59). To address this issue, our lab teamed up with Dr. Steven Sucheck’s lab at the University of Toledo with the goal of searching for potent derivatives of a marine natural product called (+)-puupehenone, which has known antimicrobial activity against C. difficile (60). This dissertation chapter documents this collaborative effort in C. difficile drug discovery. This joint work would not have been possible without the Sucheck group, as they were responsible for: (i) synthesis of the (+)-puupehenone series chemical library and creation of Figure 1 (61), (ii) determination of MICs for all bacteria excluding C. difficile in Table 1, and (iii) writing and editing the manuscript. All work done by our lab was as follows: (i) MIC determination for C. difficile in Table 1, (ii) western blot assays for TcdA and creation of Figure 2, and (iii) writing and editing the manuscript. The results of the aforementioned manuscript and the additional details therein were ultimately published in ACS Omega (Appendix A) and are presented below.

Introduction

Healthcare-associated infections (HAIs) pose a significant risk to patients undergoing treatment in hospitals and other healthcare facilities (62). These infections lead to thousands of
deaths and cost the U.S. healthcare system several billions of dollars each year (63, 64). There is undoubtedly a high prevalence in the United States as approximately 1 in 31 hospital patients carried an HAI in 2015 (65). The most common HAIs reported from that survey were pneumonia, gastrointestinal infections, and surgical-site infections; moreover, the most common pathogens responsible for these HAIs were found to be Clostridioides (formerly Clostridium) difficile, Staphylococcus aureus, and Escherichia coli (65). Several other pathogens were also reported, such as Pseudomonas aeruginosa and species belonging to the Klebsiella, Enterobacter, and Enterococcus genera (65). The challenge in treating HAIs stems from the fact that several of these clinical isolates are resistant to multiple antibiotics; indeed, 45% of S. aureus isolates from the aforementioned survey were methicillin resistant (MRSA) while 3% of E. coli, Klebsiella, and Enterobacter isolates were resistant to at least one carbapenem (65). According to the Centers for Disease Control and Prevention (CDC), over 2.8 million infections and 35,000 deaths are caused by antibiotic-resistant pathogens each year (66). Therefore, new therapeutics must be developed in order to successfully treat these HAIs.

C. difficile is a Gram-positive, spore-forming anaerobe that causes antibiotic-associated diarrhea, generally representing 15–25% of all known cases (3, 5, 8, 67). C. difficile infection (CDI) follows the clearance or disturbance of the normal gut flora, usually after antibiotic treatment (8). The disease manifestation of CDI is caused by two virulence factors, toxins A (TcdA) and B (TcdB), which target intestinal epithelial cells and inactivate host Rho proteins via glucosylation, ultimately resulting in the disruption of the actin cytoskeleton and tight junctions; additionally, both exotoxins cause severe inflammation and are known to induce programmed cell death pathways, such as apoptosis and necrosis (12, 68-72). The severity of CDI underscores its significance as an HAI; for example, the CDC reported that C. difficile was responsible for
approximately 223,900 infections in hospitalized patients and 12,800 deaths in the United States in 2017 (66). In addition to its severity, CDI is difficult to treat since the disease recurrence rate (15–35%) increases after each subsequent treatment (16, 17). The primary recommended antibiotics for CDI are fidaxomicin and vancomycin (14, 15, 58, 59). Though metronidazole was previously recommended as the first-line treatment for CDI, it has since become obsolete due to the high frequency of treatment failures and recurrence; similar data has been reported from studies of vancomycin as well (20, 73). Compared to metronidazole and vancomycin, fidaxomicin is typically associated with a lower rate of recurrence (18, 19). Furthermore, while the frequency of antibiotic resistance in C. difficile is relatively low for all three drugs (74, 75), resistant isolates have been reported in the literature (73, 76); for example, a fidaxomicin-resistant clinical isolate with a minimum inhibitory concentration (MIC) of >64 µg/mL has recently been characterized (77). These issues are further complicated with the emergence of a hypervirulent strain of C. difficile, designated as North American pulsed-field gel electrophoresis type 1 (NAP1). Isolated from several outbreaks in the early 2000s, NAP1 produces more exotoxins due to a mutation in tcdC, which encodes a negative regulator of toxin production; exhibits higher resistance to several antibiotics with a particular emphasis on fluoroquinolones; forms spores at a higher frequency; and produces an additional binary toxin called CDT (69). Thus, there is an urgent need to identify new antimicrobials that not only possess activity against C. difficile but lower the rates of CDI recurrence and antibiotic resistance.

Historically, the ocean has been recognized as a potential resource for chemically diverse compounds, as many novel marine natural products exhibiting antimicrobial activity against drug-resistant microbes have been identified (78, 79). We recently discovered that (+)-puupehenone, a meroterpenoid isolated from deep water marine sponges (80, 81), exhibited antimicrobial activity
against multiple strains of *C. difficile* (60). Based on that report, we created a chemical library of (+)-puupehenone derivatives with the intent of finding a more potent compound that could serve as a foundation for further investigation and structure-activity relationship studies (61). We then challenged *C. difficile* and other bacterial species with this library in order to evaluate their effects on organisms implicated in HAIs. Additionally, knowing that fidaxomicin inhibits *C. difficile* toxin production (22), we wanted to investigate if these compounds could modulate the expression of these toxins in a similar fashion. In this present study, we report the activity of (+)-puupehenone derivatives against *C. difficile* NAP1 and other Gram-positive bacteria. We also report that several derivatives with activity against NAP1 were able to substantially decrease toxin production in a concentration-dependent manner.

**Results and Discussion**

We previously synthesized a library of 20 compounds to be screened for activity against several Gram-positive and Gram-negative bacteria (61). The structures of these compounds are shown in Figure 1. Several of these compounds had promising activity against *C. difficile* and other Gram-positive bacteria as seen in Table 1. Our (+)-puupehenone (compound 13) exhibited an MIC of 2.0 µg/mL against *C. difficile* NAP1, which was more potent compared to a previous report where (+)-puupehenone obtained from a commercial supplier exhibited an MIC of 8.0 µg/mL against the same strain (60). Against *Bacillus subtilis, Enterococcus faecalis,* and *S. aureus,* (+)-puupehenone curiously had no observable activity. By comparison, compound 1 was less potent against *C. difficile* at 4.0 µg/mL but inhibited *B. subtilis, E. faecalis,* and *S. aureus* at 3.9, 1.9, and 7.8 µg/mL, respectively. Interestingly, the addition of the methoxy group to compound 1 (subsequently generating compound 2) rendered it inactive against all species except *B. subtilis.* Moreover, we observed that compounds 6 and 8 also possessed activity against all Gram-positive
bacteria at varying degrees. Compound 6 inhibited *C. difficile* at 16.0 µg/mL and also inhibited *B. subtilis, E. faecalis,* and *S. aureus* at 7.9, 15.8, and 16.9 µg/mL, respectively; however, compound 8 was more potent against *C. difficile* at 8.0 µg/mL but exhibited similar MICs against *B. subtilis* (8.2 µg/mL), *E. faecalis* (16.2 µg/mL), and *S. aureus* (16.6 µg/mL). Compound 3 did not inhibit *C. difficile* or *E. faecalis* but did inhibit the growth of *B. subtilis* and *S. aureus* at an MIC of 33.2 µg/mL for both organisms. Oddly enough, while compound 4 is a structural isomer of compounds 3 and 8, it did not exhibit any activity against our panel of organisms, demonstrating that the placement of groups on the aryl ring is critical for the activity of these compounds. The ester compounds 15 and 19 both inhibited *C. difficile* at 4.0 µg/mL while only compound 19 exhibited an MIC of 22.23 µg/mL against *B. subtilis* and *E. faecalis*. The other ester compounds did not inhibit the growth of each organism and may be hindered by the steric bulk of the ester group. Finally, we observed that compound 20 had an MIC of 2.0 µg/mL against *C. difficile*, similar to (+)-puupehenone. None of these compounds were active against *E. coli* or *P. aeruginosa* when tested at concentrations up to 100 µM, which we found to be consistent with published *in vitro* activity against these two organisms (80). These data support the idea that this library harbors a strict specificity for Gram-positive bacteria.
Figure 1: Synthetic meroterpenoid library.
Table 1: MICs of compounds against Gram-positive bacteria

<table>
<thead>
<tr>
<th>Compounds</th>
<th>C. difficile</th>
<th>B. subtilis</th>
<th>E. faecalis</th>
<th>S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fidaxomicin</td>
<td>0.125</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>1</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>1</td>
<td>4.0</td>
<td>3.9</td>
<td>1.9</td>
<td>7.8</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>8.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>33.2</td>
<td>-</td>
<td>33.2</td>
</tr>
<tr>
<td>6</td>
<td>16.0</td>
<td>7.9</td>
<td>15.8</td>
<td>16.9</td>
</tr>
<tr>
<td>8</td>
<td>8.0</td>
<td>8.3</td>
<td>16.2</td>
<td>16.6</td>
</tr>
<tr>
<td>(+)-Puupehenone (13)</td>
<td>2.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>4.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>4.0</td>
<td>22.23</td>
<td>22.23</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>2.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

MICs for *B. subtilis*, *E. faecalis* and *S. aureus* were determined visually by REMA assay. The MICs for *C. difficile* were determined by a modified broth microdilution assay. *Clostridioides difficile* NAP1, *Bacillus subtilis* ATCC 23857, *Enterococcus faecalis* ATCC 29212, and *Staphylococcus aureus* ATCC 25923. (-) = no activity seen. N/A = not applicable.
Because the disease state of CDI is primarily due to the pathogen’s toxins, we focused only on the subset of meroterpenoids that exhibited activity against *C. difficile* NAP1 and assessed each compound’s ability to reduce toxin production in the organism. To do so, we sampled the spent medium of each NAP1 culture challenged with 0.5×, 0.25×, and 0.125× the MIC of each compound (sub-MICs) and immunoblotted for TcdA. To account for changes in biomass, we performed a semi-quantitative analysis of TcdA production by normalizing the target bands from western blots to the amount of total protein in each sample. We observed no significant difference in the amount of TcdA produced by NAP1 in supplemented brain-heart infusion (BHIS) medium and BHIS augmented with the compound vehicle, 5% dimethyl sulfoxide (DMSO) (Fig. 2A). For this experiment, we employed fidaxomicin (MIC = 0.125 µg/mL, Table 1) as a positive control and subsequently noted a substantial reduction in TcdA levels at all sub-MICs (Fig. 2B), which is consistent with its reported toxin-suppressing activity (22, 82). Additionally, we were curious to see if vancomycin (MIC = 1 µg/mL, Table 1)—a CDI therapeutic that is not expected to modulate toxin production—would have any effect on TcdA levels in our assay. Treating vancomycin as a type of negative control, we repeated the experiment and were surprised to see a partial reduction in toxin levels at 1 µg/mL (Fig. 2C). Despite this unexpected result, it must be noted that the effects of vancomycin on toxin levels are wildly different in several *C. difficile* strains according to previous reports (22, 83-86). Interestingly, we found that 1 µg/mL (+)-puupehenone, while inferior to fidaxomicin, was superior to vancomycin in reducing toxin production to the point where TcdA was undetectable (Fig. 2D). Additionally, compounds 1 and 6 decreased the amount of toxin in a concentration-dependent manner (Fig. 2E and 2F) while compounds 8, 15, and 19 did not significantly change toxin levels in comparison (Fig. 2G, 2H, and 2I). At low sub-MICs, however, compounds 1 (0.5 and 1 µg/mL) and 8 (1 and 2 µg/mL) elicited an increase in toxin production.
(Fig. 2E and 2G), suggesting a complex dose-dependent regulatory mechanism. Finally, while also inferior to fidaxomicin, compound 20 was observed to substantially reduce toxin levels in a similar manner, but this effect did not significantly change at increased doses (Fig. 2J). These results strongly indicate that certain chemical modifications are important for these compounds to specifically target this virulence mechanism.

The data generated from this compound library suggest that several of these meroterpenoids, such as compound 1, show promise as potential antimicrobials for HAIs caused by certain Gram-positive pathogens, like *E. faecalis* and MRSA. Additionally, since (+)-puupehenone and some of its derivatives, such as compound 20, were successful in reducing NAP1 toxin production *in vitro*, these meroterpenoids may also have the potential for further development as therapeutics for CDI. Development will surely benefit from future experiments centered on uncovering the mechanism of action of these compounds. Specifically, assessment of gene expression at sub-MICs via transcriptomics and sequencing of mutants resistant to certain derivatives will help achieve this goal. Additionally, since spore formation is a core process in *C. difficile* transmission and pathogenesis, it would be beneficial to determine if these compounds possess any sporicidal activity or if they at least modulate processes such as sporulation and germination.
Figure 2: (+)-Puupehenone and several derivatives reduce toxin production in *C. difficile* NAP1.

After 48-h incubation of NAP1 challenged with several dilutions of each compound, extracellular toxin in spent media derived from triplicate cultures was assessed with western blots using a monoclonal antibody against TcdA. Semi-quantitative analysis of toxin levels was performed via densitometry of TcdA band intensity with respect to total protein as measured by Bradford assay. Semi-quantitative plots are shown below each representative blot. (A) Toxin production in BHIS (NAP1) and BHIS with 5% DMSO (Vehicle). (B) Toxin production from cultures challenged with sub-MICs of fidaxomicin, (C) vancomycin, and (D-J) (+)-puupehenone and selected derivatives. Data points represent the means of adjusted toxin levels derived from triplicate cultures while error bars represent standard deviations. Statistical analysis was performed in GraphPad Prism 8 using unpaired t-tests. * $P \leq 0.05$; ** $P \leq 0.01$. 
Methods

Bacterial Growth Conditions

\textit{P. aeruginosa} (ATCC 27853), \textit{S. aureus} (ATCC 25923), \textit{E. faecalis} (ATCC 29212), \textit{B. subtilis} (ATCC 23857), and \textit{E. coli} (ATCC 25922) were obtained from American Type Culture Collection (ATCC). Frozen stocks of \textit{P. aeruginosa}, \textit{S. aureus}, \textit{E. faecalis}, and \textit{B. subtilis} were grown in tryptic soy broth (TSB) at 37 °C for 12–24 h until mid-exponential phase, which corresponded to an optical density at 600 nm (OD\textsubscript{600}) of 0.6. Frozen stocks of \textit{E. coli} were cultured in Super Optimal broth with Catabolite repression (SOC) medium at 37 °C for 12 h until mid-exponential phase (OD\textsubscript{600} of 0.6) (87). All OD\textsubscript{600} readings were recorded with a Laxco MicroSpek DSM Cell Density Meter. All cultures were then diluted 1,000-fold into their respective media to prepare inocula.

For all studies regarding \textit{C. difficile}, we used a NAP1 strain isolated from several outbreaks (88). Anaerobic conditions were defined by maintaining an atmosphere of 1.0% \textsubscript{H}_2, 5% \textsubscript{CO}_2, and >90% \textsubscript{N}_2 in a Coy anaerobic chamber. NAP1 was routinely grown in BHIS broth: 37 g/L brain-heart infusion, 5 g/L yeast extract, and 0.1% (w/v) L-cysteine (89).

MIC Determination

The MICs of test compounds and ampicillin (Acros Organics) were determined by broth microdilution and resazurin microtiter assay (REMA). Freshly grown cultures of \textit{P. aeruginosa}, \textit{S. aureus}, \textit{B. subtilis}, \textit{E. faecalis}, and \textit{E. coli} were used as inocula at 1,000-fold dilutions in TSB and SOC media, respectively. After 24-h incubation of each organism challenged with two-fold serial dilutions of each compound, the MIC was scored as the lowest concentration where no growth was observed. The plates were then stained with resazurin stock solution added to each
well, incubated for 4–5 h, and observed for color change from blue to pink. The MIC was scored at the lowest concentration that retained its blue color. The assay was repeated in triplicate.

Antimicrobial susceptibility tests (ASTs) of NAP1 were performed with a modified broth microdilution procedure as per the Clinical and Laboratory Standards Institute (CLSI) standard M11 (90). Briefly, 96-well assay plates were pre-loaded with 95 µL BHIS and 5 µL 20× test compounds to achieve a final concentration range of 0.0625–16 µg/mL in 2-fold increments. Test compounds were serially diluted in 100% DMSO with the exception of vancomycin hydrochloride (Gold Biotechnology) which was dissolved and diluted in deionized water. Fidaxomicin (APExBIO Technology) was similarly diluted in 100% DMSO to achieve a final concentration range of 0.008–1 µg/mL. Assay plates were stored in the anaerobic chamber at room temperature to reduce overnight. A single colony of NAP1 was grown overnight in BHIS at 37 °C. The overnight culture was initially diluted the next day with pre-reduced saline (0.85% NaCl) to match the turbidity of a 0.5 McFarland standard. The inoculum was finally prepared with a subsequent 15-fold dilution in saline. Pre-reduced assay plates were inoculated with 10 µL of diluted cell suspension, stored in a half-sealed plastic bag to prevent evaporation, and incubated at 37 °C for 48 h. After incubation, growth in each well was measured by reading the OD_{600} using a BioTek Epoch 2 plate reader. The assay was performed in triplicate.

**Toxin Analysis**

Toxin production was determined by analyzing the amount of extracellular toxin in triplicate cultures from the ASTs described above. At 48 h, total protein of each culture was determined with the Bradford assay using bovine serum albumin (BSA) as a standard (91). Cultures were then centrifuged at 5,000 × g for 5 minutes to clear the supernatants which were
separately collected and frozen at −20 °C. After thawing at room temperature, cell-free supernatants were mixed 1:1 with 2× Laemmli buffer and incubated in a sand bath at 100 °C for 5 minutes. Twenty microliters of denatured samples were loaded onto 7.5% Tris-glycine gels and electrophoresed at 200 V for 1 h. After electrophoresis, samples were transferred to polyvinylidene difluoride membranes at 4 °C overnight at 30 V. Membranes were incubated in a blocking buffer (20 mM Tris-HCl, 150 mM NaCl, 0.01 mM ethylenediaminetetraacetic acid, 0.1% Tween 20, 1% BSA, [pH 7.5]) for 1 h at room temperature. TcdA was detected with a monoclonal mouse anti-TcdA antibody (PCG4.1, Novus Biologicals) and a rabbit anti-mouse IgG antibody conjugated with alkaline phosphatase. Blots were visualized with a ChemiDoc XRS+ imaging system (Bio-Rad).

Semi-quantitative analysis of TcdA was performed using Image Lab 6.0 software (Bio-Rad). Briefly, after subtracting background noise in each blot, the peak corresponding to the intensity of the TcdA band (arbitrary units) was selected with the software’s Lane Profile tool. The peak density was divided by the total protein amount of each respective sample, resulting in an adjusted toxin level with respect to apparent biomass. Adjusted values of triplicate cultures were averaged together and converted to a percentage using the Normalize tool in GraphPad Prism 8. To define 100%, the TcdA value from NAP1 in BHIS (5% DMSO) was chosen for fidaxomicin and all test compounds. For vancomycin, the TcdA value from NAP1 in BHIS was defined as 100%. In both cases, 0 was defined as 0%. During our investigation, we observed that the MICs of these compounds against C. difficile occasionally increased by 2-fold on different days. This variable activity prevented us from performing statistical analysis from multiple experiments, as the sub-MICs were not always the same. Since there were no changes to the methodology described, several other reasons could have accounted for decreased activity, such as compound
stability, freeze-thaw, and adherence to plastic. Nevertheless, the toxin results were reproducible, irrespective of the exact sub-MICs on different days.
CHAPTER THREE:

INHIBITION OF SELENOPROTEIN SYNTHESIS IS NOT THE MECHANISM BY WHICH AURANOFIN INHIBITS GROWTH OF CLOSTRIDIoidES DIFFICile

Preamble

Another contribution to the field of C. difficile drug discovery was auranofin, an antirheumatic with antimicrobial activity against C. difficile (92). Our lab previously hypothesized its mechanism as targeting selenoprotein synthesis (92), though this could not be genetically investigated at the time due to the infancy of C. difficile genetics. After obtaining C. difficile mutants deficient in selenoproteins from the labs of Dr. Joseph Sorg (Texas A&M University) and Dr. Abraham Sonenshein (Tufts University), we decided to revisit the auranofin project to test our original hypothesis and clarify the results of our past study. This dissertation chapter documents the findings of that study, which was published in Scientific Reports (Appendix B).

