Clostridium difficile Transcriptomics and Metronidazole Resistance

By

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List of Abbreviations

ATTC®	American Type Culture Collection®
BAK	Brucella agar plate supplemented with haemin and vitamin K and 5% laked sheep blood
BHI	Brain heart infusion broth
BLAST	Basic Local Alignment Search Tool
BSC	Biological safety cabinet
ddH_2O	Deionized double distilled H_2O
CTX	Cefotaxime
CDI	*Clostridium difficile* infection
CDMN	*Clostridium difficile* media with moxalactam and norfloxacin
CLSI	Clinical and Laboratory Standard Institute (formerly NCCLS, National Committee of Clinical Laboratory Standards)
emPCR	Emulsion PCR
EtBr	Ethidium bromide
IN/DEL	Insertion/deletion
MET/MTZ	Metronidazole
MIC	Minimum inhibitory concentration
NAP	North American pulsefield type
NGS	Next generation sequencing
NML	National Microbiology Laboratory
PBS	Phosphate buffered saline
PFGE	Pulse field gel electrophoresis
PFO	Pyruvate-ferredoxin oxidoreductase
PHAC	Public Health Agency of Canada
PPI	Proton pump inhibitor
RNA-Seq	RNA sequencing
SNP	Single nucleotide polymorphism

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ABSTRACT

This is a two-part project. Proton pump inhibitors (PPIs) have been associated with increased risk of C. difficile infections and increased toxin production when combined with antimicrobial therapy. The first part of this project involved characterization of a hypervirulent NAP1 C. difficile strain, including genome sequencing and assembly, and the development of methods to study its transcriptomics using RNA-Seq, which will enable future researchers to study different expression patterns when toxigenic C. difficile is challenged with PPIs and/or antimicrobials in vitro.

The second part of this project involved characterizing a clinical isolate of a NAP1 C. difficile displaying a markedly elevated MIC to metronidazole (MIC = 16 mg/mL), which initially exhibited MIC of 32 mg/mL. A method of obtaining a metronidazole-susceptible revertant from this isolate was developed and a revertant was obtained. The genomes of both isolates were sequenced, assembled, and aligned, then compared to each other for polymorphisms.

1 INTRODUCTION

1.1 Overview of *Clostridium difficile*

Clostridium difficile is a Gram-positive, spore forming, anaerobic rod. It was first discovered by Hall and O'Toole in 1935 in the stool of infants [1]. It had been named *Bacillus difficilis* at first due to the difficulties in its isolation and culture. Decades later, we can now isolate and culture it from stool samples with ease, but difficulties in managing *C. difficile* infections (CDI) remain.

1.1.1 Transmission and Pathogenesis

C. difficile is transmitted fecal-orally through contaminated hands and fomites, which commonly occur within the healthcare setting among staff and patients [2,3]. Sharing a hospital room with *C. difficile*-infected patients dramatically increases the likelihood of acquiring *C. difficile*, with acquisition in 3 days as compared to 19 days if sharing a room with patients without CDI [4].

C. difficile spores are extremely resilient to degradation, and can withstand the acidic environment of the stomach and ultimately germinate into vegetative forms in the gut [2,3]. In addition, *C. difficile* spores have been shown survive up to 70 days in a hospital room after a CDI-infected patient has vacated, and it is resistant to many disinfectants [5]. *C. difficile* is also found ubiquitously in the environment, such as water (tap, river, sea), meat products, and animals; however, the implication of these findings in human CDI still require further investigation [6].

It is generally accepted that disturbance of the normally protective gut flora allows pathogenic *C. difficile* to flourish, produce exotoxins, and cause mucosal damage

and inflammation which results in symptoms [3,7]. Exotoxins A (*tcdA*) and B (*tcdB*) are the major virulence factors of pathogenic *C. difficile*; toxin-negative strains are nonpathogenic [2,8-11]. These toxins likely exert their effects through disruption of the cell cytoskeleton, resulting in mucosal damage and inflammation [2]. A third toxin, called binary toxin (*cdt*), has been found in certain strains of *C. difficile* which were isolated in CDI cases with greater disease severity [12]. However, certain strains that are toxins A and B negative but binary toxin positive have been found to be nonpathogenic, and the exact pathogenic role of binary toxin is not yet well understood [8,9,11].

1.1.2 Risk Factors

The primary risk factor for CDI is antibiotic exposure, likely due to their disruption of commensal gut flora, allowing *C. difficile* to flourish [3]. In particular, clindamycin, fluoroquinolones, broad-spectrum penicillins, and cephalosporins have all been implicated with increasing the risk of CDIs [5]. Hospitalized and elderly patients are especially at risk, since many are receiving antibiotics in an environment with *C. difficile* contamination; therefore, increased length of stay in a care facility increases chance of exposure, and is another commonly identified risk factor [2,13]. Other risk factors include immunosuppression, history of inflammatory bowel disease, recent gastrointestinal surgery, and use of gastric suppressing agents such as proton pump inhibitors and H_2-receptor antagonists [14-17].

Although antibiotic use and *C. difficile* acquisition have been both regarded as essential in the establishment of CDI, the precise order and timing of these two events are not well understood [3].

1.1.3 Clinical Manifestations

Bartlett *et al.* in 1978 identified *C. difficile* as the etiological agent responsible for pseudomembranous colitis [18], characterized by thick, yellow, inflammatory lesions on the mucosal surfaces of the colon. However, *C. difficile* infections are associated with a spectrum of symptoms, from asymptomatic carriage, to self-limited watery diarrhea, colitis and bloody diarrhea, pseudomembranous colitis, fulminant colitis, and death [14]. Onset of symptoms can occur at any time during antibiotic use, and up to eight weeks post-treatment [2]. It is important to note that mild CDI can progress to moderate or severe disease quickly, making distinction between severity difficult [14].

The most important and challenging complication of CDI is recurrent infections. Approximately 20% of patients with CDI will have at least one recurrence after discontinuation of treatment, 40% after one recurrence, and more than 60% after two or more recurrences [19-22]. Individuals who have at least one recurrence can expect 50-60% chance of additional episodes [23]. One mechanism by which recurrences occur is the use of metronidazole or vancomycin in treating CDI which destroys normal, protective flora, thus preventing resistance to re-colonization by the same strain, or re-infection by another strain [10,24-26].

Aside from recurrent infections, others complications include intestinal perforation, toxic megacolon, sepsis, and suboptimal response to drug therapy [25].

1.1.4 Diagnosis

Diagnosis of CDI is performed through careful taking of history and physical exam of the patient, including recent use of antibiotics, and confirmed by presence of *C. difficile* toxins in the stool [2]. The stool-cytotoxin assay is based on the induction of cell-

rounding by toxin B in stool filtrate and is the gold standard due to its high sensitivity and specificity [24,26]. Many laboratories today screen stools for the *C. difficile* glutamate dehydrogenase antigen [27], and if positive, a confirmatory test of enzyme immunoassay to toxins A and B is performed [28].

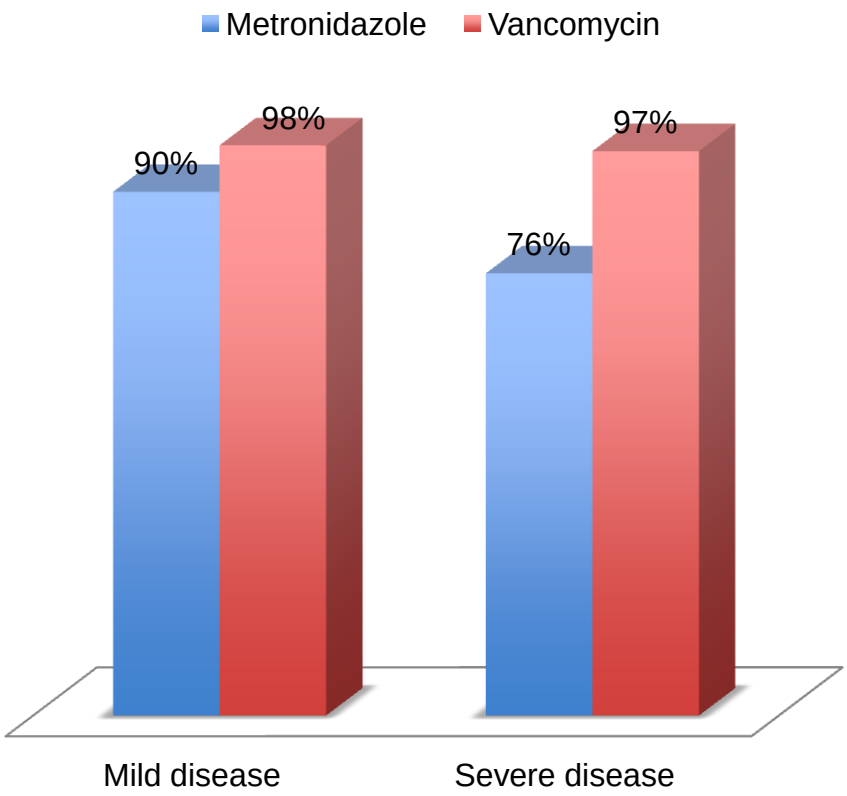
1.1.5 Treatment

The ultimate goal in the treatment of CDI is to discontinue the use of inciting antibiotic(s) and allow the normally protective flora to recover (if clinically appropriate) [5,10,21,29,30]. For severe cases, oral metronidazole and vancomycin are the treatment of choice and are given as standard therapy (alone or combined) according to the most recent guidelines [5]. Metronidazole may be preferable to vancomycin due to its lower price and the lack of selection for vancomycin-resistant enterococci [31,32]. However, since 2000, metronidazole has had increased failure rates and relapse, including the outbreak of 2003 in Quebec [21,29,30]. One recent prospective, controlled trial showed vancomycin having similar efficacy as metronidazole for mild disease, but higher efficacy than metronidazole for severe disease [20], as illustrated in Figure 1-1.

Currently, initial therapy with metronidazole is still recommended for mild to moderate disease, and vancomycin is recommended as a first-line therapy for severe disease [28]. If disease severity progresses despite treatment, additional or alternative options should be explored; for example, urgent surgical evaluation is indicated in patients with signs of toxic megacolon, perforation, or colonic wall thickening, as colectomy can be life-saving [33]. In any case, treatment should not be given for asymptomatic colonization, as no current therapy exists that eradicates colonization [14].

Unfortunately, recurrent CDI still remains a challenge to treat; no single effective therapy currently exists [28]. Pulsed or tapered dosing with vancomycin have been shown to be beneficial, which attempts to restore the protective gut flora while keeping *C. difficile* numbers low [19].

Figure 1-1. Response rates to metronidazole and vancomycin therapy according to disease severity. The difference in response rates between vancomycin and metronidazole was not significant in patients with mild infection (P=0.36), but was significant in those with severe infection (P=0.02). Data from Zar *et al*. [20] in A Comparison of Vancomycin and Metronidazole for the Treatment of *Clostridium difficile*–Associated Diarrhea, Stratified by Disease Severity. Clinical Infectious Diseases 2007;45:302–7. Reproduced with permission from Oxford University Press.



Alternative antibiotics, such as fusidic acid, nitazoxanide, teicoplanin, rifampin, bacitracin, and fidaxomicin have all been used to treat CDI. However, a 2011 Cochrane review of randomized trials of the efficacy of these antibiotics found little statistically significant difference between vancomycin and these antibiotics[1] [34].

Novel non-antibiotic therapies are emerging. A toxin binding polymer (Tolevamer) has shown promise in the treatment of mild to moderate CDI [35]. Probiotics have also been used to treat CDIs, which are intended to restore microbial balance in the gut. However, a 2008 Cochrane review found insufficient evidence to support the use of probiotics in the treatment of CDIs [36]. Fecal transplantation (fecal biotherapy) attempts to restore fecal flora by transplanting stool from a healthy, matched donor to the patient's gut. It is generally reserved for patients with multiple recurrences and refractory to other forms of therapy and has been shown to be very promising [13,37]. Other therapies such as immunoglobulins, monoclonal antibodies, and vaccination are also being studied [7].

1.1.6 Prevention

Antimicrobial stewardship is extremely important in the prevention of CDI, and should be practiced in patients of all risk groups [37,38]. Just as important, frequent hand washing can reduce the spread of *C. difficile* (alcohol-containing hand-sanitizers are ineffective against *C. difficile* spores) [3,14]. Other infection control practices, including contact precautions, use of gloves and gowns, patient isolation, and surveillance should also be utilized. In terms of environmental decontamination, chemicals such as

[1] Except for teicoplanin, which was found to be superior to vancomycin, but not as efficient due to high cost

hypochlorite and hydrogen peroxide have been shown to be effective in killing spores [39-41]. Unfortunately, Canadian health care facilities vary widely in *C. difficile* infection control practices, and opportunities still exist for better infection control and antimicrobial stewardship [5,42].

1.1.7 Epidemiology and Impact

CDIs are the most common cause of health-care-associated infectious diarrhea, affecting particularly the elderly, who have extended length of stay in hospitals and long-term care facilities [43]. Approximately 1-3% of healthy adults have been found to be asymptomatic carriers of *C. difficile*, who are potential reservoirs of the pathogen [2]. In those with recent healthcare and antibiotic exposure, 15-25% have been found to have *C. difficile* [2]. CDIs are responsible for up to 20% of antibiotic-associated diarrhea and nearly all cases of pseudomembranous colitis [5]. In addition, recent cases have been reported in nontraditional hosts, such as the otherwise healthy, non-hospitalized population, children, and those without antibiotic exposure [44,45].

Over the past decade, CDI incidence in North America increased five-fold in the community and eight-fold in the elderly; incidences in the UK, Netherlands, and France have also increased dramatically [46]. Recent surveillance data indicate that *C. difficile* has replaced methicillin-resistant *Staphylococcus aureus* as the most common nosocomial infection in certain US hospitals [47]. Equally important, the severity of CDIs has also increased [37].

This recent surge in the incidence and severity of CDIs are associated with an emerging strain of *C. difficile* first described as an isolate from a Parisian hospital in 1985 [48,49]. This strain was identified by ribotyping as ribotype 027, by pulse-field gel

electrophoresis as North American pulse-field type 1 (NAP1), by restriction-endonuclease analysis (REA) as group BI, and by toxinotyping as toxinotype III (i.e., BI/NAP1/027) [50]. BI/NAP1/027 is toxin A, B, and binary toxin positive, and has been shown to produce 16 times higher levels of toxin A and 23 times toxin B *in vitro*, in addition to producing toxins throughout its growth phases (Figure 1-2) [9]. This elevated level of toxin production is mostly likely due to a frameshift mutation in the *tcdC* gene located within the pathogenicity locus (*PaLoc*, Figure 1-3) [51], which normally suppresses toxins A and B production during early, exponential growth [3,9,10]. In addition, NAP1 may have increased sporulation rates, facilitating better survival and spread [52]. The NAP1 strain has been implicated in various outbreaks in North America and Europe [6], including the notable outbreak of 2003 in Quebec, where the incidence had quadrupled and all major hospitals in the region were simultaneously affected, accompanied by increased disease severity and mortality [53,54]. Furthermore, epidemic NAP1 strains are especially resistant to fluoroquinolones and clindamycin, which may have endowed it with a competitive advantage in a nosocomial setting [10,55-57]. Canadian data published in 2010 found that patients 60-90 years of age are twice as likely to die or experience severe outcome with a NAP1 infection compared to non-NAP1 infections [56]. In the same study, the authors found NAP1 had much higher resistance to respiratory fluoroquinolones (levofloxacin, gatifloxacin, and moxifloxacin), cefazolin, and ceftriaxone versus non-NAP1 strains. Moreover, new strains have emerged from the hypervirulent NAP1 lineage in the UK, with greater motility and antibiotic resistance than the NAP1 strain, which may be as problematic as NAP1 [48]. In the UK alone, the proportion of 027 strains rose from 25.9% to 41.3% between 2005 and 2008 [58].

The economic impact of CDI is significant, especially with the recent emergence of the NAP1/027 strain and occurrences of more severe and recurrent infections. It has been estimated that, on average, CDIs cost Canadians over $120,000 per hospital per year, in addition to suffering from the disease [55]. In the US, costs of CDI has risen from $1.1 billion per year in 2002 to over $3 billion annually in 2009 [5,59,60].

Figure 1-2. *C. difficile* NAP1/027 was demonstrated to produce 16x toxin A and 23x toxin B compared to non-NAP1 strains by Warny *et al.* [9]. Reprinted from The Lancet, Warny *et al.* in Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe, 2005; 366: 1079–84, with permission from Elsevier.

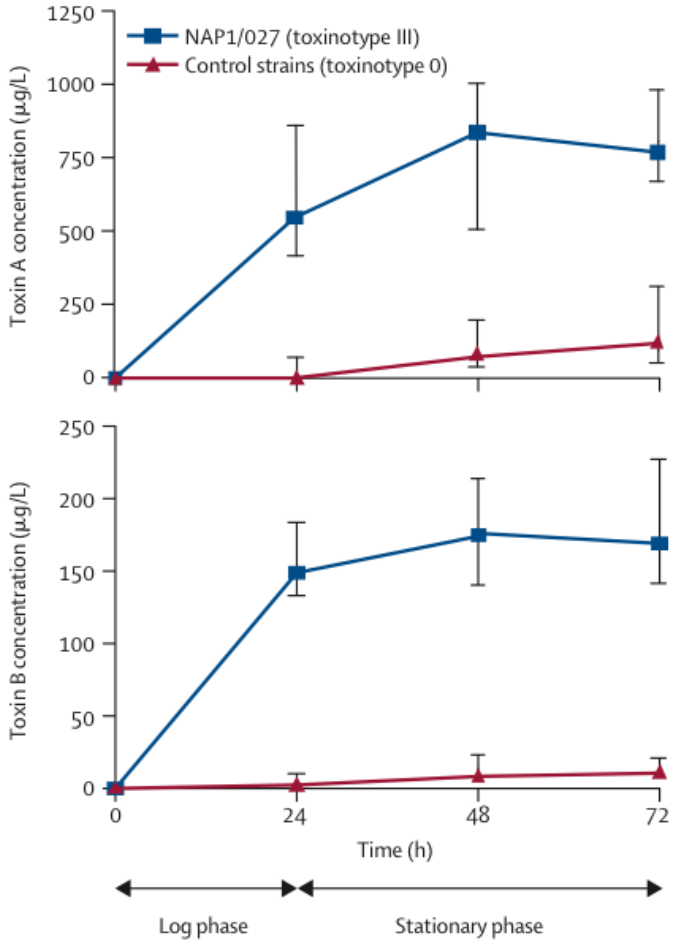
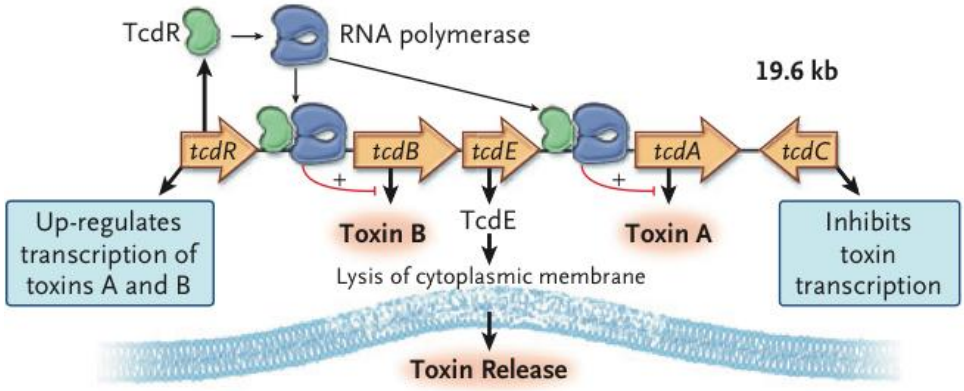


Figure 1-3. The pathogenicity locus (*PaLoc*) of *C. difficile*. Reprinted with permission from *Clostridium difficile* – More Difficult than Ever. Kelly CP & LaMont JT, N Engl J Med 2008;359:1932-40.