Introduction

Clostridioides difficile (formerly Clostridium difficile) is a Gram-positive, endospore-forming strict anaerobe and the leading cause of antibiotic-associated diarrhea (~15–25% of cases) (5, 67). C. difficile infections (CDIs) typically occur in patients with gut dysbiosis and can lead to severe clinical complications such as pseudomembranous colitis and toxic megacolon (1). During infection, C. difficile causes disease and induces inflammation by producing two large exotoxins, TcdA and TcdB, which damage the intestinal lining through the glucosylation of Rho-family GTPases in host epithelial cells (68). According to a recent CDC report, CDIs were responsible
for approximately 223,900 hospitalized patient cases and 12,800 deaths in 2017 (66). Moreover, CDIs have contributed to approximately $1 billion in U.S. healthcare costs (66).

The standard-of-care antibiotics for treating CDI are fidaxomicin and vancomycin (15, 93). If neither drug is available, metronidazole is recommended as an alternative (15, 93), though this former first-line antibiotic is regarded as obsolete due to its high rates of treatment failure (20). In fact, CDI recurrence occurs in ~15–30% of patients treated with metronidazole and vancomycin despite their effectiveness in inhibiting *C. difficile* growth (16, 20). On the other hand, fidaxomicin is a narrow-spectrum antimicrobial with greater potency and is typically associated with comparatively lower recurrence rates (18, 19), though treatment failure has also been reported (94). Moreover, while not the primary issue encountered in CDI management, antimicrobial resistance is still a cause for concern as drug-resistant clinical isolates have been reported for all three antibiotics (77, 95-97). Overall, the current repertoire for treatment is quite limited, especially since fidaxomicin was the last CDI drug approved by the U.S. Food and Drug Administration (FDA) in 2011 (98). If no new alternatives are added to the current list of standard-of-care antibiotics, the rising rates of recurrence and antibiotic resistance could outpace efforts to keep CDI under reasonable control.

Auranofin is an FDA-approved anti-rheumatic gold (Au) compound that possesses antimicrobial activity against *C. difficile* (92, 99). Many reports have highlighted its inhibitory activity against *C. difficile* vegetative cells and sporulation, its ability to reduce toxin levels and protect Caco-2 cells against their lethal effects, and its efficacy in preventing CDI and disease recurrence in mouse and hamster models (100-104). While the mechanism of action is still unknown, our group hypothesized that auranofin’s antimicrobial activity against *C. difficile* stemmed from its unique ability to halt the biosynthesis of selenoproteins (i.e., proteins that contain
the 21st amino acid selenocysteine) (92). In *C. difficile*, the established selenoproteins are selenophosphate synthetase (SelD), D-proline reductase (Prd), and glycine reductase (Grd) (28, 92). SelD possesses the highly specific role of converting toxic selenide to selenophosphate, a selenium (Se) donor that is required for selenoprotein synthesis (45, 48, 105). Prd and Grd are involved in Stickland metabolism, an important clostridial bioenergetics scheme centered on amino acid redox reactions (28, 29). Our group has previously shown via mass spectrometry and X-ray absorption spectroscopy that auranofin forms a Au-Se adduct with selenide in the culture medium; additionally, we have demonstrated via $^{75}$Se radiolabeling that auranofin inhibits uptake and incorporation of Se into selenoproteins in *C. difficile* (92). Based on these data, we assumed that auranofin’s mechanism of action against *C. difficile* involved blocking Se transport through the formation of the Au-Se adduct, thereby crippling the production of crucial selenoproteins such as Prd and Grd (92).

However, despite the perceived importance of selenoproteins in *C. difficile*, it is now known that they are not essential since *C. difficile* selD mutants derived from two different ribotypes are both clearly viable (48). These findings prompted us to revisit our previous work on auranofin and determine if the compound’s activity against the pathogen is truly from its inhibition of Se metabolism (92). Since that publication, the rapid advancement of genetic techniques to study clostridia has allowed for more precise investigations into the role of selenoproteins in *C. difficile* (29, 48, 49, 106, 107). In this study, we determined the auranofin sensitivity of a panel of *C. difficile* mutants deficient in some or all selenoproteins in order to gain more insight on the compound’s mechanism of action.
**Results**

*Wild-type C. difficile and mutants lacking selenoproteins exhibit similar sensitivity to auranofin*

To determine if auranofin inhibits *C. difficile* by targeting Se metabolism, we evaluated the auranofin sensitivity of an array of *C. difficile* strains deficient in some or all selenoproteins (Table 2) using a modified version of the Clinical and Laboratory Standards Institute (CLSI) broth microdilution method (90). While CLSI recommends the agar dilution method for antimicrobial susceptibility testing of anaerobes (90), we chose broth microdilution because of its practicality and less cumbersome methodology. Moreover, broth microdilution has been reported to perform similarly to agar dilution in susceptibility tests of *C. difficile* (108, 109), though we are aware that others have observed substantial differences between both methods and argue against routine testing with broth microdilution (110, 111). Since our goal was to compare relative differences in minimum inhibitory concentrations (MICs) between strains rather than report standardized values that could be translated to the clinic, broth microdilution was therefore deemed appropriate for this study. Briefly, we cultured each strain in supplemented brain heart infusion (BHIS) broth containing varying concentrations of auranofin at 37 °C for 48 h. At the end of the growth period, we established each strain’s MIC of auranofin by measuring the optical density of each culture at 600 nm (OD$_{600}$). With this method, we first determined the auranofin sensitivity of wild-type strains R20291 (MIC = 2 µg/mL) and JIR8094 (MIC = 8 µg/mL) (Fig. 3A and 4A). The standard-of-care CDI therapeutics, fidaxomicin and vancomycin, were also included as positive controls for the assay. Accordingly, R20291 was inhibited by 0.125 µg/mL fidaxomicin and 1 µg/mL vancomycin while JIR8094 was inhibited by 0.016 µg/mL fidaxomicin and 4 µg/mL vancomycin (Appendix C: Fig. 19).
Based on our laboratory’s previous work, it was proposed that auranofin inhibits the growth of *C. difficile* by forming a complex with Se, thereby depleting the amount of bioavailable Se for trafficking and eventual incorporation into selenoproteins (92). Thus, if auranofin’s activity arises from the inhibition of selenoprotein biosynthesis, a strain lacking selenoproteins (i.e., a *selD* mutant) would theoretically be resistant to the compound and harbor a significantly elevated MIC compared to wild type. Despite this assumption, we surprisingly found that the MICs for R20291 (Fig. 3A), KNM6 (Δ*selD*) (Fig. 3B), and KNM9 (Δ*selD::selD*+) (Fig. 3C) were all equally 2 μg/mL auranofin, suggesting that the compound’s activity does not stem from targeting selenoproteins.

To determine if this phenomenon was strain dependent, we repeated the assay with JIR8094 and LB-CD7 (*selD::ermB*) and likewise saw no increase in the MIC. However, while the wild-type strain JIR8094 exhibited an MIC of 8 μg/mL (Fig. 4A), the *selD::ermB* strain was actually more susceptible to auranofin as it failed to grow at 4 μg/mL (Fig. 4B). This slight increase in sensitivity was surprising as it seemed to suggest a complex relationship between auranofin’s antimicrobial activity and the selenoproteins in JIR8094. Out of curiosity, we evaluated two Prd mutants, LB-CD4 (*prdB::ermB*) and LB-CD8 (*prdR::ermB*), and one Grd mutant, LB-CD12 (*grdA::ermB*), using the same assay in order to determine which reductase plays a greater role in this phenomenon, if any. Interestingly, we discovered that all three mutants exhibited the same MIC of 4 μg/mL as the *selD::ermB* strain (Fig. 4C, 4D, and 4E). While these data seem to suggest that a mutation in either of these selenoproteins renders *C. difficile* JIR8094 more sensitive to auranofin, a simple two-fold difference in MIC is likely not enough evidence for this. Regardless, these data clearly show that auranofin inhibits the growth of *C. difficile* in the absence of selenoproteins.
<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Description (relevant genotype)</th>
<th>Reference / Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>R20291</td>
<td>Wild type, ribotype 027</td>
<td>(48)</td>
</tr>
<tr>
<td>KNM6</td>
<td>R20291 (ΔselD) CRISPR-Cas9 mutant</td>
<td>(48)</td>
</tr>
<tr>
<td>KNM9</td>
<td>KNM6 (ΔselD::selD+) CRISPR-Cas9 mutant</td>
<td>(49)</td>
</tr>
<tr>
<td>JIR8094</td>
<td>Wild type, ribotype 012, Erm^S derivative of strain 630</td>
<td>(29)</td>
</tr>
<tr>
<td>LB-CD4</td>
<td>JIR8094 (prdB::ermB) TargeTron mutant</td>
<td>(29)</td>
</tr>
<tr>
<td>LB-CD7</td>
<td>JIR8094 (selD::ermB) TargeTron mutant</td>
<td>(48)</td>
</tr>
<tr>
<td>LB-CD8</td>
<td>JIR8094 (prdR::ermB) TargeTron mutant</td>
<td>(29)</td>
</tr>
<tr>
<td>LB-CD12</td>
<td>JIR8094 (grdA::ermB) TargeTron mutant</td>
<td>(29)</td>
</tr>
</tbody>
</table>
Figure 3: A *C. difficile* ΔselD mutant has the same sensitivity to auranofin as wild type.

*C. difficile* strains (A) R20291, (B) KNM6, and (C) KNM9 were grown in BHIS broth augmented with 2.5% DMSO and varying concentrations of auranofin at 37 °C for 48 h. The OD$_{600}$ of each culture was recorded at 48 h. The experiment was performed twice. Data points represent the means of triplicate cultures while error bars represent standard deviations.
Figure 4: Mutations in selenophosphate synthetase, proline reductase, or glycine reductase do not confer resistance to auranofin.

*C. difficile* strains (A) JIR8094, (B) LB-CD7, (C) LB-CD4, (D) LB-CD8, and (E) LB-CD12 were grown in BHIS broth augmented with 2.5% DMSO and varying concentrations of auranofin at 37 °C for 48 h. The OD₆₀₀ of each culture was recorded at 48 h. The experiment was performed twice. Data points represent the means of triplicate cultures while error bars represent standard deviations.
Selenite supplementation neutralizes auranofin’s activity against C. difficile even in the absence of selenoproteins

We previously demonstrated that supplementing the culture medium with Se (either as sodium selenite or L-selenocysteine) exhibits a protective effect against auranofin, which we had interpreted as excess Se overcoming the apparent nutritional deficiency caused by the formation of Au-Se adducts (92). Since auranofin still inhibits the growth of selD mutants as well as wild-type strains, we wanted to determine if selenite supplementation would still influence auranofin’s antibacterial activity. When we repeated the previous assay using BHIS broth augmented with 5 μM selenite, we surprisingly observed a two-fold increase in the MICs of all strains (with the exception of JIR8094) (Fig. 5 and 6), suggesting that excess selenite dampened auranofin’s activity regardless of whether selenoproteins were present. To verify if this response could be exacerbated at higher doses, we repeated the same assay with 50 μM selenite. Under these conditions, all strains grew regardless of the auranofin concentration (Fig. 5 and 6). These results clearly demonstrate that selenite’s protective effect against auranofin cannot be explained as simply overcoming a Se deficiency imposed by the compound. While this phenomenon could potentially be interpreted as chemical inactivation by Se, it must be noted that Thangamani et al. reported no selenite-dependent neutralization of auranofin’s activity against methicillin-resistant Staphylococcus aureus (112), suggesting that there are different species-specific mechanisms at play. Finally, given the fact that selenite exhibits varying toxicity to some bacteria (113, 114), we wanted to determine if this was potentially acting as a confounding variable in our experiments. When we cultured our strains in BHIS broth containing varying selenite concentrations, we subsequently observed no difference in growth yields even up to 100 μM (Appendix C: Fig. 20 and 21). This result correlates with a publication that reports a staggering MIC of 27 mM sodium selenite against two C. difficile isolates (115).
Figure 5: Selenite supplementation decreases auranofin sensitivity even in the absence of selenoproteins.

*C. difficile* strains (A) R20291, (B) KNM6, and (C) KNM9 were grown in selenite-supplemented BHIS broth augmented with 2.5% DMSO and varying concentrations of auranofin at 37 °C for 48 h. Sodium selenite was added to give a final concentration of 5 µM (red open circle) or 50 µM (red filled circle). The OD$_{600}$ of each culture was recorded at 48 h. The experiment was performed twice. Data points represent the means of triplicate cultures while error bars represent standard deviations.
Figure 6: Selenite supplementation decreases auranofin sensitivity in a manner independent of selenophosphate synthetase, proline reductase, or glycine reductase.

*C. difficile* strains (A) JIR8094, (B) LB-CD7, (C) LB-CD4, (D) LB-CD8, and (E) LB-CD12 were grown in selenite-supplemented BHIS broth augmented with 2.5% DMSO and varying concentrations of auranofin at 37 °C for 48 h. Sodium selenite was added to give a final concentration of 5 µM (red open circle) or 50 µM (red filled circle). The OD$_{600}$ of each culture was recorded at 48 h. The experiment was performed twice. Data points represent the means of triplicate cultures while error bars represent standard deviations.
Discussion

In this work, we unexpectedly discovered that auranofin inhibits the growth of *C. difficile* mutants lacking selenoproteins. This result was perplexing as we originally thought that auranofin’s antimicrobial activity against *C. difficile* was mainly due to the inhibition of Se metabolism (92). Our idea had been supported by several lines of evidence: (i) auranofin prevented the uptake of $^{75}$Se and its incorporation into selenoproteins in both *C. difficile* and anaerobically grown *Escherichia coli*; (ii) the anaerobic growth yield of an *E. coli ΔselD* mutant was unaffected by auranofin compared to wild type; and (iii) auranofin exhibited little to no activity against *Clostridium perfringens* and *Clostridium tetani* (i.e., clostridia that lack selenoproteins) (92). Additionally, we had found that the oral pathogen *Treponema denticola*—an organism with a strict Se requirement for growth (116)—was also susceptible to auranofin, as the compound likewise prevented the uptake and incorporation of $^{75}$Se into its selenoproteins (117). Consistent with our initial observations of auranofin’s activity against *C. difficile* (92), the compound’s growth inhibition of *T. denticola* could be attenuated by supplementation with either sodium selenite or L-selenocysteine (117). Clearly, our idea of targeting Se metabolism in *C. difficile* was predicated on the assumption that the pathogen required Se for growth in the same manner as *T. denticola*, when in reality, genetic techniques have revealed that selenoproteins are actually not essential to *C. difficile* (48). Thus, when dealing with organisms that carry dispensable selenoproteins (e.g., *E. coli* and *C. difficile*), Se metabolism becomes a poor candidate for a drug target. Moreover, it is obvious that auranofin’s effects in these bacteria are far more complex than initially assumed; for example, it is unknown why an *E. coli selD* mutant gains slight resistance to auranofin while a *C. difficile selD* mutant exhibits no appreciable change in sensitivity. Further research should focus on fully characterizing the compound’s multiple modes of action in order to truly understand their effects in different pathogens.
As of now, auranofin’s mechanism of action against \textit{C. difficile} is unknown, but a promising candidate may exist within the thioredoxin (Trx) system, which utilizes disulfide reductase activity to protect cytosolic components against oxidative stress and maintain thiol redox homeostasis (118). The Trx system is comprised of Trx, Trx reductase (TrxR), and NADPH (118). Trx reduces aberrant disulfides in the cell using a thiol-disulfide exchange mechanism that inevitably causes itself to be oxidized; TrxR utilizes electrons from NADPH to reduce Trx, allowing it to resume its surveillance of the cytosol for more oxidized substrates (118). Interestingly, auranofin is known to be a selective inhibitor of TrxR in mammalian cells and parasites (119-121). Likewise, auranofin has been shown to inhibit bacterial TrxR in some clinical pathogens such as \textit{Mycobacterium tuberculosis}, \textit{S. aureus}, and \textit{Helicobacter pylori} (122-124). Harbut et al. even proposed that auranofin’s poor activity against several Gram-negative bacteria is actually due to the presence of the glutathione system, which can provide compensatory disulfide reductase activity in the event of a compromised Trx system (122). Thus, in bacteria lacking glutathione (i.e., most Gram-positives), auranofin-dependent inhibition of TrxR is expected to be lethal. It is therefore tempting to believe that auranofin could be exhibiting a similar mechanism in \textit{C. difficile} due to two important observations: (i) a \textit{trxR} gene exists within the \textit{grd} operon (28), and (ii) the cysteine-to-glutathione biosynthesis pathway is reportedly absent from the genome (125). Alternatively, Thangamani et al. claimed that auranofin likely possesses multiple modes of action, as the compound was able to inhibit several biosynthetic pathways in \textit{S. aureus} (e.g., DNA, protein, and cell wall syntheses) (112). Moreover, the authors suggest that auranofin’s weak activity against Gram-negatives may actually be due to the presence of the outer membrane and efflux pumps, rather than the redundant activity of the glutathione system (112). Specifically, they showed that several Gram-negative pathogens were only susceptible to auranofin when the
permeabilizing agent polymyxin B nonapeptide was present; moreover, an *E. coli* double mutant lacking both TrxR (*trxB*) and glutathione reductase (*gor*) did not differ in auranofin sensitivity compared to wild type (112). Overall, these data imply that inhibition of TrxR—akin to inhibition of selenoprotein synthesis in *C. difficile*—may not be the only mechanism that this compound utilizes against bacteria. A classic technique to determine the mechanism of action of an antimicrobial involves the careful isolation of spontaneous drug-resistant mutants *in vitro*; however, numerous groups have clearly reported an inability to generate spontaneous auranofin-resistant mutants of several bacterial species using this method (112, 122, 124, 126-128). Likewise, our attempts to isolate spontaneous auranofin-resistant *C. difficile* mutants were met with failure, which further supports the idea of auranofin possessing multiple modes of action.

**Materials and Methods**

*Bacterial strains and growth maintenance*

All *C. difficile* strains used in this study are listed in Table 2. Growth experiments were performed in a Coy anaerobic chamber under an atmosphere of ~1.0% H₂, 5% CO₂, and >90% N₂. Strains were routinely maintained on BHIS agar (37 g/L brain heart infusion, 5 g/L yeast extract, 0.1% L-cysteine). When indicated, overnight cultures were prepared by inoculating 5 mL BHIS broth with single colonies of the appropriate strains followed by 16–24 hours of incubation at 37 °C.

*Broth microdilution assay*

MICs were determined using a modified broth microdilution assay as per the CLSI M11 (90). Briefly, auranofin was dissolved in 100% dimethyl sulfoxide (DMSO) and subsequently diluted to achieve working stocks at 20× concentration in 50% DMSO. Similarly, fidaxomicin was
dissolved in 100% DMSO while vancomycin hydrochloride was dissolved in deionized water. Diluted test compounds (5 μL) were mixed with 95 μL BHIS broth in triplicate in 96-well plate format. Plates were reduced overnight in the anaerobic chamber. On the day of experimentation, overnight cultures of all strains were diluted to match a 0.5 McFarland standard and were subsequently diluted again 15-fold using pre-reduced 0.85% NaCl. Diluted cells (10 μL) were used to inoculate 100-μL triplicate drug-broth mixtures in pre-reduced 96-well plates, which were then incubated in half-sealed plastic bags at 37 °C for 48 hours. After incubation, the OD₆₀₀ was recorded for all cultures and normalized by subtracting the mean OD₆₀₀ of triplicate uninoculated BHIS broth controls (blank correction). MICs were scored as the lowest concentration of compound that resulted in non-turbid cultures as compared to uninoculated controls after 48 hours. For all selenite supplementation experiments, the assay described above was performed with BHIS broth augmented with either 5 or 50 μM sodium selenite in triplicate. Blank corrections were done relative to the appropriate selenite concentration, as selenite imparts a slight red color to the medium. All experiments were performed twice. Statistical analysis (mean OD₆₀₀ ± s.d.) was performed using GraphPad Prism 9.