1.2 Proton Pump Inhibitors (PPI) and *C. difficile* Infections (CDI)

PPIs were introduced in the late 1980s; today they are one of the most prescribed drugs [61], and are the most acid-suppressing drugs available [62]. PPIs have been instrumental in the management of acid-related diseases such as gastroesophageal reflux and peptic ulcers [62] by targeting and irreversibly blocking the H^+/K^+ ATPase of acid-releasing gastric parietal cells, in turn reducing the acidity in the stomach [63].

First described in 2003 [64], there have been an increasing number of studies reporting a positive association between PPI use and acquiring CDI in both nosocomial and community settings [15,16,64-78]. In a study published in 2008, Drs. Cunningham and Dial found a positive association between the recent increase of *C. difficile* epidemics and increased use of PPIs [75]. A 2007 systemic review by Leonard *et al.* found that the use of gastric acid suppressants to be significantly associated with CDI [79]. Not only have PPIs been associated with acquiring CDIs, it has also been linked to an increase in CDI recurrences [69]. It has also been observed that the combined use of antimicrobials and PPIs increase toxin production in toxigenic *C. difficile* in a rat model [80].

There are a few theories for the apparent association between use of PPI and CDI. First, it has been well established that gastric acid is a key immune defense mechanism against potential ingested pathogens and subsequent infection. Since PPIs decrease the acidity of the stomach, this could in-turn compromise the protection barrier formed by the low gastric pH [6]. In other words, patients undergoing PPI therapies may not have sufficient gastric acidity to effectively kill vegetative *C. difficile* cells, thereby facilitating their passage through the gastrointestinal tract [73]. A 2010 study showed that as the level of acid suppression increased, the risk of nosocomial CDI increased in a dose-response

relationship [68]. Second, recent findings suggest vegetative *C. difficile* cells can survive up to six hours on moist surfaces [81]—providing ample opportunity for transmission via contaminated fomites [75]. Third, a number of authors have warned that PPIs are being over-used. Currently, upwards of 25% of patients in a hospital may be on PPIs, and anywhere between 25 to 70% of patients do not present with the appropriate indication [82-85]. In addition, the use of PPI in Canada has dramatically increased over the past few years [63].

Despite numerous studies highlighting the possible association between PPI-use and CDI, the issue remains controversial, as some studies found no association between CDI and PPI [67,86,87]. There is no data indicating that reduction of PPI-use reduces incidence of CDIs [73]. However, a 2008 review points out that many studies finding no correlation between PPI and CDI collected data from mostly elderly patients, most of whom already has high stomach pH [75].

Although there have been numerous studies suggesting a link between PPI use and CDIs, there have yet to be evidence of causation. PPIs are used commonly today to treat a variety of conditions and are given frequently as prophylaxis to patients with comorbidities, such as severely ill patients in intensive care units, which may be risk factors for CDI [6]. The development of CDI involves many factors, including poor hygiene, acquisition of a toxigenic strain, and use of antibiotics. In other words, a complex interaction of host, pathogen, and environment describes CDI. Therefore, it may be very difficult to correct for these confounders in case-control studies [6]. More research is needed with better designs, such as randomized controlled trails, and higher statistical power to definitively conclude whether PPI use is a risk factor for development

of CDIs. For now, unnecessary prescription of both PPIs and antimicrobials should be avoided, which is always a good practice, with or without risk of CDI.

1.2.1 Combined Use of Antimicrobials and PPI

Louie *et al.* have reported that toxin production was significantly increased in rats treated with both antibiotics and PPI (personal communication). Table 1-1 provides data collected from a quantitative animal challenge study. Using a rat model, subjects were inoculated with either NAP1 or non-NAP1 *C. difficile* spores on days 8-11. These rats were then given cefotaxime (CTX) every 8 hours subcutaneously and/or 2 mg pantoprazole (a PPI) intraperitoneal once per day on days 10-20.

This experiment yielded some very interesting results: the colony forming units (CFU) of *C. difficile* in all rats were approximately the same, but toxin B titres were very different. First, rats treated with the NAP1 strain of *C. difficile* and challenged with both CTX and PPI produced over twice the amount of toxin B compared to rats treated with the non-NAP1 strain treated with CTX and PPI. This is consistent with the finding that NAP1 strains produce more toxins *in vitro* compared to non-NAP1 strains, mostly likely due to a an early frameshift mutation in the negative regulator gene (*tcdC*) within the pathogenicity locus of this epidemic strain [51]. Second, between the rats infected with NAP1 strains treated with CTX alone or both CTX and PPI, the subjects treated with both CTX and PPI produced significantly much more toxin B than CTX treatment alone.

Table 1-1. Cytotoxin B titre in cecal filtrate when treated with cefotaxime (CTX) and/or PPI in a rat model. Modified using unpublished data from Louie *et al.*

Ribotype	Treatment	No. Tested	Cytotoxin B titre in cecal filtrate	Log_{10} CFU in cecum
Non-NAP1	Control	9	-	< 3
NAP1	CTX	8	4000 ± 645	8.9 ± 0.4
NAP1	CTX + PPI	8	10,625 ± 2652	8.6 ± 0.4
Non-NAP1	CTX	8	6500 ± 2666	8.6 ± 0.3
Non-NAP1	CTX + PPI	8	3750 ± 701	8.6 ± 0.4

In a similar experiment, Louie *et al.* treated rats infected with NAP1 *C. difficile* with one of four antibiotics: CTX, and one of the respiratory fluoroquinolones (levofloxacin, moxifloxacin, and gatifloxacin). One treatment group then received PPI, while the other only received antibiotic. Similar to the results of their experiment described previously, the treatment group receiving both PPI and an antibiotic produced significantly more toxin than the group that received antibiotic alone. The results are displayed in Table 1-2 (unpublished).

Table 1-2. Cytotoxin B titre in cecal filtrate in rats infected with NAP1 *C. difficile* spores, then treated with one of four different antibiotics with and without PPI. Modified using unpublished data provided from Louie *et al.*

Antibiotic	No. Tested	No PPI (toxin titre)	PPI (toxin titre)	P-value
Cefotaxime	4	1300 ± 436	6000 ± 1155	<0.03
Levofloxacin	8	702 ± 238	1750 ± 366	<0.04
Moxifloxacin	8	680 ± 246	3150 ± 1131	0.05
Gatifloxacin	8	1375 ± 183	7000 ± 655	0.0002

These findings from experiments by Louie *et al.* suggest that PPI-use may be associated with increased toxin production in subjects using antibiotics. The data from

these experiments also supplied us with our interest in studying the molecular bases for this apparent increased toxin production when CDI, antimicrobials, and PPIs are combined.

1.2.2 Transcriptomics

The transcriptome can be thought of as the entire RNA content of a cell; its understanding is essential for the interpretation of the functional elements of the genome, development, and pathogenesis [88,89]. The transcriptome changes as different genes are expressed at different developmental stages, or as a response to varying conditions. It is important to note that expressed transcripts indicate what proteins might be produced, but provides no indication on the function of these proteins or their abundance.

Gene expression analysis is one of the basic approaches for biological studies; it is fundamental in studying host-pathogen interactions. The application of transcriptomics to study host–pathogen interactions can bring important insights into the mechanisms of pathogenesis. Identification of a complete set of transcripts expressed in a genome is one of the ultimate goals of transcriptome studies [90]. Quantitative and qualitative analyses of the transcriptome can help us define and understand particular cellular processes under a variety of conditions. In the case of *C. difficile*, we may wish to analyze the genes expressed (both qualitatively and quantitatively) under certain challenges, such as antimicrobials and/or PPIs.

A number of methods currently exist for transcriptome analysis, including microarrays and sequencing methods.

1.2.2.1 Microarrays

DNA microarrays have been developed as a method to rapidly analyze the expression of all genes within a genome [89]. Microarrays based on cDNA and genome sequences has greatly contributed to transcriptome analysis in model organisms; various versions of arrays are now commercially available [91]. To this day, microarray is still considered the gold standard for global gene expression analysis [92].

Microarrays work by hybridizing the products of gene expression (cDNA transcripts) to the gene itself, fabricated either using PCR reactions to generate clones or using known database to synthesize shorter oligonucleotides (GeneChip®, Affymetrix) [93]. Labeled complementary DNA (cDNA) fragments bind to fixed strands of template DNA. These DNA fragments represent a gene from the organism under study, and are physically attached to an inert support matrix, called a "chip". Transcripts (RNA) are extracted from the growing organism, converted to cDNA with reverse transcriptase, and then labeled with fluorescent markers. These cDNA are then allowed to hybridize to the chip containing the genes of the organism under study. In this manner, simply labelling each set of transcripts with unique fluorescent makers and measuring the color intensity of the fluorescence allows us to analyze the transcription profiles of an organism under different conditions [93]. For example, using microarray technology, Emerson *et al.* were able to discover that antibiotics and environmental stress such as heat and acidity modified the transcriptional profile of *C. difficile* 630 [94].

Microarrays can vary in several ways, such as the method by which DNA fragments are affixed to the chip, and the length of the DNA.

1.2.2.2 Disadvantages of Microarrays

Although DNA microarrays have allowed us to profile expression of thousands of genes in a single experiment, they also have a number of shortcomings. First, large amounts of RNA is required to produce an adequate signal-over-noise ratio for cDNA microarrays; this is particularly problematic for low-abundance transcripts, because they may be missed even if the DNA template is affixed to the chip [95]. Second, there is the possibility of cross-hybridization of highly similar sequences. Third, the sensitivity of microarrays is low; it cannot detect minute changes in gene expression [89,92]. Low-level transcripts may go undetected during microarray analysis but could have a significant role in cellular activity, or may be lost during preparation steps due to their low concentration [92]. Fourth, constructing microarrays requires *a priori* knowledge of the genes of an organism [89]. In other words, microarrays cannot discover novel genes. Undiscovered genes cannot be affixed to the chip, and novel transcripts will remain unhybridized. In addition, since oligonucleotide microarray's probes are synthesized from databases, the quality and accuracy of these probes will only be as good as information from these databases, and are therefore subject to error. Another challenge to microarray technology is the difficulties in standardization, which involves accurately comparing data collected from different microarray platforms. Post-transcriptional modifications, such as messenger RNA (mRNA) splicing, could also make microarrays problematic, as one gene could have many different splice variants [96]. It stands to reason that differential expression could be due to these splice variants instead of different genes, which may not be fully appreciated using microarray technology. Lastly,

it is difficult to quantitate levels of gene expression using microarrays, as its strength is more limited to relative expression between multiple sets of microarrays.

Due to the drawbacks of microarrays, and the recent advancement of transcriptome sequencing, it was decided the latter approach be used to study *C. difficile* transcriptome.

1.2.2.3 Next Generation Sequencing (NGS) and RNA-Sequencing (RNA-Seq)

Recent advances in sequencing technologies and assembly algorithms have provided researchers with an increasingly viable alternative to microarray technology in transcriptomic applications. Next generation DNA sequencing uses non-Sanger-based methods that substantially improves throughput. RNA-Seq uses NGS technologies to sequence and quantify RNA directly. As described previously, identifying the full set of transcripts, including novel transcripts undetected by microarrays, is fundamental to the study of an organism's transcriptome. The recent advancements in RNA-Seq and its applications in global transcriptome sequencing have allowed users to study the transcriptome on an unprecedented level; the massive depth and speed with which sequencing can be performed offers a near-complete snapshot of a transcriptome [88,97-99]. Passalacqua *et al.* recently used a high throughput sequencing-based approach in assembling the first comprehensive, single-nucleotide resolution view of *Bacillus anthracis* bacterial transcriptome [100]. In addition, by using RNA-Seq technologies Passalacqua *et al.* discovered previously non-annotated regions, which helped to refine the existing annotations. As another example, through RNA-Seq methods, Mao *et al.* [101] recently identified 20 new genes in the bacterium *Sinorhizobium meliloti* using the 454 Genome Sequencer FLX system.

Popular NGS technologies today include the Illumina Genome Analyzer (Solexa), the AB SOLiD system (Life Technologies), and the FLX pyrosequencing system by 454 Sequencing (Roche, Branford, CT). Because the Roche/454 Genome Sequencer FLX System is available in-house at the National Microbiology Lab (NML), its basic operational function will be briefly overviewed [102,103]. The Roche/454 system utilizes a massively parallel pyrosequencing technology (sequencing by synthesis). Pyrosequencing is a non-electrophoreticF, bioluminescence method that measures the release of inorganic pyrophosphate by converting it proportionally into light using a series of enzymatic reactions [104,105]. Essentially, template DNA is fragmented and processed so it can be ligated to adapters and amplified by emulsion PCR (emPCR) on a specially designed picotitre well plate containing chemiluminescent sequencing enzymes. Only one type of dNTP is added per run (i.e. A, T, C, or G). If DNA polymerase incorporates any dNTPs, pyrophosphates (PP_i) are released in the same ratio as number of dNTP incorporated. For example, incorporation of two dNTPs will result in two PP_i released. ATP sufurylase then converts PP_i to ATP, which is then used to generate light by luciferase in an amount proportional to the number of ATPs generated. The light and brightness emitted is captured by a camera and processed by a computer to determine the numbers of dNTPs added (if any). Any unincorporated nucleotides and ATPs are degraded by apyrase, after which a new cycle can begin.

Millions of picotiter-scale sequencing-reaction wells are etched into the plate surface [103], thus millions of reactions can occur simultaneously. Because only one type of dNTP is added per run, the system can determine which dNTP is incorporated and localize it to a particular well based on light emission patterns. The 454 GS FLX

pyrosequencing system can process >400,000 reads per reaction at a read length of 200-300 bases, meaning that a single run typically yields over 100 million bases in hours (http://my454.com). The GS FLX+ system has recently been announced by Roche, which can deliver sequencing reads up to 1 kb in length.

The 454 system has been applied to a number of projects for RNA-Seq purposes [106-109]. However, these projects involved only eukaryotes.

Theoretically, RNA-Seq is straightforward. First, mRNA is isolated from total nucleic acid extraction. Second, the mRNA is reverse transcribed to cDNA to generate a cDNA library. Third, the cDNA library is sequenced using NGS. The generated data are processed computationally, which involves removal of artifacts, correction of errors, reconstruction of original transcripts, and aligning reads to these transcripts to quantify (expression counting) [97,99].

Compared to traditional and alternative technologies such as microarrays, RNA-Seq obtains better sequence resolution, is able to detect novel transcripts, achieves better quantitation, and is capable of *de novo* annotation without a reference sequence [88]. For example, RNA-Seq can reveal transcription boundaries to a single base, shed light on how exons are connected, and reveal sequence variations such as SNPs [88,110,111,111]. In addition, RNA-Seq has minimal background signal, an unlimited quantification ceiling for transcripts, and is accurate and highly reproducible [88].

Table 1-3 compares RNA-Seq to microarray and cDNA/EST sequencing.

Table 1-3. RNA-Seq compared to microarray and cDNA/EST sequencing methods. Adapted by permission from Macmillan Publishers Ltd: NATURE REVIEWS GENETICS [88], copyright 2009.

Technology	Tilting microarray	cDNA/EST sequencing	RNA-Seq
Specifications			
Principle	Hybridization	Sanger sequencing	High-throughput sequencing
Resolution	Up to 100 bp	Single base	Single base
Throughput	High	Low	Very high
Reliance on genomic sequence	High	None	Moderate
Background noise	High	Low	Low
Application			
Simultaneously map transcribed regions and gene expression	Yes	Limited to gene expression	Yes
Dynamic range to quantify gene expression level	Up to few hundred-fold	Not practical	>8,000-fold
Distinguish isoforms	Limited	Yes	Yes
Distinguish allelic expression	Limited	Yes	Yes
Practicality			
RNA amount required	High	High	Low
Cost for larger genomes	High	High	Moderate (decreasing)

New NGS methods are currently being developed. For example, the Helicos system has the ability to sequence RNAs directly without cDNA library construction [112].

There are three assembly strategies in RNA-Seq: (1) assembly based on a reference genome; (2) *de novo* assembly without a reference genome; and (3) combination of *de novo* assembly and using a reference genome. Like its name suggests, the reference-based strategy utilizes a reference genome sequence to which the transcriptome assembly can be aligned [99]. Advantages of the reference-based strategy include fewer problems with contamination and artifacts, and the option of using the

reference sequence to fill in gaps caused by lack of read coverage [107,113]. The reference-based method is also easier to perform on prokaryotes due to their relatively simple transcriptomes (e.g. few introns and few alternative splicing) [99]. The *de novo* strategy leverages redundancy of short-reads to find overlaps and assembles them into transcripts without the use of a reference sequence. Advantages of the *de novo* strategy include not requiring a reference genome (or for organisms that do not have a high-quality genome sequence), independence of correct alignment of reads [114], detection of transcripts missing from an immature reference genome, and being unaffected by long introns [99].

The combination method using both a reference genome and *de novo* assembly attempts to overcome the drawbacks of both methods (described below) and to create a more comprehensive transcriptome, starting with either alignment or assembly. To choose between the two starting points depends on many factors, such as quality of the reference genome, and computing resources [99].

Overall, the choice of an RNA-Seq strategy depends on many factors, including the existence and quality of a reference genome, equipment access, time, and funds.