_Selenite sensitivity assay_

Overnight cultures of all strains were diluted 100-fold in BHIS broth augmented with 0, 5, 10, 25, 50, or 100 μM sodium selenite in triplicate. Diluted cultures were grown for 24 hours at 37 °C. The OD₆₀₀ was recorded for all cultures at the end of the growth period. Blank corrections were done relative to the appropriate selenite concentration as described above. The experiment was performed twice. Statistical analysis (mean OD₆₀₀ ± s.d.) was performed using GraphPad Prism 9.
CHAPTER FOUR:
D-PROLINE REDUCTASE UNDERLIES PROLINE-DEPENDENT GROWTH OF CLOSTRIDIIOIDES DIFFICILE

Preamble

*C. difficile* must colonize the large intestine to cause disease, though the specific mechanisms by which this occurs are still unclear. Recent publications from other groups have shown that toxin-mediated inflammation results in the generation of host-originating nutrients such as sorbitol (38) and collagen-derived amino acids such as proline, glycine, and hydroxyproline (39). Interestingly, these three amino acids are highly preferred electron acceptors in *C. difficile* energy metabolism. Since the selenoproteins D-proline reductase and glycine reductase are required for this process, we were interested in probing the relationship between these amino acids and selenium metabolism by evaluating their effects on growth using rich and minimal media and toxin production using western blot assays. This dissertation chapter documents the results of that study, which was published in *Journal of Bacteriology* (Appendix D).

Introduction

*Clostridioides difficile* (formerly *Clostridium difficile*) is a Gram-positive, rod-shaped, spore-forming anaerobe and the leading cause of antibiotic-associated diarrhea, often representing 15–25% of all cases (5, 67). Depending on the severity, symptomatic *C. difficile* infections (CDI) manifest in various disease states ranging from mild diarrhea to life-threatening fulminant colitis (1). *C. difficile* primarily thrives in the dysbiotic gut environment, which is typically induced by treatment with broad-spectrum antibiotics (1). Following the disruption of the intestinal flora,
ingested *C. difficile* spores travel through the small intestine and germinate into vegetative cells after encountering certain primary bile acids and amino acids (11, 129). These vegetative cells proliferate and eventually release two glucosyltransferases, toxin A (TcdA) and toxin B (TcdB), which are the causative agents of disease; both toxins glucosylate the host’s small Rho-family GTPases in intestinal epithelial cells, ultimately resulting in massive inflammation and colonic injury (12). Though, despite the necessary role that toxins play in establishing CDI, the rationale behind creating incredibly intense gut inflammation and the metabolic strategies that *C. difficile* employs to thrive in this environment are both poorly understood.

Inflammation is a key element of the host’s immune system, especially in the context of bacterial infections; the complex events that underlie this response have been thoroughly defined (130, 131). However, despite the fact that the immune response is expected to eliminate invading microbes, an unintended consequence of inflammation is the generation of various host-derived metabolites that instead drive the selective expansion of many enteric pathogens in the gut lumen (31-36). Likewise, this concept has recently been demonstrated in the context of CDI, as toxigenic *C. difficile* was shown to utilize sorbitol generated by aldose reductase, a host enzyme in immune cells that was upregulated during toxin-dependent inflammation (38). Another study using *in vivo* transcriptomics reported that toxin-mediated inflammation remodels the nutritional environment of the gut in a way that favors the metabolic preferences of *C. difficile*, particularly amino acid catabolism (39). Specifically, Fletcher et al. (39) suggest a mechanism where toxin-dependent inflammation releases nutrients for *C. difficile* in the form of amino acids via degradation of host collagen. Indeed, in infected mice, genes encoding collagen-degrading enzymes (e.g., matrix metalloproteinases) are upregulated while certain members of the microbiota that potentially compete for collagen-derived proline and hydroxyproline (e.g., Bacteroidaceae) are suppressed.
Additionally, the nutrients liberated by this mechanism could help satisfy the strict amino acid requirements for *C. difficile* growth (132, 133). In support of this idea, *C. difficile* uniquely excels at performing the Stickland reaction, a biochemical pathway that directly harvests energy from amino acids (28, 29, 134). Therefore, if Stickland metabolism is a relevant strategy *in vivo*, collagen may likely act as an energy depot for invading *C. difficile*, thereby providing a reasonable argument for why this pathogen easily blooms in the inflamed colon.

In many proteolytic clostridia, Stickland metabolism is a core bioenergetic scheme defined by the coupled oxidation of one amino acid (Stickland donor) and the reduction of another (Stickland acceptor) (40, 41). Briefly, Stickland donors are either oxidatively deaminated or decarboxylated to yield reducing equivalents (NADH) and ATP through substrate-level phosphorylation; Stickland acceptors are reduced or reductively deaminated in an NADH-dependent manner, ultimately regenerating NAD$^+$ for further oxidations (29, 40, 41). While Stickland donors are typically aliphatic amino acids (e.g., alanine, leucine, isoleucine, valine) (40, 41, 135), a variety of other nutrients and metabolic pathways may also functionally act as Stickland donors, since reducing power (e.g., NADH) can be derived from sugar oxidation or possibly even gaseous H$_2$ through hydrogenases (136, 137). In contrast, Stickland acceptors are comparatively less diverse, including only proline, glycine, and leucine; however, others have shown that phenylalanine, tyrosine, and methionine can be reduced at a lower efficiency (40, 135, 138-144). Therefore, Stickland metabolism is theoretically limited by its narrow selection of electron acceptors, which potentially forces *C. difficile* to occupy a niche where these amino acids are prioritized for rapid disposal of reducing equivalents. This niche, however, may be attainable through the degradation of host collagen, given that most of the amino acids found in high abundance are proline, glycine, and hydroxyproline (145). Although not a true Stickland acceptor,
hydroxyproline can be converted to proline in *C. difficile* by 4-hydroxyproline dehydratase (HypD) and Δ¹-pyrroline-5-carboxylate reductase (P5CR) (146, 147). Thus, the metabolic rationale behind toxin-mediated inflammation might be to scavenge proline, glycine, and hydroxyproline from collagen, which *C. difficile* could use to overcome the bioenergetic hurdles imposed by Stickland metabolism.

When acting as electron acceptors, proline and glycine undergo reduction by the *C. difficile* enzymes D-proline reductase (Prd) and glycine reductase (Grd), respectively (28, 29). The true substrate of Prd is generated from the conversion of L-proline to D-proline via proline racemase in *C. difficile* (28). Subsequent reduction of D-proline by Prd results in cleavage of the ring, ultimately regenerating NAD⁺ and forming 5-aminovalerate as a byproduct; glycine is reductively deaminated by Grd to produce NAD⁺ and acetyl phosphate, which yields acetate and ATP through substrate-level phosphorylation (28, 29). In *C. difficile*, the Prd and Grd complexes share a unique molecular trait in that some of their respective subunits – PrdB, GrdA, and GrdB – contain selenocysteine residues, effectively classifying them as selenoenzymes (28). To synthesize selenoproteins, UGA-encoded selenocysteine must be co-translationally inserted into polypeptides; however, the synthesis of this particular amino acid requires selenophosphate, a selenium donor with high group transfer potential (45, 105). In bacterial selenium metabolism, selenophosphate is generated from selenide in an ATP-dependent manner via the activity of selenophosphate synthetase (SelD) (148). Because of the importance of proline and glycine in *C. difficile* bioenergetics, the presence of selenium in both Prd and Grd implies a critical role in physiology. We have previously demonstrated that selenium is required for optimal growth on Stickland pairs containing either proline or glycine (28). However, despite the apparent role that SelD plays during important physiological events such as vegetative cell growth, sporulation, and
outgrowth of germinated spores (48, 49), selenoproteins have been shown to be non-essential in *C. difficile* (48), calling into question the actual significance of selenium-dependent Stickland reactions. Overall, our current understanding of the role of selenoproteins in *C. difficile* physiology is unclear, and the effects of proline, glycine, and hydroxyproline have yet to be fully elucidated in the context of cells lacking Prd and Grd.

In this study, we measured the protein levels of Prd and Grd in *C. difficile* strains R20291 and JIR8094 grown in several routine culture media to better understand the prevalence of selenium-dependent Stickland reactions. Using these same media supplemented with proline, glycine, and hydroxyproline, we then evaluated the effects of these amino acids on the growth of both wild-type strains and mutants deficient in Prd, Grd, and selenoproteins. Finally, we determined the effects of these amino acids on the ability of R20291 to produce toxins. In this work, we report various growth phenotypes of *C. difficile* in rich and defined media supplemented with proline, glycine, and hydroxyproline; a SelD-dependent regulatory effect on toxin production by proline and hydroxyproline; and an unexpected discovery that proline-dependent growth of *C. difficile* is largely due to a unique dependence on Prd.

**Results**

*PrdB is present throughout extended culture in various growth media*

Proline, glycine, and hydroxyproline are among the most abundant amino acids in collagen; therefore, if *C. difficile* were to promote collagen degradation, these nutrients would be released into the colonic environment and potentially serve as a rich source of Stickland acceptors for the pathogen. In this scenario, Prd and Grd would likely play a key role in scavenging these nutrients for electrons since the former is induced by proline and hydroxyproline while the latter is induced
by glycine (28). However, the abundance of these selenoenzymes has not been critically studied in the context of a classical *in vitro* growth study, which can reveal significant and relevant information about the organism in a carefully controlled manner. Here, we measured the protein levels of Prd and Grd in *C. difficile* by labeling R20291 and JIR8094 cells with $^{75}$Se and growing them in three different routine culture media—BHIS (supplemented brain heart infusion) (89), TY (tryptone-yeast) (149), and CDMM (*C. difficile* minimal medium) (133)—at 37 °C for 72 h (Fig. 7).

The selenoprotein subunits of Prd (PrdB) and Grd (GrdA and GrdB) were effectively revealed with this radiolabeling method and were used as proxies for the overall abundance of both complexes. We first discovered in both strains that PrdB was present at 6 h and persisted throughout the entire 72 h in rich media (BHIS, TY) (Fig. 7A, 7B, 7C, and 7D). We found this surprising considering these growth media were not augmented with excess proline to induce expression of the prd operon (28, 29). There were similar observations in minimal medium (CDMM), although the levels of PrdB differed between strains (Fig. 7E and 7F). The constant presence of PrdB in three different growth media suggests a strong dependence on the Prd complex regardless of the nutritional environment. Next, we observed varying levels of GrdA and GrdB. GrdA appeared during the early stages of growth in BHIS and TY and slowly decreased in intensity over the course of the entire study (Fig. 7A, 7B, 7C, and 7D). In CDMM, GrdA presented with less intensity in R20291 compared to JIR8094 where it reached a maximum at 24 h (Fig. 7E and 7F). On the other hand, the $^{75}$Se profile of GrdB exhibited the most variability and did not correlate with GrdA in BHIS (Fig. 7A and 7B), but both selenoproteins were found in similar levels in TY (Fig. 7C and 7D). Compared to GrdA in CDMM, GrdB was almost non-existent (Fig. 7E and 7F). We also curiously spotted an intense low-molecular-weight species (*) of unknown identity that
migrated far below 10 kDa (Fig. 7E and 7F). The differential expression of GrdA and GrdB was surprising and prompted us to evaluate whether depleting glycine from the medium would yield any differences in the $^{75}$Se profile of each subunit. When we omitted glycine from CDMM, both Grd subunits disappeared from the gel, leaving only PrdB and the low-molecular-weight selenium species (*) (Appendix E: Fig. 22).

Next, in order to carefully determine the role of Prd and Grd in *C. difficile* physiology, we employed a panel of mutants that harbored deficiencies in either enzyme or the ability to synthesize selenoproteins (Table 3). We first verified the expected phenotypes of these mutants by analyzing their selenoprotein profiles with $^{75}$Se labeling. The selenoprotein profiles of the JIR8094 mutant strains used in this study have already been verified in previous reports (29, 48). In similar fashion, we labeled the R20291 mutant strains KNM6 ($\Delta$selD) and KNM9 ($\Delta$selD::selD$^+$) with $^{75}$Se and simultaneously confirmed the absence of radiolabeled proteins in the $\Delta$selD mutant and the restoration of PrdB, GrdA, and GrdB in the $\Delta$selD::selD$^+$ mutant (Appendix E: Fig. 23). This information allowed us to interpret the resulting phenotypes from future experiments with high confidence. Overall, these findings painted a clearer picture of cellular selenoprotein levels in routine culture media which we could then reference when conducting controlled growth studies with proline, glycine, and hydroxyproline.
Figure 7: D-Proline reductase persists throughout the entirety of *in vitro* culture.

In the presence of 2 µCi $^{75}$Se per mL of culture and 50 nM sodium selenite, *C. difficile* strains R20291 (A, C, E) and JIR8094 (B, D, F) were grown in BHIS (A, B), TY (C, D), and CDMM (E, F) at 37 °C for 72 h and harvested at the indicated time points. After harvest and lysis of cells, approximately 7.5 µg (BHIS), 2.5 µg (TY), and 1.0 µg (CDMM) of soluble cellular protein were resolved on 15% acrylamide gels by SDS-PAGE. The known selenoproteins GrdB, PrdB, and GrdA are indicated with arrows based on previous literature (28, 29, 48). The asterisk (*) denotes a low-molecular-weight selenium species of unknown identity.
<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Description (Relevant Genotype)</th>
<th>Reference / Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>R20291</td>
<td>Wild-type, ribotype 027</td>
<td>(48)</td>
</tr>
<tr>
<td>KNM6</td>
<td>R20291 ($ΔselD$) CRISPR-Cas9 mutant</td>
<td>(48)</td>
</tr>
<tr>
<td>KNM9</td>
<td>KNM6 ($ΔselD::selD^+$) CRISPR-Cas9 mutant</td>
<td>(49)</td>
</tr>
<tr>
<td>JIR8094</td>
<td>Wild-type, ribotype 012, Erm&lt;sup&gt;S&lt;/sup&gt; derivative of strain 630</td>
<td>(29)</td>
</tr>
<tr>
<td>LB-CD4</td>
<td>JIR8094 ($prdB::ermB$) TargeTron mutant</td>
<td>(29)</td>
</tr>
<tr>
<td>LB-CD7</td>
<td>JIR8094 ($selD::ermB$) TargeTron mutant</td>
<td>(48)</td>
</tr>
<tr>
<td>LB-CD8</td>
<td>JIR8094 ($prdR::ermB$) TargeTron mutant</td>
<td>(29)</td>
</tr>
<tr>
<td>LB-CD12</td>
<td>JIR8094 ($grdA::ermB$) TargeTron mutant</td>
<td>(29)</td>
</tr>
</tbody>
</table>
**Proline and hydroxyproline enhance growth yields in rich media, but the latter does not require proline reductase**

To determine the physiological effects of the three Stickland acceptors, we grew a panel of *C. difficile* strains (Table 3) in rich media (BHIS and TY) augmented with either 30 mM L-proline, glycine, or L-4-hydroxyproline at 37 °C and monitored the growth of each culture by measuring the optical density at 600 nm (OD₆₀₀) over a 48-h period. We then performed analyses of the resulting growth plots to identify significant changes in growth rates and growth yields, as these parameters typically define the organism’s ability to compete within the host gut.

In both rich media, the parent strains R20291 and JIR8094 grew to substantially higher yields with added proline and hydroxyproline (Fig. 8A, 8D, 9A, and 9D). Interestingly, compared to base media without supplemented amino acids, the growth yield of the ΔSELD mutant did not change with added proline (Fig. 8B and 9B). In the ΔSELD::SELD⁺ strain, the proline-dependent growth yield was similar to that of R20291 (Fig. 8C and 9C), demonstrating that this effect was due to the presence of SelD. In JIR8094, we observed a similar SelD-dependent phenotype since the yield of LB-CD7 (*selD::ermB*) was likewise unaffected by the addition of proline (Fig. 8D, 8E, 9D, and 9E), confirming that this phenomenon was not strain dependent. Based on these results, we hypothesized that these growth yield enhancements were due to proline reduction by Prd.

We have previously demonstrated that the addition of proline to a growth medium increases the levels of Prd (28), and it is now known that this phenomenon requires the transcriptional regulator PrdR (29). We found that Prd was indeed responsible for proline-dependent growth stimulation, as either the disruption of the enzyme complex in LB-CD4 (*prdB::ermB*) (Fig. 8F and 9F) or PrdR in LB-CD8 (*prdR::ermB*) (Fig. 8G and 9G) was sufficient to remove the organism’s ability to respond to added proline. On the other hand, glycine apparently had no beneficial effect
on growth yield. We found both of these observations to be consistent with another report (29). However, we unexpectedly noticed that glycine occasionally lowered the growth yields of *C. difficile*. Since Grd levels are modulated by the presence of glycine (28, 29), we assumed that Grd activity might have been somehow responsible for the reduction in growth yield; however, this was not the case as the apparent biomass of LB-CD12 (*grdA::ermB*) still dropped in the presence of added glycine (Fig. 8H and 9H). Finally, we found that hydroxyproline consistently gave better growth yields than the other two Stickland acceptors. In surprising contrast to proline, we found that these growth enhancements were not due to either SelD or Prd (Fig. 8 and 9), suggesting that the role of hydroxyproline in *C. difficile* physiology is not simply limited to its ability to act as an additional source of proline for Prd.

Overall, our analyses indicated that the maximum growth yield of each 48-h culture (interpreted as the highest OD$_{600}$ of each curve) was significantly enhanced with proline and hydroxyproline supplementation in a Prd-dependent and Prd-independent manner, respectively (Appendix E: Fig. 24). In comparison, the doubling times of each strain (calculated as the inverse of growth rate) did not substantially change when proline, glycine, and hydroxyproline were added in excess (Appendix E: Table 6 and 7). Finally, we noted that the growth effects due to proline and hydroxyproline were greatly exacerbated in TY compared to BHIS, suggesting that nutrient status of the culture medium influences the organism’s ability to use both amino acids as growth substrates. This line of reasoning is supported by the fact that *C. difficile* produces substantially less 5-aminovalerate in Eggerth-Gagnon medium compared to BHIS, demonstrating that the rate of proline reduction changes in response to nutrient composition (150).
Figure 8: Proline and hydroxyproline increase growth yield in BHIS, but the latter does not rely on D-proline reductase.

The following *C. difficile* strains were grown in BHIS at 37 °C for 48 h: (A) R20291, (B) KNM6, (C) KNM9, (D) JIR8094, (E) LB-CD7, (F) LB-CD4, (G) LB-CD8, and (H) LB-CD12. When indicated, BHIS was supplemented with proline (Pro), glycine (Gly), and hydroxyproline (Hyp) at 30 mM. The experiment was repeated twice. Data points represent the means of triplicate cultures while error bars represent standard deviations. The first 24 h are shown to better visualize relevant phenotypes.
Figure 9: Growth yield stimulation from proline and hydroxyproline is enhanced in TY.

The following *C. difficile* strains were grown in TY at 37 °C for 48 h: (A) R20291, (B) KNM6, (C) KNM9, (D) JIR8094, (E) LB-CD7, (F) LB-CD4, (G) LB-CD8, and (H) LB-CD12. When indicated, TY was supplemented with proline (Pro), glycine (Gly), and hydroxyproline (Hyp) at 30 mM. The experiment was repeated twice. Data points represent the means of triplicate cultures while error bars represent standard deviations. The first 24 h are shown to better visualize relevant phenotypes.
In the absence of proline reductase, proline is no longer required for growth in a defined minimal medium

While our observations in rich media are potentially interesting, both of these peptide-based media unfortunately contain substantial amounts of the amino acids we were studying. Instead, in order to clearly define the effects of each Stickland acceptor, we found it advantageous to use a defined minimal medium (i.e., CDMM) as it allowed us to control the amount of each variable accurately and reproducibly. When we first grew R20291 and JIR8094 in CDMM at 37 °C, we found that they exhibited very distinct growth profiles compared to rich media (Fig. 10A and 10D). Specifically, after both strains reached maximum culture density, the growth behavior was characterized by a sharp decrease in turbidity, a phenomenon that has been reported in other physiological studies of *C. difficile* (144, 151).

To cleanly determine the effects of the three Stickland acceptors, we then grew R20291 and JIR8094 in several versions of CDMM lacking either proline, glycine, or both amino acids. For studies with hydroxyproline, we used CDMM deficient in both amino acids and substituted proline with equimolar hydroxyproline. When proline was removed from CDMM, R20291 and JIR8094 behaved as proline auxotrophs (Fig. 10A and 10D), which we expected since it has been previously established that proline is required for growth in minimal media (132, 133). However, based on these results, we believed that the experiments to determine the effects of proline were therefore confounded by the fact that it apparently must be present in every preparation of the medium to guarantee growth of *C. difficile*. Despite this assumption, we surprisingly found that the ΔselD mutant grew very well in the absence of proline (Fig. 10B), whereas the proline-dependent phenotype from R20291 was restored in the ΔselD::selD+ mutant (Fig. 10C). Moreover, this behavior was not specific to cells with the R20291 genetic background since the selD::ermB strain (with the JIR8094 genetic background) also grew in the absence of proline (Fig. 10E). Since
C. difficile cells lacking SelD gained the ability to grow without added proline, we hypothesized that selenoproteins were somehow playing a major role in establishing this absolute growth requirement. Considering that the effect was related to proline, we suspected that Prd was the selenoenzyme responsible for this phenotype. Indeed, additional growth experiments revealed that the prdB::ermB and prdR::ermB strains also grew in the absence of proline (Fig. 10F and 10G). On the other hand, the grdA::ermB strain failed to grow unless proline was present (Fig. 10H), verifying that Prd (not Grd) is the selenoenzyme responsible for establishing the growth requirement. Overall, these data suggest that Prd activity forces the organism to strictly depend on environmental proline, which likely explains why wild-type strains behave as proline auxotrophs.

In contrast, we found that the removal of glycine had little to no effect on growth, possibly substituted by threonine present in the medium (133). The grdA::ermB strain, however, gave a heavily distorted growth profile compared to JIR8094 (Fig. 10H), implying that Grd plays an important role in physiology that is not inherently obvious from studies using rich media. Finally, all C. difficile strains were still able to grow even when proline was replaced with hydroxyproline, likely explained by conversion of hydroxyproline to proline via HypD and P5CR (146, 147). However, while strains with the R20291 background grew similarly as in regular proline-containing CDMM (Fig. 10A, 10B, and 10C), most strains with the JIR8094 background comparatively exhibited reduced growth yields and highly unusual growth curves that varied drastically with each mutation (Fig. 10D, 10E, 10F, and 10H). Curiously, while hydroxyproline distorted the growth curve of the prdB::ermB mutant, it did not appear to have any negative effect on the prdR::ermB mutant as its growth curve remained similar to the proline-containing CDMM control (Fig. 10G). These data suggest that PrdR plays a Prd-independent regulatory role in the organism’s response to hydroxyproline.
Figure 10: Proline-dependent growth of C. difficile requires the presence of D-proline reductase.

The following C. difficile strains were grown in CDMM at 37 °C for 48 h: (A) R20291, (B) KNM6, (C) KNM9, (D) JIR8094, (E) LB-CD7, (F) LB-CD4, (G) LB-CD8, and (H) LB-CD12. Proline (Pro), glycine (Gly), and hydroxyproline (Hyp) were omitted (-) from or added (+) to CDMM as indicated. Refer to Materials and Methods for amino acid concentrations. The experiment was repeated twice. Data points represent the means of triplicate cultures while error bars represent standard deviations.
Proline and hydroxyproline addition leads to a decrease in toxin production that is diminished in a ΔselD mutant strain

Toxin production in *C. difficile* can be modulated by various environmental factors, including nutrient status of the growth medium (83, 152). Since amino acids are known to have varying effects on toxin synthesis (152-158), it is likely that the three Stickland acceptors play a role in regulating toxin levels in *C. difficile*. Indeed, proline negatively affects *tcdA* expression on a transcriptional level via PrdR (29). Thus, we sought to determine the effects of proline, glycine, and hydroxyproline on toxin production. We first assessed the ability of R20291 and JIR8094 to produce toxin in BHIS, TY, and CDMM at 37 °C for 72 h (Fig. 11A). We then estimated overall toxin production by measuring the levels of TcdA in spent media by immunoblot with a TcdA-specific antibody. We found that R20291 made a substantial amount of TcdA at 48 h in BHIS, whereas in TY, toxin saturation began much earlier at 24 h. In contrast, TcdA produced by JIR8094 was not detectable in rich media until 48 h. Toxin production in CDMM was incredibly low for both strains and could not be reliably analyzed.

Based on these results, we similarly probed for TcdA using the spent media from the previous BHIS growth studies (Fig. 8) and evaluated toxin production in response to excess proline, glycine, and hydroxyproline at 48 h. We found that proline and hydroxyproline substantially reduced toxin production in R20291, though only the hydroxyproline-dependent effect was deemed to be significant in our analysis. Comparatively, glycine did not have any noticeable effect on toxin levels (Fig. 11B and 11C). Interestingly enough, the toxin profile in the ΔselD mutant was strikingly different, as TcdA levels did not significantly change in response to added proline and hydroxyproline. In the ΔselD::selD* mutant, the toxin-reducing effects from supplemented proline and hydroxyproline were restored, and both were found to be significant compared to the BHIS control. These results indicate that the activity of selenoproteins, likely Prd,
leads to conditions in the cell in which one or more regulators downregulate toxin production. Overall, our findings suggest some involvement of these Stickland acceptors in controlling toxin production, but further experiments are required in order to fully map out the regulatory pathways involved.
Figure 11: Proline and hydroxyproline suppress toxin production in a SelD-dependent manner in BHIS.

Culture supernatants were harvested, resolved on 7.5% acrylamide gels via SDS-PAGE, and probed with a TcdA-specific monoclonal antibody. (A) Toxin profiles from 72-h time courses of R20291 and JIR8094 in BHIS, TY, and CDMM, as estimated by representative immunoblot against TcdA. (B) Modulation of toxin production by proline, glycine, and hydroxyproline in 48-h BHIS cultures. Proline (Pro), glycine (Gly), and hydroxyproline (Hyp) were added at 30 mM as indicated. The experiment was repeated twice. Representative TcdA immunoblots are shown. (C) TcdA band intensity as quantified by densitometry and with respect to the amount of total protein in each culture. Data points represent the means of normalized band volumes derived from triplicate cultures (including Fig. 5B) while error bars represent standard deviations. Statistical analysis was performed in GraphPad Prism 8 using two-way ANOVA with Dunnett’s multiple comparisons test in which all comparisons were made to BHIS. ns, not significant; ** P ≤ 0.01; *** P ≤ 0.001; **** P ≤ 0.0001.
Discussion

The strict physiological dependence on Prd and evidence for a hierarchy of electron acceptors

Our results demonstrated that proline enhances the growth of *C. difficile* in a manner dependent on Prd (Fig. 8, 9, and Appendix E: Fig. 24), which is consistent with a previous report (29), though we did not observe substantial changes in the growth rates with our method of analysis (Appendix E: Table 6 and 7). The Prd-dependent biomass increase *in vitro* likely provides an advantage *in vivo*, especially since the prdB::ermB mutant has been shown to exhibit a colonization defect in mice transplanted with a dysbiotic human gut microbiota (159). According to a recent report suggesting that proline reduction plays a larger role in establishing colonization resistance compared to bile acid metabolism (27), current therapies may benefit from a greater understanding of the role of proline reduction in *C. difficile*.

The historical incapability of *C. difficile* to grow in the absence of proline has long been interpreted as proline auxotrophy (132, 133, 159, 160), but this term is a misnomer since it generally describes a genetic inability to synthesize the nutrient of interest. Recently, *C. difficile* strain 630 was shown to grow in a glucose-containing medium lacking proline and leucine—another amino acid assumed to be “essential” for the organism (132, 133)—only after repeated subcultures in glucose media containing decreasing concentrations of these Stickland acceptors (151). Based on that report and the fact that several mutants in our study grew in the absence of proline (Fig. 10B, 10E, 10F, and 10G), *C. difficile* therefore must have the ability to biosynthesize its own proline for anabolic processes (e.g., protein synthesis). Indeed, *C. difficile* can generate proline from ornithine using ornithine cyclodeaminase; moreover, ornithine can be produced from intermediates derived from other non-essential amino acids like arginine and glutamate via ornithine carbamoyltransferase and acetylornithine aminotransferase, respectively (161). Though,
if this biosynthetic pathway is active, it is clearly unable to support the growth of wild-type cells. Therefore, we propose that this unusual behavior stems from a unique “addiction” to using proline as an electron acceptor based on several observations. First, PrdB was abundant throughout almost all stages of growth in several different culture media (Fig. 7), suggesting that vegetative cells highly depend on the Prd complex. Second, Prd⁻ cells can grow in the absence of proline (Fig. 10B, 10E, 10F, and 10G), implying that the presence of this enzyme significantly raises the minimum amount of proline needed for growth. Third, proline is one of the first amino acids rapidly consumed before entering stationary phase, accompanied by the simultaneous production of 5-aminovalerate (142, 144, 151). Lastly, proline reduction lowers the NADH/NAD⁺ ratio, thereby stimulating Rex, a redox-sensing transcriptional regulator, to repress alternative NAD⁺-regenerating pathways (162). We also find it very intriguing that strict proline dependence can be eliminated either by genetically disrupting Prd or by biochemically “weaning” cells off of proline (151). However, in the latter strategy, Gencic and Grahame (151) demonstrated via enzyme assays of 630 extracts that Prd activity was unexpectedly higher in the proline-deficient glucose-containing medium compared to BHIS; the authors reasoned that glucose may have induced expression of the prd operon via activation by CcpA, a transcriptional regulator involved in carbon catabolite repression (163). This observation conflicts with our idea of “proline addiction” as this is a clear example of Prd⁺ cells growing in the absence of proline, suggesting that the presence of Prd may not always force the organism to depend on proline; however, it must be noted that their glucose-only medium was also deprived of leucine, another preferred Stickland acceptor that was present in our preparations of CDMM. Therefore, we believe that the adaptation to conditions deprived of two preferred Stickland acceptors required for growth (proline and leucine) ultimately induces a metabolic reprogramming that is radically different from Prd⁻ cells growing in a culture
medium lacking only proline (151). An investigation into the molecular mechanisms behind this adaptation is certainly warranted.

The ability for *C. difficile* to maintain intracellular redox poise is potentially hampered due to the limitation of electron acceptors in Stickland metabolism. Proline reduction is likely the organism’s most preferred method to dispose of reducing equivalents, but since Prd- cells can grow in the absence of proline, an alternative NAD\(^+\)-regenerating pathway must be compensating for the loss of proline reduction. While the identity of the predominant electron-accepting pathway in Prd- strains is unknown, a possible candidate may be the Wood-Ljungdahl pathway (WLP), a metabolic scheme used by acetogens to reduce two molecules of CO\(_2\) to form acetate (151, 164). Though minimally active in rich media containing substantial amounts of Stickland acceptors (e.g., BHIS), the WLP was found to be highly upregulated in 630 after it had successfully adapted to glucose-containing media lacking proline and leucine (151). In these conditions, the WLP generates acetate in order to fuel the production of butyrate (termed “acetobutyrogenesis”), thereby simultaneously removing electrons and generating ATP for *C. difficile*. Gencic and Grahame (151) proposed that the coupling of the WLP to butyrate fermentation creates an efficient electron sink even with low flux through the pathways, as the former disposes of reducing equivalents (e.g., reduced ferredoxin) generated by the latter. In glucose-only media, it seems rational for this coupled metabolism to dominate as it would make use of the CO\(_2\) and electrons generated from the oxidation of glucose-derived pyruvate. Since our growth experiments were conducted in glucose-supplemented CDMM under an atmosphere containing 5% CO\(_2\) (Fig. 10), it is possible that the Prd- cells shifted to “acetobutyrogenesis” to compensate for the loss of proline reduction. Furthermore, this metabolic shift is likely controlled by Rex, as it has been shown that genes involved in butyrate fermentation are derepressed in the prdR::ermB and rex::ermB mutants (162).
In support of this idea, Gencic and Grahame (151) even noted several Rex-binding sites in the WLP promoter. Based on these studies, evaluating whether the \textit{rex::ermB} mutant could grow in proline-deficient conditions may help outline the unknown hierarchy of electron acceptors in \textit{C. difficile}.

Our proline experiments provide further evidence that \textit{C. difficile} harbors distinct preferences for different electron acceptors (Fig. 12). While reducing power can be derived indiscriminately from multiple sources such as classical Stickland donors (i.e., aliphatic amino acids), sugars and sugar alcohols, or even H$_2$, the availability of certain electron acceptors mainly determines the process of NAD$^+$ regeneration. While this concept has been studied heavily in the model organism \textit{Escherichia coli} (165), little is known about the electron acceptor hierarchy in \textit{C. difficile} other than the fact that proline and leucine are the most preferred acceptors and that the former is consumed first before the latter (142, 144, 151). In the event that both preferred acceptors are absent, it is likely that one or more Rex-mediated alternative pathways will attempt to compensate for the decrease in NAD$^+$ regeneration (162), though the preferential order of these pathways is unknown. An interesting example of a potential alternative electron sink involves H$_2$ metabolism, as \textit{C. difficile} possesses various hydrogenases that mediate the consumption or production of this molecule (166). It must be noted, however, that H$_2$ is almost always present within the anaerobic chamber due to its requirement for palladium-catalyzed O$_2$ decontamination. Physiological experiments are unfortunately confounded by the presence of H$_2$ in the environment, especially if the levels of H$_2$ are inconsistent between experiments. Indeed, McAllister et al. (49) observed that plasmid complementation of \textit{selD} only occurred under low (~1.7%) H$_2$ and not high (4%) H$_2$. Because of this, we deliberately performed experiments at an atmosphere containing a low level of H$_2$ (~1.0%) that was still sufficient to remove O$_2$ from our chamber. Further
investigations of the electron acceptor hierarchy should be conducted at a low and consistent level of \( \text{H}_2 \) to lessen the impact of this confounding variable and to allow for direct comparisons between experiments.
Reducing power (NADH) is generated from various electron donors (red). To regenerate NAD$^+$ for continued oxidation of fuel sources, certain enzymes dispose of electrons using specific molecules as electron acceptors in a set order (blue). *C. difficile* harbors preferences for electron acceptors based on a poorly understood hierarchy. The most preferred substrate is proline. Leucine is also a preferred substrate but is probably lower on the hierarchy. Prd elimination, which cures “proline addiction” as demonstrated in this study, may inadvertently activate alternative NAD$^+$-regenerating pathways in an unknown order (indicated by blue circles with dashed outlines). Electron acceptors that are the focus of this study are in bold. Hydroxyproline (*) is not a true Stickland acceptor but serves as an additional source of proline for Prd. NAD(H) was chosen to represent electron flow for simplicity, but it must be noted that additional carriers besides NAD(H) exist (e.g., ferredoxin). The donors and acceptors listed here are representative and by no means comprehensive. Abbreviations: NAD(H), nicotinamide adenine dinucleotide (oxidized/reduced); SCFA, short-chain fatty acids; WLP, Wood-Ljungdahl pathway.
Our growth experiments revealed that glycine offered no obvious benefit to vegetative *C. difficile*. Furthermore, in rich media supplemented with 30 mM glycine, the growth yields of several strains decreased in a Grd-independent manner (Fig. 8, 9, and Appendix E: Fig. 24). Although the idea of glycine-mediated toxicity seemed puzzling considering the amino acid’s role in Stickland bioenergetics, it is well established that glycine exhibits an inhibitory effect as a result of its misincorporation into the peptidoglycan of several bacteria (167). Even in *C. difficile*, glycine likely functions as a cell wall-weakening agent when in excess, as Bhattacharjee and Sorg (168) demonstrated its requirement for the generation of competent cells; indeed, the authors observed that R20291 and 630 cells curiously adopted a curved morphology after growth in BHIS supplemented with 1% glycine (~133 mM) (168). On the basis of these studies, the glycine-dependent decrease in growth yield could possibly be explained as cell lysis due to the weakening of cell walls resulting from glycine misincorporation. Previous studies, however, did not always describe growth inhibition at 30 mM glycine (28, 29), so our interpretation is that this concentration approaches—but does not necessarily equate to—a reproducibly toxic amount of glycine for *C. difficile*.

Despite the fact that glycine reduction supports the generation of ATP via substrate-level phosphorylation, we observed no change in the growth of the *grdA::ermB* mutant compared to JIR8094 in rich media (Fig. 8H and 9H), an observation consistent with another report (29). While appearing to have no obvious phenotype in those media, this strain exhibited a highly distorted growth profile in CDMM compared to wild-type (Fig. 10H), in that there was no rapid loss in turbidity immediately after achieving maximum culture density (which decreased substantially compared to wild-type). It is interesting that the loss of Grd caused a growth defect compared to
Prd⁻ strains which still grew as well as wild-type (Fig. 10E, 10G, and 10H), indicating that Grd plays an important physiological role in this medium that is not readily compensated by another process in *C. difficile*. Although, while the significance behind its role as a substrate for Grd is still unknown, glycine has been well established to be a known co-germinant for *C. difficile* spores (11). Recently, Leslie et al. (169) demonstrated that gnotobiotic mice pre-colonized with non-lethal strain 630 were protected by lethal strain VPI 10463 in that the former apparently prevented germination of the latter by depleting glycine, though the study did not examine whether this was due to a Grd-dependent mechanism. Given the fact that a recent report suggests the importance of glycine reduction in establishing colonization resistance (27), an interesting link between Grd and spore biology may likely exist in CDI. McAllister et al. (49) reported that, while the presence of SelD apparently plays no role in spore germination, it does specifically affect sporulation and outgrowth from germinated spores. If Grd is one of the selenoenzymes responsible for those phenotypes, the ATP and NAD⁺ yield from glycine reduction would likely be more relevant during spore outgrowth as this process relates to vegetative cell development. Indeed, since Grd⁻ cells exhibited a growth defect in CDMM (Fig. 10H), it would be interesting to evaluate the outgrowth kinetics of the *grdA::ermB* mutant to determine if glycine, in addition to acting as a co-germinant, plays a larger role in spore biology than originally assumed. Investigating the potential relationship between Grd and spore-related processes may reveal critical mechanisms that could be exploited by therapeutics.

Our growth experiments using ⁷⁵Se revealed vast differences in the levels of labeled GrdA and GrdB (Fig. 7). We found this puzzling considering that *grdA* and *grdB* are adjacent to each other in the *grd* operon and are likely under the control of the same unknown promoter. This discrepancy could be explained by a difference in the molar ratio of GrdA to GrdB in the Grd
complex; however, as there no reports that describe the biochemical characterization or modeling of Grd, the true stoichiometry of the native complex is unknown. Unfortunately, interpretation of the $^{75}\text{Se}$ profile of GrdA is additionally confounded by what appears to be a similarly-sized band that migrated slightly higher than GrdA—this band is most obvious within the range of 6 h to 12 h in BHIS (Fig. 7A and 7B). We are currently unaware if GrdA undergoes a chemical modification that slightly changes its molecular weight or if a different selenoprotein of similar size to GrdA (~16.8 kDa) appears only during early growth.

The varying physiological effects of hydroxyproline in different strains

The addition of hydroxyproline to rich media increased the growth yield of *C. difficile* even in the absence of Prd (Fig. 8, 9, and Appendix E: Fig. 24). This result was surprising as we initially assumed that hydroxyproline would primarily fuel Prd through full conversion to proline via HypD and P5CR; furthermore, it has recently been demonstrated that hydroxyproline-dependent growth yield enhancement requires HypD (170). Given that hydroxyproline is not a true substrate for Prd (28), its conversion to proline would mainly fuel non-Stickland processes in Prd– strains; however, it is interesting to note that P5CR also regenerates NAD$^+$ in the process of generating proline (147), allowing Prd– strains to fulfill the bioenergetic requirement with hydroxyproline using a Stickland-independent mechanism. Thus, in an infection, *C. difficile* potentially benefits from scavenging hydroxyproline from collagen (in which it is a major constituent), by providing itself with two unique opportunities to dispose of electrons: (i) conversion to proline in a Stickland-independent manner using P5CR, and (ii) Stickland-dependent reduction of converted proline by Prd.

Our experiments in CDMM curiously revealed that the effect of hydroxyproline varied greatly between strains. In the case of R20291 and related mutants, the growth yields in regular
proline-containing CDMM and proline-deficient CDMM supplemented with hydroxyproline were approximately similar (Fig. 10A, 10B, and 10C). On the other hand, JIR8094 and several of its mutants grew very strangely and at substantially lower yields in comparison (Fig. 10D, 10E, 10F, and 10H), implying that hydroxyproline poorly compensates for the absence of proline in this strain. A recent report on the role of HypD in CDI likely sheds some light on this phenomenon as it was demonstrated that a ΔhypD mutant exhibited a significant growth defect in CDMM when proline was replaced with hydroxyproline (compared to its parent strain 630Δerm which grew better in the same medium) (170). Moreover, Reed et al. (170) found that hypD expression varied in several strains of C. difficile; for example, as determined by qRT-PCR, the expression of hypD (reported as relative copy number) increased in R20291 and VPI 10463 in the presence of hydroxyproline (between 1 and 10 copies), whereas 630 did not exhibit any change in its minimally-expressed hypD in comparison (between 0.1 and 1 copy). Based on these observations, there may be a rational explanation for why R20291 and JIR8094 respond differently to hydroxyproline. On one hand, R20291 probably expresses enough HypD to facilitate a 1:1 conversion of hydroxyproline to proline, allowing for optimal growth. On the other hand, one might assume that JIR8094 barely expresses HypD even in the presence of hydroxyproline (similar to its parent strain 630), likely resulting in inefficient conversion and, ultimately, substandard growth. In the context of an infection, these results suggest that not all strains have the same capacity to benefit from hydroxyproline released from degraded collagen.