1.2.2.4 Disadvantages of RNA-Seq

One disadvantage of RNA-Seq is its multitude of nucleic acid manipulation stages. Also, sequence reads from NGS platforms are often very short—from 35 to 500 bp[2]—which necessitates transcriptome assembly (piecing together short, low-quality reads) [99,102]. Due to this limit, long RNA sequences (some mRNA) need to be digested into

[2] New GS FLX+ System announced by Roche has increased read lengths up to 1,000 bp (http://my454.com)

smaller fragments, which could introduce bias depending on the enzyme. However, sequencing DNA molecules from both ends and joining the sequences together computationally can improve this flow by forming longer reads [115]. Heavy computational resources, sophisticated software and a multitude of complex algorithms are involved in the processing of the deluge of data from RNA-Seq methods. In addition, unlike DNA sequencing, where both strands are sequenced, strand-specificity could create difficulties in RNA-Seq. Use of strand-specific RNA-Seq protocols can mitigate this problem of overlapping transcripts, but are laborious [116]. Furthermore, the possibility of transcript variants from the same gene (e.g. exon-sharing) could be difficult to resolve, therefore reconstruction of the transcripts including all of the variants has been difficult, and uncertainty could arise regarding which read belongs to which transcript [117]. Due to the extensive variability in abundance of transcripts, detection of low-level transcripts can be challenging. However, this can be alleviated through rRNA subtraction and removal of highly abundant transcripts (computationally after sequencing). These methods increase detection and assembly of low-level transcripts through reduction of highly abundant rRNAs [118]. Another disadvantage of RNA-Seq is the possibility of transcripts matching to multiple genomic locations, which becomes increasingly likely in larger genomes [88]. While this may be true, sophisticated computer algorithms have somewhat mitigated this problem.

There are disadvantages specific to a reference-based strategy. Obviously, this strategy can only be used if there is a reference sequence available. Using sequences from closely related strains is possible (e.g. *C. difficile* 630 instead of a NAP1 strain), but transcripts from divergent regions would be missed [99] (e.g. *C. difficile* 630 does not

produce binary toxin, unlike NAP1). In addition, quality and success relies on the quality of the reference genome sequence. This can be problematic because most genome assemblies (excluding model organisms) contain many errors [119]. Therefore, the reference-based method is more suitable when a quality reference strain is available, or can be obtained. There are also disadvantages to the *de novo* system. First, de novo assembly requires extensive computing power. Second, much higher sequencing depth is required (less than 10-fold coverage for reference-based strategy compared to 30-fold for *de novo*) [120]. Lastly, *de novo* methods are error-prone [121].

1.2.3 Objective One – Transcriptomics and *C. difficile*

As discussed in section 1.2.1, Louie *et al.* reported significantly elevated toxin production by a NAP1 *C. difficile* in the gut of rat models treated with both PPI and antibiotic when compared to just antibiotic alone. Gene expression analysis is fundamental to studying host-pathogen interactions, helping us gain insight into cellular processes, toxin production, and ultimately pathogenesis. Comparative gene expression analysis can enable us understand differential gene expression under a variety of conditions, such as exposure to PPI and/or antibiotics.

Although thousands of bacterial genomes have been sequenced, we still do not have a good understanding of the bacterial transcriptome [100]. To the best of my knowledge, no prior published studies have used sequencing technologies to analyze the transcriptome of the hypervirulent *C. difficile* 027/NAP1 strain. In addition, existing NAP1 genomic data is neither closed (multiple contigs) nor annotated.

The recent advancement of RNA-Seq technologies has made it possible to study the transcriptome with much higher power than ever before, which makes it an attractive

option to study the *C. difficile* NAP1 genome. As described previously, Passalacqua *et al*. recently used the ABI SOLiD and Illumina/Solexa NGS systems to assemble the first comprehensive, single-nucleotide resolution view of the *Bacillus anthracis* transcriptome, in which they discovered previously non-annotated regions and improved current annotations [100]. One factor in the decision regarding which sequencing system to use is availability [99]. Fortunately, the 454 Genome Sequencer FLX pyrosequencing system from 454 Life Sciences (Roche, Branford, CT) was available in-house at the DNA Core (National Microbiology Laboratory), which can be used in both genome sequencing and RNA-Seq.

There are four objectives for this first-of-two-part project: (1) carry out PCR, PFGE, and growth studies to characterize two *C. difficile* isolates received from Louie *et al.* (NAP1 and non-NAP1) used in their toxin assays described previously; (2) develop methods for the efficient extraction and cleanup of *C. difficile* NAP1 and non-NAP1 DNA and RNA based on current protocols; (3) sequence genomes of both NAP1 and non-NAP1 *C. difficile* received from Louie *et al.* using the 454 GS FLX, and submit for contig-closure using reference strains; and (4) develop methods for the efficient extraction and concentration of mRNA and its conversion to cDNA in quantities acceptable for RNA-Seq, again using the 454 GS FLX sequencing system.

Uses of antimicrobials and PPIs have both been associated with CDI. The ultimate goal of our study is to provide a framework for the study of *C. difficile* transcriptomics using NGS and RNA-Seq technologies, which are unencumbered in their ability to detect novel transcripts (unlike microarrays), to make it possible to qualitatively and

quantitatively study the different expression patterns when NAP1 *C. difficile* is challenged with PPI and/or antibiotic *in vitro*.

1.3 Metronidazole and *C. difficile*—Second Study

Having been used for over 45 years, metronidazole still remains as a mainstay for the treatment of anaerobic infections, particularly for patients with mild to moderate CDI [122,123]. The indication of metronidazole in the treatment of CDI was discussed previously in section 1.1.5.

Metronidazole is active against a spectrum of bacterial anaerobes, protozoa, and microaerophilic bacteria [124]. Metronidazole achieves high serum concentrations following oral administration, and has excellent tissue penetration. It is also well distributed into body tissues and effectively penetrates the blood-brain barrier [125]. Metronidazole does not bind well to proteins; 80% of circulating metronidazole is protein-free. It is metabolized in the liver to glucuronide and oxidative products, with a half-life of six to nine hours that is minimally affected by renal function [126,127]. However, in patients with compromised liver function the half-life may increase seven-fold [128-130].

The most common side effects metronidazole-use are gastrointestinal: nausea, vomiting, diarrhea, abdominal pain, and constipation [131]. Less common side effects include candida growth, central nervous system symptoms (e.g. seizures, vertigo, irritability, headache, irritability) [132-134], and allergic reactions [135].

Most drug interactions with metronidazole occur in the liver, where it is metabolized. For example, alcohol consumption during metronidazole therapy can result in a disulfiram-like reaction (flushing, tachycardia, palpitations, nausea, vomiting) [136], and use of disulfiram can result in acute psychosis [211]. Other potentially dangerous

interactions include lithium, warfarin, amiodarone, cyclosporin, rifampin, and prednisone [137].

1.3.1 Mechanism of Action

Metronidazole is cytotoxic to both facultative (e.g. *Helicobacter pylori*) and obligate (e.g. *C. difficile*) anaerobic bacteria, but the mechanism of action remains incompletely understood for facultative anaerobes [138].

It has been elucidated that metronidazole's activity against anaerobes occurs through a four-step process[3] [124,138-143]:

1. Entry – Metronidazole is a low molecular weight compound, and it is able to diffuse across the membrane of microbes. However, it is not toxic to aerobes, therefore the host is spared.
2. Reductive activation – Metronidazole is reduced by the pyruvate-ferredoxin oxidoreductase system (PFOS) within the mitochondria of obligate anaerobes in Eukaryotic organisms. The PFOS normally functions in generation of ATP through oxidative decarboxylation of pyruvate. However, presence of metronidazole's nitro group "steals" electrons intended for H^+ ions, and is reduced instead (Figure 1-4). The reduction of metronidazole changes its chemical structure and effectively eliminates the concentration gradient, in turn allowing more uptake of the drug.
3. Cytotoxicity – The formation of intermediate compounds and free radicals as metronidazole becomes reduced and as it accumulates inflicts cytotoxic damage

[3] Modified from Johnson, M. *Metronidazole: An overview*. UpToDate, Basow, DS (Ed), UpToDate, Waltham, MA, 2011

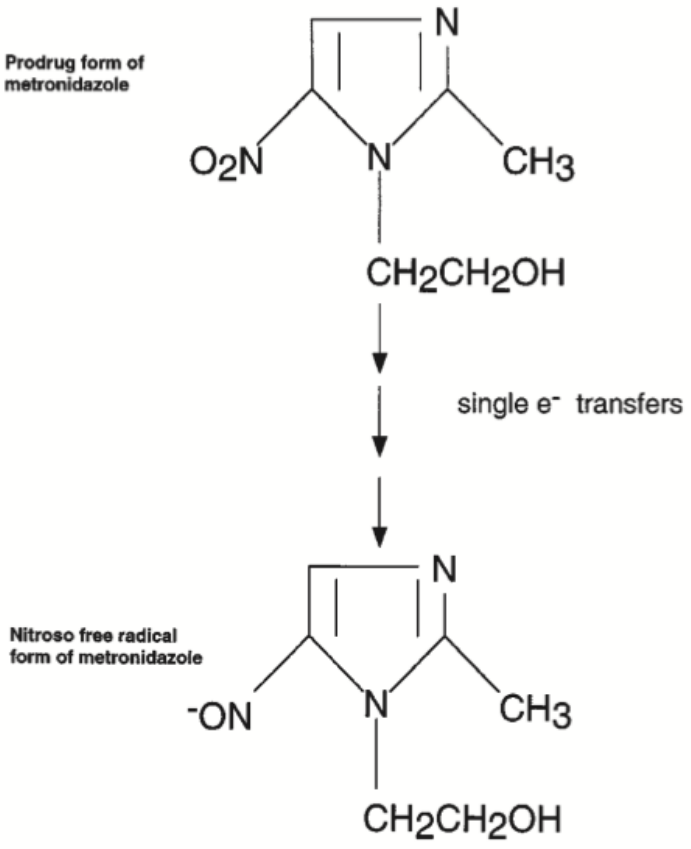
by interactions with DNA, resulting in strand breakage and destabilization of the helix.

4. Metabolism – Cytotoxic intermediary products decay into inactive products, and are cleared by the host's system.

Aerobic cells lack electron transport proteins with sufficient redox potential, therefore they are not affected by metronidazole [123].