Another element to consider is the fact that the prdR::ermB mutant grew better in hydroxyproline-containing CDMM compared to JIR8094; in fact, the mutant growth curves in this medium and the proline-containing control were almost identical, as if disrupting prdR somehow alleviated the growth defect (Fig. 10G). Since this effect was not observed in the prdB::ermB
mutant, it may be that PrdR performs another function unrelated to the expression of prd. The binding capability of PrdR has not been characterized, but it is assumed that it binds proline; therefore, if PrdR also had an affinity for hydroxyproline based on its structural similarity to proline, one could assume that the transcriptional regulator functions to “sense” the levels of hydroxyproline in the cell for an unknown process. Whether or not PrdR directly regulates other genes besides the prd operon is unknown, but an investigation of the potential effects of PrdR on the hydroxyproline utilization genes (e.g., hypD) may yield interesting results.

**Toxin production and the impact of selenoproteins on its regulation**

We found that the addition of proline and hydroxyproline to BHIS caused a reduction in the amount of TcdA, but this effect was abolished in a ΔselD mutant (Fig. 11B and 11C). These results suggest that toxin production is not affected by the presence of these amino acids but, rather, by their involvement in pathways regulated by selenoproteins. Since Prd is a selenoenzyme, it is tempting to think that proline reduction creates a condition that results in the downregulation of toxins. In support of this, tcdA expression was shown to decrease in JIR8094 when grown in proline-supplemented TY; moreover, the prdR::ermB mutant expressed higher levels of tcdA regardless of whether proline was present (29). Additionally, this same mutant was more virulent than JIR8094 in a hamster model (162). On the basis of these observations, Bouillaut et al. (162) suggested that PrdR indirectly influences Rex to downregulate toxin production; specifically, Prd would lower the NADH/NAD⁺ ratio, thereby causing Rex to repress the pathway involved in the synthesis of butyrate, a toxin-inducing molecule (158). Indeed, a rex::ermB mutant had a higher abundance of tcdA transcript compared to JIR8094 (162). While these results indicate a role for Prd in toxin suppression, the prdB::ermB mutant paradoxically harbored lower levels of tcdA transcript in TY regardless of proline addition; moreover, the authors found no significant
difference in the relative abundance of \textit{tcdA} mRNA from this mutant cultured in TY and JIR8094 cultured in proline-supplemented TY (29). Furthermore, in mice, the \textit{prdB::ermB} strain made considerably less TcdB compared to JIR8094 (159), while the \textit{rex::ermB} strain was actually less virulent in hamsters than JIR8094 (162). To help make sense of these issues, the authors proposed that PrdR likely regulates toxin production via a mechanism independent of Prd and Rex (162). Given the fact that PrdR is an enhancer-binding protein for the alternative sigma factor $\sigma^{54}$, an additional layer of regulation may be influencing toxin production. In a recent investigation of SigL ($\sigma^{54}$) in \textit{C. difficile}, Clark et al. (171) reported pleiotropic effects on toxin production and other processes in two \textit{sigL::erm} mutants from strains of two different ribotypes. Since a SigL-dependent promoter does indeed exist within the \textit{prd} operon, it would be interesting to understand how PrdR and SigL specifically influence this operon in different strains, which may give us insight into the mechanism behind proline-mediated suppression of toxin production.

Our experiments to determine the effects of amino acids on toxin production were performed mainly with R20291 and its related mutants (Fig. 10B and 10C). Evaluating the amount of TcdA secreted from the \textit{prdB::ermB} and \textit{prdR::ermB} strains in the presence and absence of proline and hydroxyproline may help determine the nature of the toxin phenotype in the $\Delta selD$ mutant and clarify any strain-dependent discrepancies and issues mentioned in the previous section; however, these strains did not produce enough TcdA to be detected even up to 72 h, regardless of the medium. This is likely due to the fact that JIR8094 has been reported as a poor producer of toxins (172, 173). Moreover, since our experiments with R20291 were performed in BHIS, it is possible that the toxin profile differs based on the medium. Unfortunately, we were unable to obtain reproducible results in similar experiments using TY. Finally, because the \textit{prdR::ermB} and \textit{rex::ermB} strains were reported to express higher levels of \textit{tcdA} transcript in
CDMM compared to JIR8094 (162), we attempted to evaluate TcdA levels in CDMM with our method. However, in this medium, none of our strains produced enough TcdA to be detected even at 72 h.

Due to the recent discovery that *C. difficile* can degrade collagen for nutrients in an infection (39), our findings potentially describe an intriguing mechanism for nutrient scavenging. According to our results, the release of proline and hydroxyproline from collagen would simultaneously boost the growth of *C. difficile* and suppress toxin production. While this downregulation is puzzling considering that toxin-mediated inflammation is required for collagen degradation, uncontrolled toxin expression would likely cause the death of the host, essentially depriving the pathogen of a vital source of amino acids. We propose that the metabolism of collagen-derived proline and hydroxyproline signals a “fed state” in *C. difficile*, which responds by downregulating toxin production in order to avoid killing the host, thereby maintaining its niche. Overall, our findings describe a CDI model in which the organism makes key decisions in several important scenarios depending on the presence or absence of specific nutrients (Fig. 13). As this model is based on the limited information regarding the pathogen’s electron acceptor preferences, investigations focused on uncovering the specific order of these electron acceptors in the hierarchy will prove beneficial. Understanding the nature of this hierarchy will likely paint a clearer picture of *C. difficile* pathogenesis and the organism’s ability to thrive in infected patients.
Figure 13: A model depicting *C. difficile* infection as a series of decisions based on nutrient environment.

Newly ingested *C. difficile* spores travel to the small intestine and will germinate into vegetative cells if taurocholate and glycine are present; depletion of either nutrient would result in no spore germination. Early colonization of vegetative *C. difficile* requires proline, likely due to a forced “addiction” imposed by the presence of Prd. If proline is absent, the pathogen may rely on alternative NAD$^+$-regenerating pathways mediated by Rex. In this scenario, *C. difficile* scans the environment for other electron acceptors. If leucine or glycine is present, reductive Stickland metabolism occurs. If Stickland acceptors are absent, *C. difficile* may resort to utilizing non-Stickland bioenergetic schemes such as the Wood-Ljungdahl pathway coupled with butyrate fermentation. On the other hand, the presence of proline likely promotes effective colonization and growth of vegetative cells. In order to maintain the niche and thrive in the host, the pathogen will produce toxins to obtain host-derived nutrients (e.g., Stickland acceptors from collagen). Although not fully understood, the lifestyle of *C. difficile* is ultimately defined by a delicate balance of controlled growth, toxin production, and sporulation. Box colors indicate the following: optimal infection scenarios (green), suboptimal infection scenarios (yellow), and a failure to colonize (red).
Materials and Methods

Bacterial strains and culture media

All *C. difficile* strains used in this study are listed in Table 3. Experiments were routinely performed under an atmosphere of ~1% H₂, 5% CO₂, and >90% N₂ using a Coy anaerobic chamber. H₂ was maintained within a difference of 0.2%, as measured by a Coy anaerobic monitor (CAM-12). For physiological studies in rich media, strains were grown in either BHIS (37 g/L brain-heart infusion [Oxoid], 5 g/L yeast extract, 0.1% L-cysteine) or TY (30 g/L tryptone, 20 g/L yeast extract, 0.1% mercaptoacetic acid) (89, 149). For physiological studies in a defined minimal medium, strains were grown in CDMM (133). The amino acids (mg/L) in CDMM were as follows: L-tryptophan (100), L-valine (100), L-isoleucine (100), L-leucine (1,000), L-cysteine hydrochloride monohydrate (500), L-proline (800), L-arginine hydrochloride (100), L-histidine monohydrochloride monohydrate (100), L-methionine (100), glycine (100), and L-threonine (100). When indicated, L-proline and glycine were omitted while L-4-hydroxyproline was substituted for L-proline at equimolar concentration. The vitamins (mg/L) in CDMM were as follows: calcium-D-pantothenate (1), pyridoxine (0.1), and biotin (0.01). Glucose was present in CDMM at 0.2%. The following salts and metals (mg/L) in CDMM were as follows: potassium phosphate monobasic (300), sodium phosphate dibasic (1,500), sodium chloride (900), calcium chloride dihydrate (26), magnesium chloride hexahydrate (20), manganese chloride tetrahydrate (10), ammonium sulfate (40), ferrous sulfate heptahydrate (4), cobalt chloride hexahydrate (1), and sodium bicarbonate (5,000). Additionally, the recipe was modified to include nickel chloride hexahydrate (1 mg/L), sodium selenite (1 µM), sodium molybdate dihydrate (1 µM), and sodium tungstate dihydrate (1 µM).
Growth studies and analysis

Briefly, single colonies of each strain were used to inoculate 5-mL broths (BHIS, TY, or CDMM) and were expanded overnight at 37 °C. Overnight cultures were then diluted 100-fold the following day in their respective media and transferred to 96-well plates in 200-µL volumes in triplicate. Diluted cultures were grown at 37 °C in a BioTek Epoch 2 Microplate Spectrophotometer, and the OD$_{600}$ of each culture was automatically recorded every 30 min over a 48-h period. Before every read, cultures were resuspended for 5 sec using the double orbital function on the fast speed.

Radiolabeling studies with $^{75}$Se

Overnight cultures were diluted 100-fold in their respective media, each containing 2 µCi $^{75}$Se per mL medium along with “cold” sodium selenite at 50 nM. Cultures were then grown at 37 °C for 72 h. At several time points, culture aliquots (2 mL) were harvested at 12,000 × g (BHIS, TY) or 16,000 × g (CDMM) for 5 min. After discarding supernatants, cell pellets were frozen at −80 °C until ready for use. Cell pellets were resuspended in 50 µL cold lysis buffer (50 mM Tris-HCl, 0.5 mM EDTA, 0.1 mM benzamidine, [pH 8.0]) and homogenized with a sonic dismembrator (Fisher Scientific, Model 1000). Lysates were clarified at 16,100 × g for 5 min and subsequently loaded onto Tris-glycine gels (15% acrylamide) to achieve either 7.5 µg (BHIS), 2.5 µg (TY), or 1.0 µg (CDMM) soluble cellular protein each lane, as estimated by Bradford assays calibrated with bovine serum albumin (BSA) (91). Proteins were resolved at 200 V for 50 min and stained with GelCode Blue for 1–2 h. After overnight destaining with dH$_2$O, gels were soaked with a drying solution (30% methanol, 5% glycerol) for 15 min and then dried overnight. Dry gels were exposed to a phosphor screen for at least 48 h and subsequently analyzed using a Personal Molecular Imager (Bio-Rad).
Analysis of TcdA production

Toxin production of 48-h cultures derived from each growth study were analyzed by TcdA immunoblot. Briefly, total protein was estimated with the Bradford assay as described above. Triplicate cultures were harvested at 2,500 × g and 4 °C for 20 min (Hermle Z400K, Labnet). Cell-free supernatants were collected and frozen at −20 °C until day of use. Thawed supernatants were mixed with equal volumes of 2× Laemmli buffer (1:1) and subsequently incubated in a 100 °C sand bath for 5 min. Denatured samples (20 µL) were loaded onto Tris-glycine gels (7.5% acrylamide) for SDS-PAGE and resolved for 1 h at 200 V. Transfer of electrophoresed samples to polyvinylidene difluoride membranes was performed at 4 °C overnight at 30 V. Membranes were incubated in a blocking buffer (20 mM Tris-HCl, 150 mM NaCl, 0.01 mM EDTA, 0.1% Tween 20, 1% BSA, [pH 7.5]) for 1 h at room temperature. Detection of TcdA was performed using a monoclonal mouse anti-TcdA antibody (PCG4.1, Novus Biologicals) and a rabbit anti-mouse IgG antibody conjugated with alkaline phosphatase. Blots were visualized with a ChemiDoc XRS+ imaging system (Bio-Rad).

Semi-quantitative analysis of TcdA was performed using Image Lab 6.0 software (Bio-Rad). Briefly, after subtracting background noise, peaks corresponding to the target band were selected with the software’s Lane Profile tool, in which the peak density (arbitrary units) was normalized to the amount of total protein measured from each sample. Normalized TcdA values derived from identical triplicate cultures were averaged together and subsequently converted to percentages using the Normalize tool in GraphPad Prism 8. The mean TcdA value from R20291 in BHIS was set as 100% while 0 was set as 0%.
CHAPTER FIVE:

CLOSTRIDOIDES DIFFICILE EXPLOITS XANTHINE AND URIC ACID AS NUTRIENTS BY UTILIZING A SELENIUM-DEPENDENT CATABOLIC PATHWAY

Preamble

While some selenocysteine-containing selenoproteins have defined roles in C. difficile physiology (e.g., D-proline reductase and glycine reductase), there are other lesser-known pathways of selenium metabolism that have not been elucidated. The least understood biological utilization of selenium is the insertion of a selenium cofactor in the active sites of certain molybdoenzymes (174), which are involved in purine catabolism. Interestingly, recent work has introduced the idea of host-derived purines as potential nutrients that can be scavenged by C. difficile during infection (26). Whether or not this process is catalyzed by selenoenzymes is still unknown. In this work, we attempted to genetically characterize the selenium cofactor trait by examining the selenium-dependent effects of purines on the growth of C. difficile. This dissertation chapter documents the results of that study and are presented below.

Introduction

Selenium is an important trace element generally involved in catalyzing redox reactions in all domains of life. The biological utilization of selenium is achieved by its specific incorporation into molecules with unique chemical properties (Fig. 14). The best characterized use of selenium is the biosynthesis of the 21st amino acid selenocysteine and its subsequent incorporation into special oxidoreductases known as selenoproteins, which are generally involved in redox
homeostasis and energy metabolism (105). First characterized in *Salmonella typhimurium* and *Escherichia coli* (175-178), bacterial selenoprotein synthesis is a co-translational process that requires the combined actions of the selenocysteine synthase SelA (selA), the selenocysteine-specific elongation factor SelB (selB), the selenocysteine-specific tRNA^{Sec} (selC), and the *cis*-acting selenocysteine insertion sequence (SECIS) element (50, 51, 176). Besides selenoproteins, selenium is also incorporated into nucleic acids and small molecules through lesser-known pathways mainly illustrated in bacteria to date. Regarding nucleic acids, the selenouridine synthase SelU (selU) is required to replace 2-thiouridine with 2-selenouridine in the wobble positions of bacterial and archaeal tRNAs (43, 46, 179). In terms of small molecules, the production of selenoneine was recently characterized *in vitro* using the purified enzymes selenoneine synthase SenA (senA) and selenosugar synthase SenB (senB) from *Variovorax paradoxus* (44), though this has not been demonstrated *in vivo*. While the role of 2-selenouridine and selenoneine in bacterial physiology is still unclear, the gene products required for each utilization trait clearly manipulate selenium in a biologically purposeful manner. Specific incorporation of selenium ultimately requires formation of the activated selenium donor selenophosphate (45, 180), which is produced from the ATP-dependent phosphorylation of selenide via the selenophosphate synthetase SelD (selD, alternatively *senC* in *V. paradoxus*) (47). Indeed, selenophosphate plays a central role in the biological utilization of selenium as it is ultimately essential for the production of selenocysteine (175-178), selenouridine (43, 175-178), and selenoneine (44).
The biological insertion of selenium requires the ATP-dependent formation of selenophosphate (SePO$_3^-$) which is an activated selenium donor necessary for the biosynthesis of selenocysteine (pathway #1) (175-178), selenouridine (pathway #2) (43, 175-178), and selenoneine (pathway #4) (44). Of all four pathways, the insertion of selenium as a labile cofactor into SDMH enzymes (pathway #3) is the least understood. While not experimentally proven, the $smhA$ and $smhB$ genes are assumed to be necessary for this selenium utilization trait (181, 182). For each pathway, the genes required for selenium insertion and the organism in which the system was characterized are indicated above and below each arrow, respectively. Adapted from (174).
In addition to the above utilization traits, selenium is uniquely found as a labile cofactor in the active sites of some bacterial molybdoenzymes, specifically xanthine dehydrogenase (XDH) (53, 54), purine hydroxylase (PH) (54, 55), and nicotinic acid hydroxylase (52, 56). Originally purified and characterized from *Gottschalkia acidurici* (formerly *Clostridium*) (183, 184), *Clostridium cylindrosorum* (183, 185), *Eubacterium barkeri* (formerly *Clostridium*) (53, 186, 187), and *Gottschalkia purinilytica* (formerly *Clostridium purinolyticum*) (54, 55), these proteins belong to a class of enzymes known as selenium-dependent molybdenum hydroxylases (SDMHs), which are involved in the catabolism of nicotinic acid and purines (e.g., hypoxanthine, xanthine, and uric acid) for carbon and nitrogen (186, 188, 189). Molybdenum hydroxylases, such as the bovine xanthine oxidoreductase (XOR), catalyze the hydroxylation of carbon substrates (e.g., purines and nicotinate) using water as the hydroxyl oxygen donor (57). Generally, molybdenum hydroxylases contain multiple redox centers including a molybdenum center, flavin adenine dinucleotide (FAD), and iron-sulfur (FeS) clusters (57, 190). In most XORs, the molybdenum center typically coordinates a terminal sulfur atom which is essential for catalytic activity but also extremely labile (191). Notably, cyanide inactivates the enzyme through forced release of the sulfur (known as the “cyanolyzable sulfur”) from the active site as thiocyanate; moreover, reconstitution of enzymatic activity can be achieved by incubation of the cyanolyzed enzyme with excess sulfide (191, 192). Analogous to the cyanolyzable sulfur, the labile selenium cofactor in SDMHs is also required for catalysis (53, 54, 193), though it is not entirely clear why the clostridial enzymes utilize selenium in place of sulfur. Based on the significant difference in turnover numbers between sulfur-dependent enzymes (e.g., bovine XOR: 15 s\(^{-1}\)) (194) and selenium-dependent enzymes (e.g., *G. purinilytica* PH: 412 s\(^{-1}\)) (55), it is thought that selenium offers a superior catalytic advantage likely exploited by the purinolytic clostridia (56). Nevertheless,
despite the wealth of biochemical information on SDMHs, there are still several considerable gaps in knowledge about the nature of the labile selenium cofactor, specifically regarding the unknown mechanism of its integration into SDMHs and whether the selenium is even derived from selenophosphate.

A proper genetic model for the biological utilization of selenium is necessary to elucidate the mechanism of insertion and maturation of the labile selenium cofactor in SDMHs, though the purinolytic clostridia are poor candidates due to their limited genetics. Furthermore, while *E. coli* serves as a model organism for many biological processes, it likely does not make SDMHs and only possesses an incomplete purine degradation pathway (195, 196). An alternative organism must therefore be considered. The nosocomial pathogen *Clostridioides difficile* (formerly *Clostridium*) is the leading cause of antibiotic-associated diarrhea (5). *C. difficile* colonizes the large intestine and causes disease by the action of its exotoxins TcdA and TcdB (12). We believe that *C. difficile* is an appropriate organism to serve as a genetic model to probe this pathway because of its clear reliance on selenium (48, 49), increasing genetic tractability (197), and similarity to the purinolytic clostridia from which SDMHs were first characterized. In this study, we take the first steps towards genetic characterization of the selenium cofactor utilization trait by providing genetic evidence for a selenium-dependent purine degradation pathway in *C. difficile*. Moreover, we contribute further knowledge to the field by investigating the role of two genetic markers for the selenium cofactor trait (*yqeB* and *yqeC*) in *C. difficile* purine catabolism.
Results

*C. difficile* contains gene clusters that putatively encode molybdenum hydroxylases

To verify if *C. difficile* possesses the genetic potential to utilize the labile selenium cofactor, we first sought out putative SDMH genes in the *C. difficile* genome. Having previously shown that the EF2570 gene in *Enterococcus faecalis* V583 encodes a selenium-dependent XDH with purinolytic activity (198), we performed tblastn of EF2570 against the *C. difficile* 630 and R20291 genomes to search for open reading frames (ORFs) encoding potential SDMHs. From our BLAST analysis, we identified five loci each consisting of genes encoding putative subunits for multiple molybdenum hydroxylases (Appendix F: Fig. 25). Genes in these loci have been previously reported by our group and others via different methods of computational biology (181, 182, 199, 200). Molybdenum hydroxylases are generally considered to harbor subunits with a molybdenum cofactor, FAD, and FeS clusters (57, 190). In *E. coli*, the genes encoding these subunits are typically annotated as *xdhA*, *xdhB*, and *xdhC*, respectively (195). Since the *C. difficile* gene products have not yet been characterized, we tentatively used this *E. coli* nomenclature for convenience. All five loci contained genes predicted to encode for molybdenum cofactor-binding subunits (annotated as *xdhA1* through *xdhA5*), but not all of them contained genes encoding FAD-binding and FeS-containing subunits. In fact, only three genes encoding hypothetical FAD-binding subunits (annotated as *xdhB1* through *xdhB3*) were found to colocalize with *xdhA1*, *xdhA2*, and *xdhA4*. Similarly, three genes encoding hypothetical FeS-containing subunits (annotated as *xdhC1* through *xdhC3*) were found in our analysis, and they colocalized with *xdhA2* (and *xdhB2*), *xdhA3*, and *xdhA4* (and *xdhB3*). The gene IDs and genomic locations of these ORFs are listed in Table 8 (Appendix F). Four out of five loci (*xdhA1* through *xdhA4*) were found within close proximity to each other while the fifth locus (*xdhA5*) was isolated elsewhere on the chromosome. Additionally,
*xdhA5* is uniquely predicted to simultaneously encode both a molybdenum center and an FeS-containing subunit based on sequence identity to EF2570. The diversity of these gene clusters suggests that some molybdenum hydroxylases may exhibit unique substrate specificities depending on the presence or absence of certain redox centers (e.g., FAD and FeS). Overall, the presence of these molybdenum hydroxylase gene clusters caused us to speculate that *C. difficile* could utilize selenium to modify these putative molybdoenzymes into SDMHs.

*Hypoxanthine, xanthine, and uric acid enhance C. difficile growth in a minimal medium lacking glycine and threonine*

Based on the fact that various soil clostridia catalyze purinolytic reactions using SDMHs (189), we wondered if *C. difficile* could likewise catabolize known SDMH substrates such as hypoxanthine, xanthine, and uric acid. However, when grown in rich (BHIS) or minimal (CDMM) media separately augmented with each purine at 1 mM, *C. difficile* strains R20291 and JIR8094 exhibited no significant differences in growth pattern (Appendix F: Fig. 26). While this initially suggested that *C. difficile* does not benefit from the addition of these purines, we reasoned that significant purine-dependent changes in physiology could not be observed under these growth conditions. In the seminal report that describes the original recipe for CDMM, Karasawa et al. (201) observed that *C. difficile* VPI 10463—which was found to grow poorly in the absence of glycine and threonine—could use adenine to compensate for both amino acids. This finding inspired us to design growth experiments centered on this unique physiological phenomenon in order to study the effects of purine catabolism, so we first evaluated whether this adenine-dependent effect occurred in our strains as well. Indeed, we successfully recapitulated the finding with R20291 and JIR8094: both suffered similar growth defects in the absence of glycine and threonine while substitution of both amino acids with adenine sufficiently resulted in growth levels
identical to the CDMM controls (Fig. 15). Using this unique growth behavior as a proxy for growth on purines, we repeated the assay with hypoxanthine, xanthine, and uric acid and indeed observed similar enhanced growth with each purine in the absence of glycine and threonine (Fig. 15), suggesting that they were also compensating for the deficiency of these amino acids. Interestingly, while R20291 responded identically to every tested purine (Fig. 15A), JIR8094 did not grow as well on uric acid as compared to the other purines (Fig. 15B). Nevertheless, these results suggest that C. difficile utilizes hypoxanthine, xanthine, and uric acid as growth substrates.
Figure 15: Hypoxanthine, xanthine, and uric acid induce rapid growth of *C. difficile* in a minimal medium devoid of glycine and threonine.

*C. difficile* wild-type strains (A) R20291 and (B) JIR8094 were grown in CDMM at 37 °C for 48 h. The turbidity (OD$_{600}$) of each culture was recorded every 0.5 h over the 48-h period. When indicated, glycine and threonine were omitted (Gly$^{-}$ Thr$^{-}$) and substituted with either 1 mM adenine (A$^{+}$), hypoxanthine (HX$^{+}$), xanthine (X$^{+}$), or uric acid (UA$^{+}$). The experiment was repeated twice. Data points represent the means of triplicate cultures while error bars represent standard deviations.
Selenophosphate synthetase plays a major role in growth with xanthine and uric acid but not hypoxanthine

To determine if these purine-dependent growth patterns relied on the production of selenophosphate, we repeated the assay with selD mutant strains KNM6 (ΔselD; R20291 background) and LB-CD7 (selD::ermB; JIR8094 background). We found that both selD mutants were unable to utilize uric acid for rapid growth compared to wild-type strains independent of genetic background (Fig. 16A and 16C). Moreover, growth on xanthine was severely impaired but not completely abolished (Fig. 16A and 16C). The restored selD mutant strain KNM9 (ΔselD::selD⁺; KNM6 background) did not suffer a growth defect in the presence of xanthine and uric acid, instead exhibiting a behavior similar to the wild-type R20291 (Fig. 16B). In contrast, mutation of selD did not affect growth on hypoxanthine as all mutants grew as well as wild-type strains (Fig. 16). These results suggest that selenophosphate synthetase is absolutely required for uric acid utilization, partially required for xanthine utilization, and not required at all for hypoxanthine utilization.
Figure 16: Selenophosphate synthetase is required for rapid growth with xanthine and urate but not hypoxanthine in the absence of glycine and threonine.

*C. difficile* selD mutant strains (A) KNM6, (B) KNM9, and (C) LB-CD7 were grown in CDMM at 37 °C for 48 h. The turbidity (OD$_{600}$) of each culture was recorded every 0.5 h over the 48-h period. When indicated, glycine and threonine were omitted (Gly·Thr') and substituted with either 1 mM hypoxanthine (HX$^+$), xanthine (X$^+$), or uric acid (UA$^+$). The experiment was repeated twice. Data points represent the means of triplicate cultures while error bars represent standard deviations.
smhA and smhB are putative genetic determinants for the maturation of the labile Se cofactor

Given that SelD plays a role in enhancing growth on purines, we speculated that SDMHs may be involved in this process. While the mechanism by which the labile Se cofactor is inserted into these molybdoenzymes is unknown, our lab and another group previously identified two uncharacterized genes—yqeB and yqeC—that appear to act as markers for the SDMH trait based on their co-localization with selD and gene clusters encoding molybdenum hydroxylases in several bacterial species including \textit{E. faecalis} (181, 182). Both genes were also identified in the \textit{C. difficile} genome (181, 182), though they surprisingly did not co-localize with each other or with selD (Fig. 17). Perplexingly, yqeB (CD630_34780; CDR20291_3314) was flanked by two genes predicted to encode enzymes apparently involved in pyrimidine metabolism (\textit{upp}, uracil phosphoribosyltransferase; \textit{comEB2}, deoxycytidylate deaminase) (Fig. 17A). In a clearer association, yqeC (CD630_20710; CDR20291_1978) was located closely downstream of the \textit{xdhA1-xdhB1} gene cluster and upstream of genes encoding proteins putatively involved in molybdenum cofactor biosynthesis (\textit{mocA}, molybdopterin-guanine dinucleotide biosynthesis protein; CDR20291_1976, molybdopterin cofactor biosynthesis protein) (Fig. 17B). Because of their reported association with the SDMH utilization trait (181, 182), we hereby propose that yqeB and yqeC be renamed to \textit{smhA} and \textit{smhB} (selenium-dependent molybdenum hydroxylase), respectively.
Figure 17: *smhA* and *smhB* are located within gene clusters associated with pyrimidine and purine metabolism in *C. difficile*.

The markers for the SDMH trait (*smhA* and *smhB*) are present within the *C. difficile* genome, but they do not co-localize with each other or with *selD* as in other bacterial genomes (181, 182). (A) *smhA* is flanked by two genes putatively involved in pyrimidine metabolism. (B) *smhB* is located amongst genes associated with the biosynthesis of a molybdenum hydroxylase. Numbers on each side of the gene cluster indicate the location within the R20291 genome.
To determine the role of these two genes in purine catabolism, we used a recently published dual-plasmid CRISPR-Cas9 system to delete \textit{smhA} and \textit{smhB} from the R20291 genome \cite{202}. After conjugation of the Cas9-encoding plasmid pJB06 into R20291, this strain was then used as the recipient for subsequent conjugations of newly constructed targeting plasmids for \textit{smhA} (pMJ18) and \textit{smhB} (pMJ21). Xylose induction of CRISPR-Cas9 machinery resulted in the generation of mutant strains MAJ2 (Δ\textit{smhA}), MAJ3 (Δ\textit{smhB}), and MAJ4 (Δ\textit{smhA ΔsmhB}) as verified by colony PCR with primers flanking the mutation sites in the chromosome (Appendix F: Fig. 27). Growth of these mutants in BHIS and CDMM revealed no obvious growth phenotypes (Appendix F: Fig. 28). However, in CDMM lacking glycine and threonine, all mutants suffered the same growth defect as R20291 (Fig. 18). The addition of uric acid was unable to enhance the growth of the Δ\textit{smhA} mutant (Fig. 18A), suggesting that it could no longer utilize the purine as a growth substrate. Moreover, growth of the Δ\textit{smhA} mutant in the presence of xanthine was severely diminished, though it could still fully benefit from hypoxanthine (Fig. 18A). These Δ\textit{smhA} growth phenotypes curiously mirrored the phenotypes exhibited by the \textit{selD} mutants (Fig. 16A and 16C), indicating that both genes are equally necessary for this process. Indeed, the xanthine and uric phenotypes of the Δ\textit{smhA} mutant were fully complemented by a plasmid containing a wild-type copy of \textit{smhA} under the control of its native promoter (pMJ23) compared to the empty vector control (pHN149) (Appendix F: Fig. 29A and 29B). In contrast, the Δ\textit{smhB} mutant showed no appreciable growth change in all tested conditions (Fig. 18B), implying that the \textit{smhB} gene product does not play a necessary role in these growth conditions. In further support of this idea, the double mutant Δ\textit{smhA ΔsmhB} was identical to the Δ\textit{smhA} mutant in that it did not benefit from uric acid, barely grew better with xanthine, and fully exploited hypoxanthine (Fig. 18C). However, we surprisingly observed only partial complementation of the xanthine and uric acid phenotypes in
the ΔsmhA ΔsmhB mutant containing pMJ23 compared to empty vector control (Appendix F: Fig. 29C and 29D), hinting that smhB is still required for optimal utilization of these purines. Overall, these results heavily suggest that smhA plays a necessary role in selenium-dependent purine catabolism while the role of smhB remains uncertain.
Figure 18: The product of \textit{smhA} but not \textit{smhB} is necessary for rapid growth with uric acid or xanthine in the absence of glycine and threonine.

\textit{C. difficile} \textit{smh} mutant strains (A) MAJ2, (B) MAJ3, and (C) MAJ4 were grown in CDMM at 37 °C for 48 h. The turbidity (OD\textsubscript{600}) of each culture was recorded every 0.5 h over the 48-h period. When indicated, glycine and threonine were omitted (Gly\textsuperscript{-} Thr\textsuperscript{-}) and substituted with either 1 mM hypoxanthine (HX\textsuperscript{+}), xanthine (X\textsuperscript{+}), or uric acid (UA\textsuperscript{+}). The experiment was repeated twice. Data points represent the means of triplicate cultures while error bars represent standard deviations.
Discussion

The biological utilization of selenium is a well-established genetic system present in all domains of life. Although many pathways of selenium incorporation have been genetically characterized such as selenocysteine biosynthesis, the biological mechanism by which molybdenum hydroxylases are modified with selenium is poorly understood (Fig. 14). Despite decades of biochemical and spectroscopic research on SDMHs, the field has remained stagnant due to the fact that the selenium cofactor trait lacks genetic characterization. In this work, we lay the foundation for further characterization of this trait by providing genetic evidence of selenium-dependent purine degradation in *C. difficile*. Specifically, we observed that two *selD* mutants from different strain lineages exhibited impaired growth on xanthine and uric acid in the absence of glycine and threonine (Fig. 16A and 16C). If *C. difficile* does indeed use SDMHs to degrade these purines, our results strongly suggest that selenophosphate synthetase is required for this process.

As it is still unknown whether the labile selenium cofactor even originates from selenophosphate, our observation of SelD-dependent growth on two known SDMH substrates may help to answer this fundamental question.

In this study, we examined the role of *smhA* (*yqeB*) and *smhB* (*yqeC*) in purine-dependent growth of *C. difficile*. We found that *smhA* was required for optimal growth on xanthine and uric acid in the absence of glycine and threonine (Fig. 18A), suggesting that the *smhA* gene product is just as important as selenophosphate synthetase for this process. Comparatively, deletion of *smhB* gave no apparent phenotype (Fig. 18B) while the Δ*smhA* Δ*smhB* double mutant exhibited a phenotype that was no different from the Δ*smhA* mutant (Fig. 18C), implying that the *smhB* gene product is dispensable for this catabolic system. Given the fact that the mere co-existence of *smhA* and *smhB* appears to function as a genetic marker for the selenium cofactor trait (181, 182), we
found this apparent inequality between the two genes puzzling. Several studies, however, appear to hint at a physiological “preference” of \textit{smhA} over \textit{smhB}. For example, in multidrug-resistant \textit{E. faecalis} MMH594, EF2563 (\textit{smhA}) was one of many selenium- and molybdenum-associated genes essential for growth in Mueller-Hinton broth according to transposon insertion sequencing (203). \textit{E. faecalis} is a selenium-utilizing organism that exclusively harbors the selenium cofactor trait based on the presence and co-localization of \textit{smhA}, \textit{smhB}, and \textit{selD} (181, 182), so it is intriguing that only \textit{smhA} was deemed essential in that study (203). In addition, several groups have identified a SigL-dependent (\(\sigma^{54}\)) promoter upstream of \textit{smhA} but not \textit{smhB} in \textit{C. difficile} (171, 204). This SigL-dependent promoter is predicted to be recognized by an uncharacterized bacterial enhancer-binding protein known as DioR (204). Interestingly, the proposed regulon of DioR includes \textit{pbuX} (xanthine-specific purine permease) and \textit{pyrC} (dihydroorotase) (204), suggesting a regulatory role in \textit{C. difficile} purine and pyrimidine metabolism. While these observations seem to emphasize the cell’s preference of \textit{smhA} over \textit{smhB} in physiology, it must be noted that the \(\Delta\textit{smhA} \Delta\textit{smhB}\) double mutant was only partially complemented by a wild-type copy of \textit{smhA} (Appendix F: Fig. 29D), suggesting that \textit{smhB} may still be required for full utilization of xanthine and uric acid. Further biochemical studies are needed to identify the function of each gene product and clarify these observations. Unfortunately, because SmhA and SmhB are hypothetical proteins with no known predicted domains, it is difficult to envision a clear mechanism of how they contribute to the maturation of the selenium cofactor. However, we tentatively observe that \textit{C. difficile} SmhA is 29.3\% identical to an acetyl-CoA carboxylase biotin carboxyl carrier protein encoded by a gene annotated as \textit{accB} in the genome. In \textit{E. coli}, AccB binds biotin and presents it as a substrate for carboxylation by the acetyl-CoA carboxylase (205). If the identical region in SmhA also functions as a binding domain, it may be that this protein aids in SDMH modification by binding some to-
be-determined substrate (e.g., the molybdenum cofactor or a selenium intermediate). Further biochemical studies centered on this hypothesis may help elucidate the mechanism of selenium cofactor integration.

During our investigation, we observed impaired growth of selD and smhA mutants on xanthine and uric acid but not on hypoxanthine (Fig. 16 and 18), implying the presence of selenium-dependent and selenium-independent catabolic enzymes with varying substrate specificities. While hypoxanthine is a preferred substrate of selenium-dependent PH in *G. purinilytica* (206), *C. difficile* may instead oxidize hypoxanthine using a sulfur-dependent molybdenum hydroxylase or a completely different pathway. Additionally, we observed that *C. difficile* selD and smhA mutants completely lost the growth-enhancing effect from uric acid but still partially benefited from xanthine (Fig. 16 and 18). We speculate that uric acid decomposition may require initial catalysis by an SDMH while xanthine may function as a substrate for both sulfur-dependent and selenium-dependent enzymes. It must also be noted that the clostridial XDHs are reversible SDMHs that can oxidize xanthine to uric acid but also reduce urate back to xanthine (53, 185, 207). Therefore, if *C. difficile* encodes an SDMH with XDH activity that targets both substrates, an alternate hypothesis would be that urate reduction is strictly a selenium-dependent reaction while xanthine oxidation can still proceed independently of selenium albeit at a lower rate of activity. Overall, we believe these purine-dependent growth phenotypes may arise from varying substrate specificities exhibited by different molybdenum hydroxylases. The diversity of FAD-binding and FeS-containing subunits in each molybdenum hydroxylase gene cluster may produce enzymes each with unique redox chemistry (Appendix F: Fig. 25), which certainly supports this hypothesis. Biochemical characterization of each putative molybdenum hydroxylase will aid in mapping out the metabolic pathways of purine catabolism in *C. difficile*.
While purine catabolism has been thoroughly studied across various clostridia and enterobacteria (188, 208), the role of purines as a nutrient source for *C. difficile* during infection has not been defined. Purines are likely to be relevant nutrients for *C. difficile* during gut colonization according to several observations. First, it is well known that the gut functions as a reservoir for uric acid considering that approximately one-third of the uric acid produced in the body is eliminated via the gastrointestinal tract (209). Second, a recent study demonstrated that hypoxanthine and xanthine are gut microbiota-derived products that are present in the intestinal lumen (210). Lastly, Girinathan et al. (26) found that various cecal nutrients including hypoxanthine were significantly enriched in gnotobiotic mice co-colonized with *C. difficile* and either *Clostridium sardiniense* or *Paraclostridium bifermentans*. In that same study, genes for xanthine metabolism and transport were found to be upregulated in *C. difficile* (26). These observations strongly reinforce the idea of host-derived purines as available nutrients for *C. difficile* during infection. Interestingly, for the purinolytic clostridia, purine degradation seems to begin at the point of xanthine, resulting in the eventual breakdown to ammonia, acetate, carbon dioxide, and formate (189). Supposing a similar biochemical scheme in *C. difficile*, xanthine could potentially be generated from hypoxanthine oxidation and urate reduction via molybdenum hydroxylases, though further experiments are needed to thoroughly characterize this pathway. In support of our findings describing enhanced growth on uric acid (Fig. 15), evidence of this purine’s decomposition by *C. difficile* was recently reported by two groups attempting to identify and characterize the human gut commensals responsible for anaerobic uric acid degradation (211, 212). In the study by Kasahara et al. (211), a panel of purine-degrading bacteria enriched from human feces contained a *C. difficile* isolate (CD196) that grew on agar overlaid with saturating uric acid. Likewise, in a different panel of human gut bacteria studied by Liu et al. (212), three *C. difficile*
strains (ATCC BAA-1801, M68, and 630) were able to sufficiently deplete uric acid from a carbohydrate-limited chopped meat medium; curiously, xanthine accumulated in the culture supernatants during uric acid consumption. While it is clear from these studies that *C. difficile* catabolizes uric acid, it is still unclear whether SDMHs participate in this process. If *in vivo* decomposition of uric acid proceeds via a selenium-dependent manner as inferred by our *in vitro* data (Fig. 16), it would serve as compelling evidence for the selenium cofactor trait serving a role in *C. difficile* infection. In summary, our findings provide a basis for further study of purine catabolism in *C. difficile* and may help delineate new selenium-dependent and selenium-independent mechanisms for scavenging purines.