Overall, metronidazole exerts bactericidal effects against obligate anaerobic bacteria with a rate proportional to the drug concentration [144,145].

Figure 1-4. Reduction and activation of inactive metronidazole to its cytotoxic form via electron transfer. Reprinted from Drug Resistance Updates {Land:1999ji}, Vol. 2, No. 6, Land KM & Johnson PJ, Molecular basis of metronidazole resistance in pathogenic bacteria and protozoa, Copyright 1999, with permission from Elsevier.



1.3.2 Resistance

Acquired resistance to metronidazole among anaerobic bacteria is rare [124]. One study found no resistance in *Bacteroides spp.* in over 4,000 strains between 1990 and 1996 in the US [146]. A similar study in Canada also found no change in resistance rate to metronidazole in *Bacteroides spp.* between 1992 and 1997 [147].

In *Bacteroides spp.*, resistance has been shown to be transferable by both plasmid and chromosomal mechanisms [148,149] through specific nitroimidazole (*nim*) resistance genes [150]. *Nim* genes are believed to encode a nitroimidazole reductase, which converts metronidazole to a nontoxic derivative instead of a toxic one [123,151]. However, *nim* genes have been found in *Bacteroides spp.* that are susceptible [152], suggesting that while *nim* genes have been shown to confer resistance, possession of *nim* does not necessarily mean resistance. This may be due to a mutation in the *nim* gene and/or altered upstream regulation [151]. Decreased PFO activity has also been shown to play a role in resistance [124], which eliminates the concentration gradient across the cell membrane, thus decreasing metronidazole entry into the microbe. Therefore, the level of PFO activity has an inverse relationship with resistance [124]. For example, downregulation of PFO has been associated with resistance to metronidazole in the protozoan *Giardia lamblia* [153], demonstrating that mechanisms of resistance are shared across species.

H. pylori utilize a different resistance mechanism compared to anaerobes such as *Bacteroides* [155]. In *H. pylori*, reduction and activation of metronidazole occurs through a two-electron transfer step mediated by a gene (*rdxA*) encoding an oxygen-insensitive NADPH nitroreductase. The reason for this different enzyme is due to the microaerophilic environment in which *H. pylori* survive (presence of oxygen). It stands to

reason that metronidazole-resistant isolates, both clinically isolated and induced, have typically been found to have mutated *rdxA* genes. Indeed, Δ*rdxA* has been found in *H. pylori* resistant to metronidazole [154-156]. However, other factors may be involved as well [157]; for example, resistant isolates of *H. pylori* have been found to exhibit reduced levels of metronidazole uptake and accumulation [158,159]. Table 1-4 lists many of the proposed mechanisms for metronidazole resistance.

Table 1-4. Proposed mechanism of metronidazole resistance in anaerobic bacteria. Obtained from Löfmark *et al.* in Metronidazole Is Still the Drug of Choice for Treatment of Anaerobic Infections [123], Clinical Infectious Diseases, 2010;50:S16–23, by permission of Oxford University Press.

Mechanism of resistance
Reduced drug activation (i.e. downregulation of enzyme)
Alternative pathway – "activated" drug is non-toxic (*nim* genes)
Prevention of entry or efflux
Altered DNA repair

1.3.3 Resistance in *C. difficile*

The Clinical Laboratory and Standards Institute's (CLSI) breakpoint for metronidazole is a minimum inhibitory concentration (MIC) of >16 µg/mL [160]. Several studies have reported reduced susceptibility and/or resistance of *C. difficile* to metronidazole. In 1997, Jang *et al.* reported equine isolates of *C. difficile* with MICs of 16 µg/mL [161]. In 1999, Wong *et al.* reported the first documented case of a resistant strain isolated from a patient with CDI in Hong Kong out of a total of 100 isolates (MIC of 64 µg/mL by agar dilution) [162]. It is unknown if the aforementioned strain was

toxigenic. Then, Barbut *et al.* in 1999 identified six *C. difficile* isolates from 198 isolates with MIC ranging from 8 to 32 µg/mL metronidazole [163]. However, out of these six isolates, five were non-toxigenic and thus clinically irrelevant. In a 2001 correspondence, Brazier *et al.* reported the discovery of an UK isolate with reduced susceptibility to metronidazole *in vitro* (non-toxigenic ribotype 010; MIC = 16 µg/mL by Etest) [164]. In 2002, Peláez *et al.* published data on the resistance profiles of 2,384 isolates of toxigenic *C. difficile* obtained from patients in a Spanish hospital between 1993 to 2000 [165]. Out of 415 randomly selected samples, 3 had MIC = 32 µg/mL, and 23 had MIC >32 µg/mL (6.3% of total tested, all by agar dilution methods). No isolates had MIC <16 µg/mL, and there was no clear evidence of trends in susceptibility profile changes over the eight-year period. Despite all isolates being toxigenic, the particular molecular types to which these resistant isolates belonged are unknown.

In 2008, Peláez *et al.* published another study suggesting that metronidazole resistance in *C. difficile* is heterogenous [166]. In this study, Peláez *et al.* performed susceptibility testing on the same resistant isolates in the 2002 study after freeze-thaw cycles and prolonged exposure to metronidazole. Fourteen strains out of the original 26 were selected (all were toxigenic, but none were ribotype 027/NAP1), with MICs ranging from 16 to 64 µg/mL. After thawing, all 14 strains had become susceptible again, with MICs ranging from 0.125 to 8 µg/mL by agar dilution. It is worth noting that Peláez *et al.* also included Etest data in this study, and the MIC values obtained by Etest were significantly less than the agar dilution method—0.125 to only 2 µg/mL (up to four-fold less). In their induction study, Peláez *et al.* found that resistance was inducible by culturing with metronidazole pressure; two subpopulations were observed on Etest: (1)

subpopulation with the same MIC as before (0.125 to 2 µg/mL), and (2) another subpopulation inside the first subpopulation with elevated MICs ranging from 8 to 256 µg/mL. The authors also found that after repeated passaging without metronidazole the heterogenous population became susceptible. Furthermore, no strains were found to possess *nim* genes when checked by PCR. Overall, the conclusions were that resistance to metronidazole in *C. difficile* is unstable and inducible.

Other studies have since discovered isolates with both reduced susceptibility and resistance. Baines *et al.* demonstrated the emergence of reduced susceptibility in ribotype 001 isolates in the UK [167]. However, no reduced susceptibility was found in ribotype 027/NAP1 isolates. In a recent study by Miller *et al.*, NAP1 isolates in Canada were found to have a two-fold higher MIC than non-NAP1 strains [56], although MIC_{90} and MIC_{50} were only 2 and 1 µg/mL for NAP1, respectively. In a different study, Martin *et al.* performed susceptibility testing on 1,080 isolates of *C. difficile* in Ontario, Canada, and found 17 (1.6%) isolates to be resistant to metronidazole, 5 of which were NAP1 [168]. Two isolates exhibited MICs of >256 µg/mL by Etest (unknown molecular type). However, resistance was only transient and was lost upon repeated passaging.

Nonetheless, it is important to note that most *C. difficile* strains are still susceptible to metronidazole. In addition, of the reported isolates of metronidazole-resistant *C. difficile*, most were non-toxigenic [123], as described earlier. In a UK study, no clinical isolate among 110,000 tested strains was resistant, and only one resistant non-toxigenic strain of environmental origin was detected [169]. An increasing number of clinical failures with metronidazole treatment of *C. difficile* infection has been reported over the past few years [29,170]. However, Sanchez *et al.* [171] found no evidence

between patients with failure of metronidazole therapy and resistance in a matched retrospective study. In a ten-year prospective study of strains associated with treatment-failure, all isolates had MICs of less than 1 µg/mL, and the decreased susceptibility of the infecting *C. difficile* strains was not considered to be the cause of the failures [172]. Mechanisms for reduced susceptibility and resistance of *C. difficile* to metronidazole *in vitro* remain elusive: both Brazier *et al.* [164] and Peláez *et al.* [166] did not find *nim* genes in their UK and Spanish *C. difficile* isolates, respectively. Similarly, the reasons behind the finding that metronidazole has diminished effectiveness compared to vancomycin for severe CDI are not obvious. Currently, treatment failure has not yet been linked to resistance [6,171], and the potential clinical significance of reduced susceptibility to metronidazole remain unclear. However, given the pharmacokinetics of metronidazole, only a small elevation in the MIC could hypothetically have a dramatic effect on the treatment outcome of CDIs [6]. Since CDI is a toxin mediated disease, and *C. difficile* is not known to invade intestinal epithelium, the antibiotic needs to be in the colonic lumen to function [173]. Most of the metronidazole affecting *C. difficile* is thought to reach the gut by diffusion from serum to the intestinal mucosa [123], and poor gut levels of metronidazole and emerging evidence of reduced susceptibility in epidemic *C. difficile* strains may play a role in reduced response [174]. By the same token, observations of reduced susceptibility to metronidazole could have implications in the clinical setting due to the poor penetration of metronidazole into the colon [167] (metronidazole is highly absorbed in the small bowel). As described previously, the CLSI breakpoint for metronidazole resistance is >16 µg/mL, but this value may not necessarily reflect achievable fecal metronidazole concentrations [58]. Metronidazole concentrations

in feces average between <0.25 to 9.5 mg/kg and are dependent on the inflammatory state of the colon [175-177]. Therefore, isolates with MICs of ≥4 μg/mL may be unaffected by colonic concentrations of the drug [6]. Vancomycin, in contrast, is not well absorbed, and colonic concentrations range between 520 to 2,200 μg/mL [178]. Therefore, at these antibiotic concentrations, *C. difficile* strains with elevated MICs to vancomycin will still be affected.