**Materials and Methods**

*Bacterial strains, culture media, and growth conditions*

Bacterial strains are listed in Table 4. *C. difficile* strains were routinely cultured in an anaerobic atmosphere (~1.0% H₂, 5% CO₂, ~94% N₂) generated by a Coy anaerobic chamber. Hydrogen levels were maintained within a range of ± 0.2% based on continuous detection by a Coy anaerobic monitor (CAM-12). *C. difficile* strains were grown in 37 g/L brain heart infusion (BHI) supplemented (BHIS) with 5 g/L yeast extract and 0.1% L-cysteine (89). When necessary, the following antibiotics were supplemented to BHIS: thiamphenicol (10 µg/mL), lincomycin (20 µg/mL), kanamycin (50 µg/mL), or D-cycloserine (250 µg/mL). *E. coli* strains were cultured in lysogeny broth (LB) containing 10 g/L tryptone, 5 g/L yeast extract, and 5 g/L sodium chloride (213). When necessary, the following antibiotics were supplemented to LB: ampicillin (100 µg/mL), chloramphenicol (25 µg/mL), or erythromycin (200 µg/mL). For physiological studies, *C. difficile* strains were grown in CDMM (201), which was prepared in a manner described.
previously (107). When indicated, glycine and threonine were omitted from CDMM preparations and substituted with either adenine, hypoxanthine, xanthine, or uric acid at 1 mM from autoclaved 20 mM stock solutions. To make these solutions, solid purines were suspended in hot deionized water (containing phenol red to monitor pH) and eventually dissolved by slow addition of sodium hydroxide from a 1 M solution. For *smhA* complementation tests, thiamphenicol was included in all growth media to maintain complementation plasmids.
Table 4. Bacterial strains used in Chapter Five.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Relevant genotype or description</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| NEB 5-alpha       | \( fhUA2 \Delta(argF-lacZ)U169\ \text{pha}\ \text{glnV44} \)  
|                  | \( \Phi80\Delta(lacZ)M15\ \text{gyrA96}\ \text{recA1}\ \text{relA1}\ \text{endA1}\ \text{thi-1}\  
|                  | \( hsdR17\)             | New England Biolabs |
| HB101 pRK24       | \( lacYI\ \text{galK2}\ \text{xyl-6}\ \text{mtl-I}\ \text{repsL20}\ \text{pRK24 (Amp^R)}\) | (48) |
| **C. difficile**  |                                  |                  |
| R20291            | Wild type, ribotype 027         | (48) |
| KNM6              | R20291 (\( \Delta selD \)) CRISPR-Cas9 mutant | (48) |
| KNM9              | KNM6 (\( \Delta selD::selD^+ \)) CRISPR-Cas9 mutant | (49) |
| MAJ2              | R20291 (\( \Delta smhA \)) CRISPR-Cas9 mutant | This study |
| MAJ3              | R20291 (\( \Delta smhB \)) CRISPR-Cas9 mutant | This study |
| MAJ4              | MAJ2 (\( \Delta smhA\ \Delta smhB \)) CRISPR-Cas9 mutant | This study |
| JIR8094           | Wild type, ribotype 012, Erm^5 derivative of strain 630 | (29) |
| LB-CD7            | JIR8094 (\( selD::ermB \)) TargeTron mutant | (48) |
**Growth studies and analysis**

Growth studies were performed as done previously with slight modifications (107). Briefly, single colonies of *C. difficile* strains were inoculated into 5-mL BHIS broths and grown at 37 °C for 16–24 h. Overnight BHIS cultures were diluted 100-fold the following day in 5-mL CDMM broths, which were then grown at 37 °C for 16–24 h. Overnight CDMM cultures were diluted 100-fold the following day in test media which were then transferred to sterile 96-well plates in 200-µL triplicate volumes. Diluted cultures were incubated at 37 °C for 48 h in a BioTek Epoch 2 Microplate Spectrophotometer. Growth was monitored by turbidity measurements (OD$_{600}$) recorded every 0.5 h over the 48-h period. Before each OD$_{600}$ measurement, cultures were rapidly resuspended for 5 sec using the double orbital function on the fast setting.

**Plasmid construction**

All plasmids are listed in Table 5. Primers are listed in Table 9 (Appendix F). The targeting plasmids for *smhA* and *smhB* were constructed using pJB07 as template (202). In order to generate clean in-frame deletions of *smhA* and *smhB* in the R20291 chromosome (i.e., deletion of entire ORF including start and stop codons), the pre-existing *pyrE* homology arms in pJB07 were first deleted via ‘Round-the-horn site-directed mutagenesis (214) using primers pJB07 empty FWD and pJB07 empty REV, ultimately producing a linear vector lacking homology arms. The linear vector was treated with DpnI to eliminate remaining plasmid template. The *smhA* homology arms were generated by PCR amplification of R20291 genomic DNA with primers flanking the 500-bp regions directly upstream (yqeB-UA-fwd and yqeB-UA-rev) and downstream (yqeB-DA-fwd and yqeB-DA-rev) of *smhA*. Likewise, the *smhB* homology arms were PCR-amplified from the R20291 genome with primers flanking the 500-bp upstream regions (yqeC-UA-fwd and yqeC-UA-rev) and downstream regions (yqeC-DA-fwd and yqeC-DA-rev) of *smhB*. In separate three-
fragment assembly reactions for *smhA* and *smhB*, the upstream and downstream homology arms were fused together and inserted into the linear targeting vector using NEBuilder HiFi DNA Assembly (New England Biolabs) according to the manufacturer’s instructions. Assembly reactions were subsequently transformed into *E. coli* NEB 5-alpha to generate pMJ15 (for *smhA*) and pMJ19 (for *smhB*). In order to direct Cas9 to target *smhA* and *smhB*, we utilized ‘Round-the-horn site-directed mutagenesis to mutate the pre-existing pyrE guide RNA (gRNA) sequence in pMJ15 and pMJ19 to new gRNA sequences corresponding to *smhA* and *smhB*, respectively. Specifically, pMJ15 was mutated with primers yqeB gRNA 3 RTH and pJB07 gRNA rev RTH while pMJ19 was mutated with primers yqeC gRNA 2 RTH and pJB07 gRNA rev RTH. Prior to these PCRs, ‘Round-the-horn primers used for gRNA mutation were phosphorylated with T4 polynucleotide kinase (New England Biolabs) to allow for eventual ligation of PCR products with T4 DNA ligase (New England Biolabs) as done previously (215). Linear ‘Round-the-horn PCR products were treated with DpnI to digest remaining plasmid template, ligated overnight at room temperature using T4 DNA ligase, and subsequently transformed into NEB 5-alpha to yield pMJ18 (for *smhA*) and pMJ21 (for *smhB*). Plasmid constructs were confirmed via Sanger sequencing (GENEWIZ).

To generate the *smhA* complementation plasmid, the *smhA* gene under the control of its native promoter was subcloned into pHN149 (216). Briefly, the multiple cloning site of pHN149 was deleted with ‘Round-the-horn site-directed mutagenesis using primers pHN149 empty fwd and pJB07 empty REV in order to generate an empty vector. The empty ‘Round-the-horn product was treated with DpnI. PCR amplification of the *smhA* gene (plus 300 bp directly upstream and 100 bp directly downstream) from the R20291 genome was achieved using primers yqeB comp fwd and yqeB comp rev. The *smhA* fragment was subcloned into the empty vector using NEBuilder
HiFi DNA Assembly as described above. The assembly reaction was transformed into NEB 5-alpha to generate the *smhA* complementation plasmid pMJ23.
Table 5. Plasmids used in this study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant genotype or features</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJB06</td>
<td>Cas9-encoding plasmid, <em>catP</em></td>
<td>(202)</td>
</tr>
<tr>
<td>pJB07</td>
<td>Homology- and gRNA-encoding plasmid (<em>pyrE</em> homology arms, <em>pyrE</em> gRNA), <em>ermB</em></td>
<td>(202)</td>
</tr>
<tr>
<td>pMJ15</td>
<td>pJB07 (<em>smhA</em> homology arms, <em>pyrE</em> gRNA)</td>
<td>This study</td>
</tr>
<tr>
<td>pMJ18</td>
<td>pMJ15 (<em>smhA</em> homology arms, <em>smhA</em> gRNA)</td>
<td>This study</td>
</tr>
<tr>
<td>pMJ19</td>
<td>pJB07 (<em>smhB</em> homology arms, <em>pyrE</em> gRNA)</td>
<td>This study</td>
</tr>
<tr>
<td>pMJ21</td>
<td>pMJ19 (<em>smhB</em> homology arms, <em>smhB</em> gRNA)</td>
<td>This study</td>
</tr>
<tr>
<td>pHN149</td>
<td>Shuttle vector used for complementation, <em>catP</em></td>
<td>(216)</td>
</tr>
<tr>
<td>pMJ23</td>
<td>pHN149 (<em>smhA</em> complementation plasmid)</td>
<td>This study</td>
</tr>
</tbody>
</table>
**Conjugation into C. difficile**

Plasmids intended for conjugation were first transformed into *E. coli* HB101 pRK24 to be used as donors. In all conjugations, *E. coli* HB101 pRK24 harboring each plasmid was grown overnight at 37 °C in LB with ampicillin and either chloramphenicol for pJB06 or erythromycin for pMJ18 and pMJ21. For conjugation of pJB06, *C. difficile* R20291 was the recipient and was grown overnight at 37 °C in BHIS. For conjugation of *smhA* and *smhB* targeting plasmids, *C. difficile* R20291 pJB06 was the recipient and was similarly grown overnight at 37 °C in BHIS with thiamphenicol. Conjugation of CRISPR-Cas9 plasmids into *C. difficile* was performed using a filter-mating technique as described previously (217). Briefly, *E. coli* donor cells from overnight cultures were harvested (0.5 mL) at 5,000 × g for 1 min and washed with 1 mL LB to remove antibiotics. Washed donor pellets were transferred into the anaerobic chamber. *C. difficile* recipient cells were aliquoted (200 μL) into 1-mL microcentrifuge tubes, harvested at 5,000 × g for 1 min, and washed with an equal volume of BHIS if necessary to remove antibiotics. This harvest and wash step was skipped entirely if *C. difficile* was grown without antibiotics. *C. difficile* culture aliquots were incubated in a water bath at 48 °C for 5 min to heat-shock the cells (217, 218). Afterwards, heat-shocked *C. difficile* cultures were transferred back to the anaerobic chamber and were used to resuspend (100 μL) the donor pellets. Mixed cultures were then plated (100 μL) directly onto sterile 0.45-μm mixed cellulose filters (Millipore; HAWP02500) aseptically placed onto BHI agar as described previously (217). Once the 100-μL spots dried, the inoculated BHI plates were incubated at 37 °C overnight. The following day, the resulting growth was resuspended with 0.5 mL BHIS, plated directly onto BHIS agar (with selection for the *C. difficile* transconjugants and counterselection for the *E. coli* donor), and subsequently incubated at 37 °C for 24–72 h. For conjugation of pJB06 into R20291, the growth resuspension was plated onto BHIS agar with thiamphenicol, kanamycin, and D-cycloserine. For conjugation of pMJ18 and
pMJ21 into R20291 pJB06, the growth resuspension was plated onto BHIS agar with the same antibiotics plus lincomycin. In both cases, transconjugants were re-streaked onto identical antibiotic-supplemented BHIS and were subsequently tested for the presence of pJB06 and each targeting plasmid via colony PCR. pJB06 was confirmed via amplification of plasmid-born catP using primers catP pJB06 FWD and catP pJB06 REV. pMJ18 and pMJ21 were confirmed via amplification of plasmid-born ermB using primers ermB pJB07 FWD and ermB pJB07 REV. Colonies were confirmed to be *C. difficile* via amplification of genomic tcdB using primers tcdB FWD and tcdB internal rev. Confirmed transconjugants were used for downstream experimentation. For conjugation of complementation plasmids pHN149 and pMJ23, the above procedure was performed as previously stated, but the recipients were *C. difficile* MAJ2 and MAJ4. pHN149 and pMJ23 were maintained with chloramphenicol in *E. coli* and thiamphenicol in *C. difficile*. Conjugation of each plasmid was confirmed via amplification of plasmid-born catP as above using primers catP pJB06 FWD and catP pJB06 REV.

**CRISPR induction**

To induce Cas9-mediated deletion of smhA and smhB, single colonies of *C. difficile* R20291 pJB06 containing either pMJ18 or pMJ21 were streaked onto BHIS supplemented with thiamphenicol, lincomycin, and 1% xylose. Plates were incubated at 37 °C until isolated colonies arose (typically ~24–72 h). Colonies were continually passaged onto identical media up to five times until normal growth rates were restored. At this stage, colonies were screened for successful mutation using colony PCR. Deletion of smhA was confirmed using primers yqeB mutation check fwd and yqeB mutation check rev. Deletion of smhB was confirmed using primers yqeC mutation check fwd and yqeC mutation check rev. Confirmed mutant colonies were cured of CRISPR-Cas9 plasmids through inoculation of BHIS broth supplemented with 2% xylose and subsequent
overnight growth at 37 °C. A loopful of overnight culture was streaked for isolation on non-selective BHIS and grown overnight at 37 °C, and resulting colonies were pick-and-patched onto BHIS with and without thiamphenicol and lincomycin. Thiamphenicol- and lincomycin-sensitive isolates were confirmed for plasmid loss via colony PCR using primers catP pJB06 FWD, catP pJB06 REV, ermB pJB07 FWD, and ermB pJB07 REV. If plasmids were still present after overnight growth, the culture was passaged again via 100-fold dilution in BHIS broth with 2% xylose, grown overnight at 37 °C, and screened once more via the above method. All mutants were continually passaged until colonies successfully cured of both plasmids were obtained. Construction of the MAJ4 strain was performed by deleting *smhB* from an isolate of MAJ2 containing pJB06 using the same methodology. Specifically, MAJ2 pJB06 was cured of pMJ18, conjugated with pMJ21, and plated on xylose. The resulting mutation was confirmed via colony PCR, and both plasmids were cured as described above.
CHAPTER SIX: CONCLUSION

Despite the advent of modern medicine, *C. difficile* remains an important antibiotic-associated pathogen. In fact, it is clear that the expansion of this clinical bacterium is largely due to antibiotic overexposure in the 1970s (4). To help manage the disease and avoid exacerbating the issue with broad-spectrum drugs, antibiotics with activity specific to *C. difficile* (“narrow spectrum”) are preferred. This dissertation presented two studies in drug discovery to address the scarcity of CDI therapeutics.

In Chapter Two, we reported the antimicrobial activity of chemically synthesized derivatives of (+)-puupehenone against *C. difficile* (219). Moreover, many of these derivatives were found to modulate the production of toxin in diverse ways (Fig. 2). We found that derivative 20 possessed potent activity and suppressed toxin in a substantial manner (Table 1 and Fig. 2). Interestingly, Dr. Kyle Rohde’s lab (University of Central Florida) has observed similar potency of these same derivatives against *Mycobacterium tuberculosis* (61). Specifically, derivative 20—referred to as compound 14 and 15-α-acetoxypuupehenol triacetate in the publication by Si et al. (61)—had high activity against replicating and dormant *Mtb* cells while also exhibiting low toxicity against mammalian cell lines HepG2 and J774. Considering the adequate activity against both *Mtb* and *C. difficile*, one cannot help but speculate the mechanism of action. Further studies with RNA-seq technology may help point investigators towards an obvious targeted pathway. Moreover, it would be interesting to assess this compound’s activity against spores. While much more study is needed, it is clear that (+)-puupehenone and derivative 20 may be good candidates for further CDI drug development.
In Chapter Three, we evaluated the activity of auranofin against *C. difficile* mutants unable to utilize selenium (220). Auranofin’s safety profile in humans is well-documented (221), and its antimicrobial activity against *C. difficile* is significant. However, despite our initial hypothesis of auranofin’s ability to inhibit the utilization of selenium (92), it appears that this was simply a red herring since *C. difficile* selD mutants exhibit equivalent sensitivity to the drug as wild-type strains (Fig. 3 and 4). Clearly, the drug targets something else in *C. difficile*, though this is still unknown and should be investigated with gene expression experiments. Considering that auranofin’s capabilities are currently being assessed in CDI animal models (103, 104), it is imperative that researchers clear up any confusion regarding the purported mechanism of the drug. Our clarification of the previous mechanism will help investigators to ponder alternative mechanisms of action as this antirheumatic drug is being considered for repurposing.

The utilization of selenium by *C. difficile* is a fascinating biochemical system that has clear implications for physiology. Known uses of selenium include the Prd and Grd enzymes utilized in Stickland reactions while other unexplored uses of selenium include the purine-hydroxylating SDMHs. This dissertation presented two explorations of these biological uses of selenium.

In Chapter Four, we investigated the effects of collagen-derived Stickland acceptors on the growth and physiology of *C. difficile* (107). During our attempts to map out the effects of glycine, proline, and hydroxyproline on the growth of *C. difficile* (Fig. 8, 9, and 10), we surprisingly found that the inability to grow on proline (often misinterpreted as auxotrophy) was actually due to a unique “addiction” to its specific use as an electron acceptor (Fig. 10). In other words, the mere presence of Prd forces the organism to depend on proline as if it were auxotrophic. Genetic inactivation of *selD, prdB*, or *prdR* alleviated the pseudo-auxotrophic effect, resulting in strains that could grow in the absence of proline. Interestingly, Behlendorf et al. (222) recently reported
that inactivation of *prdH*—which encodes a newly identified maturation enzyme for the Prd complex—also resulted in a strain that could grow without proline, corroborating our idea of Prd-dependent “proline addiction.” Though, because the mutants still grow without their most preferred electron acceptor (as currently believed), it is clear that there are other mechanisms to manage this shift in bioenergetics. As an example, recent work by Gencic and Grahame (151) hints at a possible shift to butyrate fermentation coupled with CO$_2$ fixation via Wood-Ljungdahl. Additionally, our observation of selenophosphate-dependent toxin suppression by proline and hydroxyproline was interesting (Fig. 11), suggesting a potential collagen-dependent mechanism to gradually shut off toxin (e.g., a metabolic “fader” or “rheostat”) and thereby prevent loss of the nutrient niche due to accidental host death. While these results suggest selenoproteins are involved in modulating toxin production, it is too early to speculate since experiments were conducted in undefined media, so further investigations using minimal media may help in this regard. Overall, based on the results presented here, we developed a simplified CDI model centered on the concept of nutrient availability within the host (Fig. 13); however, three limitations of this model must be addressed. First, we recognize that taurocholate-dependent germination as a prerequisite for colonization is merely an assumption based on *in vitro* data (11). Because it has been recently shown that cholate derivatives are not required for CDI in germ-free mice (27), there is a possibility that germination in the human gut may proceed in a bile acid-independent manner as well. Second, toxin-dependent nutrient acquisition is not essential for colonization because nontoxigenic *C. difficile* strains can still colonize the gut (223). Delineating the colonization mechanisms between nontoxigenic and toxigenic *C. difficile* may be beneficial to a greater understanding of disease. Lastly, the idea that *C. difficile* can still colonize in the absence of proline by switching to alternative metabolic strategies has not been clearly demonstrated *in vivo*. Recent studies using
germ-free mice suggest that proline depletion by other competing microbes confers resistance against CDI (26, 27), implying that *C. difficile* is truly “proline-addicted” *in vivo* and might not be able to compensate with other gut nutrients. In sum, the results offer an interesting perspective into the organism’s bioenergetic preferences and how it relates to collagen as an energy depot during infection.