Routine antibiotic susceptibility screening has not been performed by most institutions, because it has been broadly accepted that *C. difficile* is regularly and predictably susceptible to metronidazole and vancomycin; the assay is time-consuming, and breakpoints are based on serum levels, not gut lumen [165].

1.3.4 Objective Two – Metronidazole and *C. difficile*

Despite numerous reports of elevated MICs to metronidazole, to the best of our knowledge there has only been one report on NAP1 *C. difficile* with resistance (transient) to metronidazole by Martin *et al.* in 2008 [168], who documented five *C. difficile* NAP1 isolates that were transiently resistant to metronidazole by Etest. Both Martin *et al.* [168] and Peláez *et al.* [166] reported that metronidazole resistance by *C. difficile* was transient and easily lost by serial passages and/or freeze-thawing. To date, I do not know of any studies characterizing resistant strains both before and after loss of resistance.

The Antimicrobial Resistance and Nosocomial Infectious Group (National Microbiology Laboratory, Winnipeg, MB, Canada) have recently discovered a NAP1 strain obtained from feces of a patient in a western Canadian hospital exhibiting reduced susceptibility to metronidazole *in vitro*.

My objectives for the second half of this two-part project are as follows: (1) characterize the *C. difficile* strain recovered from western Canada using PCR and PFGE, in addition to performing MIC testing with both Etest and agar dilution methods; (2) devise and carry out a method to obtain a metronidazole-susceptible *C. difficile* strain from the original strain with reduced susceptibility; (3) characterize the susceptible isolate and compare it with the original in terms of MIC, growth, PCR and PFGE assays; (4) sequence, compare, and contrast the genomes of both strains.

Metronidazole treatment failure rates are increasingly being reported, as well as *C. difficile* strains with decreasing susceptibility to the drug. However, there has yet to be any evidence suggesting these two findings are correlated. Nonetheless, toxigenic metronidazole-resistant *C. difficile*, such as the epidemic NAP1 strain, could have profound implications on the treatment, morbidity and mortality rates across the world. The ultimate goal of the second part of the project is to search for genomic differences between the strain with reduced susceptibility and the susceptible strain, which may give us insight into the mechanisms behind resistance. Furthermore, with knowledge gained from the transcriptomics part of this project, researchers may soon be able to study the transcriptome of metronidazole-resistant *C. difficile*, shedding light on this important, and difficult, pathogen.

1.4 Hypotheses

For the transcriptomics portion of this project, the objectives are listed in section 1.2.3. For the metronidazole resistance project, I hypothesize that *C. difficile* NAP1 resistance to metronidazole is due to genetic mutation(s) that can be localized by comparing the resistant parental strain to a susceptible revertant of the parental strain.

2 MATERIALS AND METHODS

2.1 Transcriptomics Study

2.1.1 Growth Conditions

Two anaerobic chambers were used in this study: (1) the Vinyl Anaerobic Chamber and (2) the Gloveless Anaerobic Chamber (both from Coy Laboratory Products Inc., Grass Lake, MI). The accessories used with the anaerobic chambers were as follows: (1) the palladium catalyst (unheated), air lock, and auto gas injector were all from Coy Laboratory Products Inc. (Grass Lake, MI); (2) Nitrogen (Air Liquide, Winnipeg, MB); (3) mixed gas (5% H_2, 10% CO_2, 85% N_2, from Air Liquide, Winnipeg, MB); (4) T.H.E. Desiccant (VWR, Mississauga, Ontario). All *C. difficile* cultures were incubated at 35°C inside an incubator in the anaerobic chambers.

The growth media used were as follows: BHI broth (Brain Heart Infusion, BD Bacto™, LOT 8303257, Mississauga, ON); CDMN (*C. difficile* agar base OXOID, Nepean, ON, and *C. difficile* moxalactam norfloxacin selective supplement, OXOID, Nepean, ON); BAK (Brucella agar with 5% laked sheep blood, hemin, and vitamin K, BD, Mississauga, ON).

All media (broth, agar plates, and ddH_2O) were pre-reduced for 24 h in anaerobic atmosphere prior to use (i.e. placed in anaerobic chamber for 24 h) and all plates were inverted and test tubes covered during incubation.

2.1.2 Handling of RNA

Due to the ubiquitous, active, and stable nature of ribonucleases (RNases), even minute amounts can destroy RNA (Qiagen RNAprotect® Handbook, 2005). Great care and vigilance were taken to ensure an RNase-free environment during RNA processing. First, proper microbiological and aseptic techniques were always used. Gloves were worn and changed frequently and handling equipment was done with caution. Second, gloves, bench top, and equipment (e.g. pipettes) were frequently cleaned with RNase*Zap*® (Ambion®, Applied Biosystems™ Canada, Streetsville, ON) to eliminate RNases. Containers and tubes were kept closed when not in use, and nuclease-free water (Ambion®, Streetsville, ON) and nuclease-free pipet tips (Molecular BioProducts, Fisher Scientific Canada, Ottawa, ON) were used. Third, RNA products were kept on ice whenever possible to reduce chances of degradation due to heat.

2.1.3 Origin, Storage, and Recovery of *C. difficile*

VLOO13 and VLOO18 were received from Dr. Louie *et al.* (University of Calgary) and were immediately plated onto CDMN plates and grown anaerobically at 37°C for 48 h. One colony was picked for each strain and streaked for isolation. One isolated colony was then streaked on to a BAK plate and incubated for 48 h. The cells were then stored using the Microbank™ Bacterial and Fungal Preservation System (Pro-Lab Diagnostics, Richmond Hill, ON). First, sweeps of colonies on plates were transferred to Microbank™ vials containing beads and liquid medium inside the anaerobic chamber using aseptic technique. Then, the vial was shaken by hand to distribute the cells on to the beads. Finally, excess liquid was aspirated, and vials placed in a -80°C freezer for storage.

For recovery, the Microbank™ vials were taken out of the -80°C freezer and placed on ice, then transferred into the anaerobic chamber. Using aseptic technique, one bead was removed from the vial and dropped into a pre-reduced BHI broth to incubate for 24 h at 35°C.

2.1.4 DNA Extraction and Sequencing

Methods of DNA purification were modified from Unit 5.2 of Current Protocols in Essential Laboratory Techniques (Purification and Concentration of Nucleic Acids) to obtain the best yield and purity [179]. This method is described below.

2.1.4.1 Cellular lysates

One colony from overnight growth (24 h) on a BAK plate was transferred to 5 mL pre-reduced BHI and allowed to grow for 6-8 h at 35°C. A 1 mL sample of culture was then dispensed into a sterile 1.5 mL tube and centrifuged at 15,000 x *g* for 2 min. The supernatant was then removed by aspiration. The pellet was resuspended by vortexing in 600 µL of 1X PBS (phosphate buffered saline, 0.13M NaCl, 2.68mM KCl, 10.14mM $NaHPO_4$, pH 7.4) buffer containing 2 mg/mL of lysozyme (Sigma-Aldrich, Oakville, ON), 150 U/mL mutanolysin (Sigma-Aldrich, Oakville, ON), and 2 µL of 10 mg/mL RNase (Sigma-Aldrich, Oakville, ON). The suspension was incubated for 3 h in a 37°C water bath. After incubation, 15 µL of 20 mg/mL proteinase K (Sigma-Aldrich, Oakville, ON) was dispensed into the tube, and the tube placed in a 55°C water bath until the contents became clear (1-2 min).

2.1.4.2 Phenol/chloroform extraction and ethanol precipitation

First, 600 µL of 25:24:1 phenol:chloroform:isoamyl alcohol (Sigma-Aldrich, Oakville, ON) was added to the cellular lysate, then vortexed and centrifuged at 15,000 x *g* for 3 min. The top aqueous layer was transferred to a new 1.5 mL tube, while the precipitate at the interface and bottom layer were discarded. This process was repeated two more times, until no white precipitate remained at the interface. Next, 600 µL of chloroform/isoamyl alcohol (24:1, Sigma-Aldrich, Oakville, ON) was then added and vortexed to mix, and then centrifuged at 15,000 x *g* for 3 min. The top layer was again transferred to a new tube. Next, 700 µL of 70% ice-cold isopropanol (Sigma-Aldrich, Oakville, ON) was added to the tube containing nucleic acid, which was mixed by vortexing, and then placed at -20°C for 1 h. The tube was then centrifuged at 15,000 x *g* for 5 min at room temperature. The isopropanol was removed by decanting, and 700 µL of 100% ice-cold ethanol (Sigma-Aldrich, Oakville, ON) was added to the pellet. After brief vortexing, the tube was centrifuged at 15,000 x *g* for 1 min. The ethanol was removed by aspiration with a pipet. Then, 700 µL of room temperature 70% ethanol was added to the pellet, and after brief vortexing, the tube was centrifuged again at 15,000 x *g* for 1 min. The ethanol was again removed by aspiration with a pipet. The pellet was dried using SpeedVac® Plus concentrator (SC210A, Thermo Scientific, Nepean, ON) for 5 min at 37°C. A 30-µL volume of nuclease-free water (Ambion®, Streetsville, ON) was then added, and the tube was placed in a 55°C water bath for 2 min with pipetting up and down to dissolve. Prior to placing purified DNA at -80°C for storage, a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE) was used to measure the quality and quantity of the nucleic acid sample.

2.1.4.3 Sequencing

Samples were given to the DNA Core facility at the National Microbiology Laboratory for sequencing with the 454 Genome Sequencer FLX (using the GS Rapid Library Kit (454 Sequencing, Roche, Branford, CT)), as well as library construction.