In Chapter Five, we began characterization of a selenium-dependent purinolytic pathway in *C. difficile*. While SDMHs have not been studied in *C. difficile*, the enzymatic substrates known as purines are likely relevant nutrients during an infection (26). Surprisingly, we observed the ability of *C. difficile* to enhance growth with hypoxanthine, xanthine, and uric acid at varying degrees in the absence of glycine and threonine (Fig. 15). Specifically, xanthine and uric acid were utilized in a selenophosphate-dependent manner (Fig. 16). Deletion of *smhA* mimicked the *selD* phenotype (Fig. 18), suggesting the presence of a selenium-dependent pathway involving *smhA*, though it is still unclear how the gene product plays a role in this phenomenon. Further biochemical studies are needed to characterize SmhA and map out this biological utilization of selenium. Regarding SDMHs, we presented evidence of gene clusters that likely encode putative molybdenum hydroxylases (Appendix F: Fig. 25), though the roles of these enzymes in *C. difficile* physiology have not yet been determined. Interestingly, Deshpande et al. (199) generated spontaneous metronidazole-resistant mutants that possessed a mutation in *xdhA5*, a putative SDMH gene identified in our study, though it is unknown why an SDMH would be involved in metronidazole’s activity against the organism. Further investigation of these unique selenoenzymes will help answer questions about the biological use of selenium and their role in *C. difficile* physiology.
In summary, this dissertation presents an extensive exploration in both avenues of applied science (CDI drug discovery) and basic science (selenium metabolism). While *C. difficile* is still a problem in the clinic, these discoveries may advance our efforts in attempting to eradicate this pathogen. Future investigations of (+)-puupehenone and auranofin may result in promising therapeutics that can help with management of the disease. Additionally, characterization of the organism’s use of selenium may help us understand the biology of the pathogen, especially regarding its use of different types of nutrients such as amino acids and purines.
APPENDIX A:

COPYRIGHT INFORMATION FOR CHAPTER TWO
APPENDIX B:  
COPYRIGHT INFORMATION FOR CHAPTER THREE
APPENDIX C:

CHAPTER THREE SUPPLEMENTAL INFORMATION
Figure 19: Fidaxomicin and vancomycin activity against R20291 and JIR8094.

*C. difficile* strains R20291 and JIR8094 were grown in BHIS broth with varying concentrations of (A) fidaxomicin and (B) vancomycin at 37 °C for 48 h. The OD₆₀₀ of each culture was recorded at 48 h. The experiment was performed twice. The vehicle control for fidaxomicin was 5% DMSO. A vehicle control was not included for vancomycin as it was dissolved in dH₂O. Data points represent the means of triplicate cultures while error bars represent standard deviations.
Figure 20: The R20291 strains are not sensitive to selenite up to 100 µM.

*C. difficile* strains (A) R20291, (B) KNM6, and (C) KNM9 were grown in BHIS broth supplemented with 0, 5, 10, 25, 50, or 100 µM sodium selenite at 37 °C for 24 h. The OD$_{600}$ of each culture was recorded at 24 h. The experiment was performed twice. Data points represent the means of triplicate cultures while error bars represent standard deviations.
Figure 21: The JIR8094 strains are not sensitive to selenite up to 100 µM.

*C. difficile* strains (A) JIR8094, (B) LB-CD7, (C) LB-CD4, (D) LB-CD8, and (E) LB-CD12 were grown in BHIS broth supplemented with 0, 5, 10, 25, 50, or 100 µM sodium selenite at 37 °C for 24 h. The OD$_{600}$ of each culture was recorded at 24 h. The experiment was performed twice. Data points represent the means of triplicate cultures while error bars represent standard deviations.
APPENDIX D:

COPYRIGHT INFORMATION FOR CHAPTER FOUR
d-Proline Reductase Underlies Proline-Dependent Growth of Clostridiodes difficile

Michael A. Johnstone, William T. Self

Department of Microbiology, University of Central Florida, Orlando, Florida, USA.

DOI: https://doi.org/10.1128/jb.00229-22

License ID: 1457105-1
117
APPENDIX E:

CHAPTER FOUR SUPPLEMENTAL INFORMATION
In the presence of 0.5 µCi $^{75}$Se per mL of culture and 50 nM sodium selenite, *C. difficile* strains R20291 (A) and JIR8094 (B) were grown in glycine-depleted CDMM at 37 °C for 72 h and harvested at the indicated time points. After harvest and lysis of cells, approximately 0.75 µg of soluble cellular protein was resolved on 15% acrylamide gels by SDS-PAGE. PrdB is indicated with an arrow based on previous literature (28, 29, 48). The asterisk (*) denotes a low-molecular-weight selenium species of unknown identity.
Figure 23: Confirmed selenoprotein profiles of R20291, KNM6, and KNM9.

Ten-milliliter cultures (BHIS, TY, and CDMM) of R20291 (WT), KNM6 (ΔselD), and KNM9 (ΔselD::selD*) were labeled with 10 µCi of $^{75}$Se. After 48 h of anaerobic growth at 37 °C, cultures were harvested with centrifugation, cells were lysed by sonication, and 7.5 µg of soluble cellular protein were resolved on a 15% acrylamide gel by SDS-PAGE. The known selenoproteins GrdB, PrdB, and GrdA are indicated with arrows based on previous literature (28, 29, 48).
Figure 24: Proline and hydroxyproline enhance maximum growth yields in rich media.

The highest OD$_{600}$ values from Figures 2 and 3 were selected and used a proxy for maximum biomass attained. Data points represent the means of triplicate cultures while error bars represent standard deviations. Statistical analysis was performed in GraphPad Prism 8 using two-way ANOVA with Dunnett’s multiple comparisons test in which all comparisons were made to base media (BHIS, TY) without amino acid supplementation. ns, not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$. 

### Table 6. Doubling times in BHIS supplemented with proline, glycine, and hydroxyproline.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Doubling time (min)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BHIS</td>
<td>+ Pro</td>
<td>+ Gly</td>
<td>+ Hyp</td>
</tr>
<tr>
<td>R20291</td>
<td>Wild type</td>
<td>45.23 ± 3.19</td>
<td>42.19 ± 1.37</td>
<td>41.58 ± 5.67</td>
<td>46.82 ± 1.15</td>
</tr>
<tr>
<td>KNM6</td>
<td>ΔselD</td>
<td>46.33 ± 0.51</td>
<td>45.61 ± 1.37</td>
<td>42.33 ± 5.00</td>
<td>47.24 ± 1.99</td>
</tr>
<tr>
<td>KNM9</td>
<td>ΔselD::selD⁺</td>
<td>43.24 ± 1.82</td>
<td>42.26 ± 0.50</td>
<td>40.07 ± 1.63</td>
<td>45.44 ± 1.21</td>
</tr>
<tr>
<td>JIR8094</td>
<td>Wild type</td>
<td>47.15 ± 0.30</td>
<td>43.91 ± 1.88</td>
<td>42.82 ± 1.52</td>
<td>42.64 ± 1.71</td>
</tr>
<tr>
<td>LB-CD7</td>
<td>selD::ermB</td>
<td>45.66 ± 1.70</td>
<td>46.45 ± 0.23</td>
<td>45.41 ± 0.68</td>
<td>48.08 ± 1.11</td>
</tr>
<tr>
<td>LB-CD4</td>
<td>prdB::ermB</td>
<td>48.01 ± 0.89</td>
<td>49.96 ± 1.90</td>
<td>48.13 ± 1.38</td>
<td>48.45 ± 0.41</td>
</tr>
<tr>
<td>LB-CD8</td>
<td>prdR::ermB</td>
<td>47.51 ± 0.87</td>
<td>46.05 ± 1.33</td>
<td>49.66 ± 1.16</td>
<td>45.86 ± 1.67</td>
</tr>
<tr>
<td>LB-CD12</td>
<td>grdA::ermB</td>
<td>44.41 ± 1.03</td>
<td>43.33 ± 0.80</td>
<td>44.71 ± 0.66</td>
<td>43.32 ± 0.80</td>
</tr>
</tbody>
</table>
Table 7. Doubling times in TY supplemented with proline, glycine, and hydroxyproline.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Doubling time (min)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TY</td>
<td>+ Pro</td>
<td>+ Gly</td>
<td>+ Hyp</td>
</tr>
<tr>
<td>R20291</td>
<td>Wild type</td>
<td>35.77 ± 1.36</td>
<td>34.23 ± 3.46</td>
<td>34.50 ± 1.87</td>
<td>35.37 ± 1.32</td>
</tr>
<tr>
<td>KNM6</td>
<td>ΔselD</td>
<td>39.47 ± 2.29</td>
<td>40.44 ± 2.78</td>
<td>37.41 ± 3.63</td>
<td>37.20 ± 2.85</td>
</tr>
<tr>
<td>KNM9</td>
<td>ΔselD::selD⁺</td>
<td>37.38 ± 3.42</td>
<td>34.72 ± 0.82</td>
<td>33.45 ± 2.00</td>
<td>41.85 ± 3.59</td>
</tr>
<tr>
<td>JIR8094</td>
<td>Wild type</td>
<td>46.26 ± 1.06</td>
<td>37.91 ± 1.92</td>
<td>38.40 ± 3.42</td>
<td>33.83 ± 3.97</td>
</tr>
<tr>
<td>LB-CD7</td>
<td>selD::ermB</td>
<td>55.94 ± 1.15</td>
<td>47.04 ± 3.71</td>
<td>47.99 ± 1.53</td>
<td>53.76 ± 10.17</td>
</tr>
<tr>
<td>LB-CD4</td>
<td>prdB::ermB</td>
<td>45.81 ± 7.16</td>
<td>38.19 ± 3.24</td>
<td>47.34 ± 1.25</td>
<td>44.62 ± 3.71</td>
</tr>
<tr>
<td>LB-CD8</td>
<td>prdR::ermB</td>
<td>56.44 ± 5.77</td>
<td>49.56 ± 3.94</td>
<td>54.59 ± 2.10</td>
<td>47.65 ± 4.96</td>
</tr>
<tr>
<td>LB-CD12</td>
<td>grdA::ermB</td>
<td>46.68 ± 0.48</td>
<td>42.23 ± 0.85</td>
<td>47.11 ± 0.31</td>
<td>45.26 ± 0.53</td>
</tr>
</tbody>
</table>
APPENDIX F:

CHAPTER FIVE SUPPLEMENTAL INFORMATION
Figure 25: *C. difficile* contains five gene clusters putatively encoding molybdenum hydroxylases.

Five loci containing genes encoding putative subunits of molybdenum hydroxylases were located in the R20291 genome via tblastn of *E. faecalis* V583 EF2570. The molybdenum center subunits (*xdhA*), FAD-binding subunits (*xdhB*), and FeS-containing subunits (*xdhC*) are annotated using the *E. coli* nomenclature (195). *xdhA5* is isolated from the other four loci and seems to encode a molybdenum hydroxylase with a FeS-containing domain directly fused to the molybdenum center. Numbers flanking each locus indicate the location in the R20291 genome.
Figure 26: Addition of hypoxanthine, xanthine, or uric acid does not affect growth of R20291 and JIR8094 in BHIS and CDMM.

*C. difficile* wild-type strains R20291 and JIR8094 were grown in either (A) BHIS or (B) CDMM at 37 °C for 48 h. The turbidity (OD$_{600}$) of each culture was recorded every 0.5 h over the 48-h period. When indicated, hypoxanthine (HX$^+$), xanthine (X$^+$), or uric acid (UA$^+$) were added to each medium at 1 mM. The experiment was repeated twice. Data points represent the means of triplicate cultures while error bars represent standard deviations.
Figure 27: In-frame deletions of *smhA* and *smhB* were successfully constructed in *C. difficile* R20291.

*smhA* and *smhB* were targeted and knocked-out using a dual-plasmid xylose-inducible CRISPR-Cas9 system (202). Genomic DNA of mutant isolates plated on xylose were used in colony PCRs with primers flanking the mutation sites. R20291 was used as a control. PCR products were visualized on 1% (w/v) agarose gels containing 0.5 μg/mL ethidium bromide. Amplification of full-length *smhA* and *smhB* generated slower-migrating PCR products while deleted genes gave faster-migrating PCR products.
Deletion of *smhA* and *smhB* does not affect growth of *C. difficile* in BHIS and CDMM.

*C. difficile* strains R20291, MAJ2, MAJ3, and MAJ4 were grown in either (A) BHIS or (B) CDMM at 37 °C for 48 h. The turbidity (OD$_{600}$) of each culture was recorded every 0.5 h over the 48-h period. The experiment was repeated twice. Data points represent the means of triplicate cultures while error bars represent standard deviations.
Figure 29: A wild-type copy of *smhA* provided *in trans* fully complements the Δ*smhA* mutant and partially complements the Δ*smhA* Δ*smhB* mutant.

*C. difficile* mutant strains (A, B) MAJ2 and (C, D) MAJ4 harboring either an empty shuttle vector (pHN149) or the vector containing a wild-type copy of *smhA* (pMJ23) were grown in CDMM augmented with 10 µg/mL thiamphenicol at 37 °C for 48 h. The turbidity (OD$_{600}$) of each culture was recorded every 0.5 h over the 48-h period. When indicated, glycine and threonine were omitted (Gly$^-$ Thr$^-$) and substituted with either 1 mM xanthine (X$^+$) or uric acid (UA$^+$). The experiment was repeated twice. Data points represent the means of triplicate cultures while error bars represent standard deviations.
Table 8. Genome IDs and locations for genes encoding putative selenium-dependent molybdenum hydroxylases in *C. difficile* 630 and R20291.

<table>
<thead>
<tr>
<th>Gene ID (630, R20291)</th>
<th>Gene name (<em>E. coli</em> designation)</th>
<th>Genome Location (630)</th>
<th>Genome Location (R20291)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD630_20730, CDR20291_1980</td>
<td><em>xdhA1</em></td>
<td>2,373,538..2,375,844</td>
<td>2,309,694..2,312,000</td>
</tr>
<tr>
<td>CD630_20790, CDR20291_1986</td>
<td><em>xdhA2</em></td>
<td>2,383,198..2,385,489</td>
<td>2,319,354..2,321,645</td>
</tr>
<tr>
<td>CD630_20870, CDR20291_1994</td>
<td><em>xdhA3</em></td>
<td>2,393,402..2,395,618</td>
<td>2,329,558..2,331,774</td>
</tr>
<tr>
<td>CD630_20990, CDR20291_2006</td>
<td><em>xdhA4</em></td>
<td>2,408,599..2,410,863</td>
<td>2,344,756..2,347,020</td>
</tr>
<tr>
<td>CD630_31770, CDR20291_3033</td>
<td><em>xdhA5</em></td>
<td>3,698,060..3,700,621</td>
<td>3,617,878..3,620,439</td>
</tr>
<tr>
<td>CD630_20740, CDR20291_1981</td>
<td><em>xdhB1</em></td>
<td>2,375,837..2,376,631</td>
<td>2,311,993..2,312,802</td>
</tr>
<tr>
<td>CD630_21010, CDR20291_2008</td>
<td><em>xdhB3</em></td>
<td>2,411,316..2,412,104</td>
<td>2,347,473..2,348,261</td>
</tr>
<tr>
<td>CD630_20810, CDR20291_1988</td>
<td><em>xdhC1</em></td>
<td>2,386,312..2,386,758</td>
<td>2,322,468..2,322,935</td>
</tr>
<tr>
<td>CD630_20880, CDR20291_1995</td>
<td><em>xdhC2</em></td>
<td>2,395,621..2,396,091</td>
<td>2,331,777..2,332,247</td>
</tr>
<tr>
<td>CD630_21000, CDR20291_2007</td>
<td><em>xdhC3</em></td>
<td>2,410,867..2,411,316</td>
<td>2,347,024..2,347,473</td>
</tr>
</tbody>
</table>
Table 9. Oligonucleotides used in this study.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Description</th>
<th>Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJB07 empty</td>
<td>To delete pyrE homology arms and generate empty vector for re-targeting</td>
<td>CTCGAGCCTGCAGACATGCA</td>
</tr>
<tr>
<td>FWD</td>
<td></td>
<td>GCGGCCGCGGGTCATAGC</td>
</tr>
<tr>
<td>REV</td>
<td></td>
<td>TTATCAGGAAACAGCTATGACCGCGGCC</td>
</tr>
<tr>
<td>yqeB-UA-fwd</td>
<td>To amplify 500 bp upstream of <em>smhA</em> for gene deletion</td>
<td>GCAATAGTTCTAAGGTCTAAAGGAGCT</td>
</tr>
<tr>
<td>yqeB-UA-rev</td>
<td></td>
<td>AAA</td>
</tr>
<tr>
<td>yqeB-DA-fwd</td>
<td>To amplify 500 bp downstream of <em>smhA</em> for gene deletion</td>
<td>GAGCTAATACCATTAGAGCGCAGTTGTAT</td>
</tr>
<tr>
<td>yqeB-DA-rev</td>
<td></td>
<td>TAT</td>
</tr>
<tr>
<td>yqeC-UA-fwd</td>
<td>To amplify 500 bp upstream of <em>smhB</em> for gene deletion</td>
<td>TTAATATATCATATAAACACTTTTTAATCTA</td>
</tr>
<tr>
<td>yqeC-UA-rev</td>
<td></td>
<td>CTTTCACCCTTTTATAACGTTTCTATA</td>
</tr>
<tr>
<td>yqeC-DA-fwd</td>
<td>To amplify 500 bp downstream of <em>smhB</em> for gene deletion</td>
<td>ATATAATATCATATAAACACTTTTTAATCTA</td>
</tr>
<tr>
<td>yqeC-DA-rev</td>
<td></td>
<td>AGTATATATATATATATATATATATATA</td>
</tr>
<tr>
<td>yqeB gRNA 3</td>
<td>To mutate pyrE gRNA to <em>smhA</em> gRNA</td>
<td>TTGGAGCCATACTAAACAAAGTTTTTGAAGCTAGAAATAGCAAGTAAAAATAAGGCTAGT</td>
</tr>
<tr>
<td>RTH</td>
<td></td>
<td>ATG</td>
</tr>
<tr>
<td>yqeC gRNA 2</td>
<td>To mutate pyrE gRNA to <em>smhB</em> gRNA</td>
<td>ATAAAACCTACAAAAAACCATAGTTTTTGAAGCTAGAAATAGCAAGTAAAAATAAGGCTAGT</td>
</tr>
<tr>
<td>RTH</td>
<td></td>
<td>AGT</td>
</tr>
<tr>
<td>pJB07 gRNA rev</td>
<td>To be paired with any ‘Round-the-horn forward primer for gRNA mutation</td>
<td>GGTACCCCCCTCCTTGAAATGCCC</td>
</tr>
<tr>
<td>RTH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>catP pJB06 FWD</td>
<td>To PCR check for presence of pJB06 in conjugation and plasmid curing experiments</td>
<td>GGGAACTTAGATGTTATTTGGAAAAAATGTATAAAATAGTTGG</td>
</tr>
<tr>
<td>catP pJB06 REV</td>
<td></td>
<td>CCGTTGAAGTTAATTTATCAATTCCTGCAATTCG</td>
</tr>
<tr>
<td>ermB pJB07 FWD</td>
<td>To PCR check for presence of targeting plasmid in conjugation and plasmid curing experiments</td>
<td>GAGTGATTACATGAACAAAAATATAAAATAGGCTATTTTACCTCAGTTAAATAAAATAAGGCTAGT</td>
</tr>
<tr>
<td>ermB pJB07 REV</td>
<td></td>
<td>AATTTGCTAAAAACCTTTTTAAC</td>
</tr>
</tbody>
</table>

131
<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Description</th>
<th>Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>tcdB FWD</td>
<td>To PCR check if selected colony in conjugation experiment is <em>C. difficile</em></td>
<td>ATGAGTTTAGTTAATAGAAAAACAGTTAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AAAAATGGCAA</td>
</tr>
<tr>
<td>tcdB internal rev</td>
<td></td>
<td>ATAATTGGAATGACTCCTCCACCTTTAAAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCTTCAAG</td>
</tr>
<tr>
<td>yqeB mutation check fwd</td>
<td></td>
<td>GGGTTTCTGCTGTAGCAGCT</td>
</tr>
<tr>
<td>yqeB mutation check rev</td>
<td></td>
<td>AGTAACATAAAAAAGTTGCCCATCTACA</td>
</tr>
<tr>
<td>yqeC mutation check fwd</td>
<td></td>
<td>TGGAGAGAAAAATATAGAAAATTTTATA</td>
</tr>
<tr>
<td>yqeC mutation check rev</td>
<td></td>
<td>AGAATCCATTTTGA</td>
</tr>
<tr>
<td>pHN149 empty fwd</td>
<td>To be paired with pJB07 empty REV to delete the multiple cloning site of pHN149 and generate an empty vector for <em>smhA</em> complementation</td>
<td>CTCGAGGCGCTGCAGACATGC</td>
</tr>
<tr>
<td>yqeB comp fwd</td>
<td>To amplify <em>smhA</em> plus 300 bp upstream and 100 bp downstream for complementation</td>
<td>ACAGCTATGACCCGCGCCGCAAATAAAA</td>
</tr>
<tr>
<td>yqeB comp rev</td>
<td></td>
<td>TAACTATAAAATTTATTTAGGGACTTCTTAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GCATGTCTGCAGGCTCGAGTTTACCATT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TAAATTTAAATACCCGTCATATG</td>
</tr>
</tbody>
</table>
LIST OF REFERENCES


136