2.1.5 Quantification and Qualification of Nucleic Acids

Nucleic acid quantitation and purity analyses were carried out with NanoDrop™ 1000 Spectrophotometer according to V3.7 User's Manual and software version V3.7.1 (Windows® XP). NanoDrop™ allows for the use of only 1 µL samples for measurement at a spectrum of 220 nm to 750 nm at much faster speeds than other sample containment devices such as cuvettes. The "Nucleic acid" measurement option was selected in the NanoDrop software, and either DNA-50 or RNA-40 was chosen depending on the sample. For calibration, the contact points were wiped clean with a lint-free cloth, and a 1 µL aliquot of nuclease-free H_2O (Ambion®, Streetsville, ON) was pipetted onto the lower measurement pedestal, containing a fibre optic cable (receiving fibre). Measurement of samples was performed the same way as calibration, replacing the ddH_2O with 1 of µL sample. The 260/280 nm absorbance ratio was used to assess the purity of DNA and RNA. According to the user manual, a ratio of ~1.8 is generally accepted as "pure" for DNA; a ratio of ~2.0 is generally accepted as "pure" for RNA. Lower ratios may indicate presence of protein, phenol, or other contaminants that absorb strongly at 280 nm.

Both messenger and ribosomal RNA integrity was checked using Agilent RNA 6000 Nano Kit (Agilent Technologies, Cedar Creek, TX), used with the Agilent 2100 Bioanalyzer (Agilent Technologies, Cedar Creek, TX). The manufacture's protocol was

followed. To prepare the gel-dye mix, all reagents were equilibrated at room temperature for 30 min before use. Next, 550 µL of gel matrix was transferred into the spin filter, and spun for 10 min at 1,500 x g. Aliquots of gel (65 µL) were transferred to 0.5 mL RNase-free microfuge tubes for storage at 4°C. RNA 6000 Nano dye concentrate was vortexed for 10 sec and spun down briefly to collect at the bottom of the tube. A 1-µL volume of dye concentrate was added to 65 µL of filtered gel and then mixed by vortexing. The mixture was spun for 10 min at room temperature at 13,000 x *g* and used the same day. The remaining filtered gel without dye was stored at 4°C for up to 1 month. To decontaminate the electrodes, the provided electrode-cleaning chip was filled with 370 µL of RNase*Zap*® (Ambion®, Streetsville, ON), and placed in the Agilent 2100 Bioanalyzer for 1 min. The electrode was then rinsed with another electrode-cleaning chip filled with 350 µL of RNase-free water for 10 seconds. Samples and RNA ladder were then prepared by denaturing at 70°C for 2 min (1 µL required, extra 0.2 µL to account for evaporation during heating and loss during transfers). To load the chip, 9.0 µL of gel-dye mix was transferred to the appropriate well, placed in the chip-priming station, and the plunger was pushed down until held by the clip, and then released after 30 sec. After the plunger moves back to the 1 mL position, the chip was taken out of the priming station, and 9.0 µL of the gel-dye mix was transferred into each of the marked wells. A 5-µL volume of the RNA 6000 Nano marker was then transferred to the ladder well and each sample well (6 µL to each unused sample well). One µL of RNA ladder was pipetted into the appropriate well, followed by 1 µL of samples (up to 12). The chip was inserted onto the IKA vortex mixer (IKA® Works, Inc., Wilmington, USA) and mixed for 1 min at 2,400 rpm. The “RNA - Prokaryote” assay was selected in the

provided software for RNA samples, and the "RNA - mRNA", and "dsDNA - cDNA" assays were selected for mRNA and cDNA samples, respectively

2.1.6 PCR

The presence of *tcdA*, *tcdB*, *tcdC*, *tpi*, and *cdtB* were determined using PCR, described previously, by Miller *et al.* [56]. Controls are listed in Table 2-1, and primers given in Table 2-2.

The Qiagen Multiplex PCR Kit (Mississauga, ON) was used, which includes 2X Qiagen Multiplex PCR Master Mix (HotStar Taq DNA Polymerase, 6M $MgCl_2$, 400 µM dNTPs), Q-Solution (5X), and distilled water.

Table 2-1. Controls used in PCR to assay for *C. difficile* toxins.

Control	Description
11ACD0028 (NAP7)	39 bp tcdC deletion = 637 bp product Toxin A positive (*tcdA*+) Toxin B positive (*tcdB*+) Binary toxin positive (*cdtB*+) Triose phosphate isomerase positive (*tpi*+)
11ACD0075 (NAP1)	*tcdC* deletion of 1 bp + 18 bp = 657 bp product Binary toxin positive (*cdtB*+) Toxin A positive (*tcdA*+) Toxin B positive (*tcdB*+) Triose phosphate isomerase positive (*tpi*+)
N07-01533 (toxin A-/B+)	No tcdC deletion (676 bp) Binary toxin negative (*cdtB*-) Toxin A positive (*tcdA*+) Toxin B positive (*tcdB*+) Triose phosphate isomerase positive (*tpi*+)
Sterile ddH_2O as negative control	Negative control

Table 2-2. Primers for amplification of *C. difficile* toxins.

Gene	Sequence (5'-3')	T_m (°C)	Size (bp)	Gene	Product size (bp)
Binary toxin subunit B	TGGACAGGAAGAATAATTCCTTC	68.2	23	*cdtB*	582
	TGCAACTAACGGATCTCTTGC	68.9	21		
Toxin A	AGATTCCTATATTTACATGACAATAT	65.0	26	*tcdA*	420/150
	ACCATCAATCTCGAAAAGTCCAC	70.0	26		
Toxin B	AATGCATTTTTGATAAACACATTG	63.6	24	*tcdB*	329
	AAGTTTCTAACATCATTTCCAC	63.9	22		
Triose phosphate isomerase	AAAGAAGCTACTAAGGGTACAAA	66.4	23	*tpi*	230
	CATAATATTGGGTCTATTCCTAC	66.4	23		
Negative regulator of pathogenicity locus	TCTCTACAGCTATCCCTGGT3	68.2	20	*tcdC*	637-676
	AAAAATGAGGGTAACGAATTT	61.1	21		

2.1.6.1 PCR Mix Preparation

First, 2X Qiagen Multiplex PCR Master Mix (Mississauga, ON) and primers were removed from −20°C and thawed on ice. The PCR Master Mix was prepared according to Table 2-3. Barrier pipette tips were used to prevent contamination of micropipets. All solutions were vortexed prior to preparing the master mix. The master mix was kept on ice at all times during use and stored at −20°C.

Table 2-3. PCR reaction mixture.

Multiplex I		Multiplex II	
Component	**Volume (µL)**	**Component**	**Volume (µL)**
2X Master Mix (Qiagen)	12.5	2X Master Mix	12.5
cdtB F1/R1 (10 µM)	0.75	*tpi* (10 µM)	0.50
tcdA F/A3B (10 µM)	0.75	*Pal* 15/16 (10 µM)	0.50
tcdB 3+4 (10 µM)	0.50		
H_2O (autoclaved)	8.50	H_2O (autoclaved)	9.50
DNA	2.00	DNA	2.00

2.1.6.2 DNA extraction for PCR

DNA templates used in PCR were extracted using InstaGene™ Matrix (Bio-Rad, Mississauga, ON), modified from the manufacturer's protocol, described as follows. A colony was picked from CDMN plate after 48 h growth under anaerobic conditions and suspended in 5 mL BHI broth and incubated for 6-8 h. The BHI culture was taken out of the anaerobic chamber and into the biological safety cabinet (BSC). Four hundred µL of culture was transferred to a 1.5 mL microfuge tube and centrifuged at 15,000 x *g* for 2 min (another 400 µL was used for PFGE). The supernatant was then removed through aspiration by a pipet. The InstaGene™ Matrix was shaken vigorously to mix, and 200 µL was added to the pellet. The microfuge tube was then vortexed to mix, and placed in a boiling water bath for 30 min. The tube was then centrifuged at 14,000 x *g* for 1 min. Next, the DNA-containing supernatant was used to carry out PCR reactions.

2.1.6.3 Reaction Preparation

First, a 23-µL volume of thawed and vortexed master mix was dispensed into sterilized 0.2 mL PCR reaction tubes on ice. A 2 µL aliquot of template DNA (controls and samples) was added to make a total of 25 µL.

2.1.6.4 Amplification

The thermocyclers used include GeneAmp ® 9700 and Ventri 96 well model (both from Applied Biosystems, Streetsville, ON). The thermocyclers were programmed according to Table 2-4.

The PCR tubes were either analyzed immediately or removed from the thermocyclers and stored at −4°C after the PCR reaction.

2.1.6.5 Analysis

PCR products were analyzed by gel electrophoresis. A 1.5% agarose gel was prepared by mixing 1.5 g of agarose (Fisher Scientific, Ottawa, ON) in 100 mL 0.5X TBE buffer (0.045 M Tris-borate, 0.001 M EDTA, Fisher Scientific, Ottawa, ON) and heating in a microwave to dissolve. A 2-µL volume of bromophenol blue loading dye (0.35% w/v bromophenol blue, 40% w/v sucrose, Sigma-Aldrich, Oakville, ON) was added to 25 µL of amplicon, and 10 µL of the mixture was loaded per sample well. A 100 bp ladder (Invitrogen, Burlington, ON) was run alongside the samples. The gel was electrophoresed for 1 h at 120 V/cm, and then transferred to a 0.5 µg/mL ethidium bromide solution (Sigma-Aldrich, Oakville, ON) on a gyrating shaker and incubated for 30 min. Visualization was performed in an UV Multi-Image Light Cabinet (Alpha Innotech, San Leandro, CA).

Table 2-4. PCR conditions for detection of *C. difficile* toxins.

Cycles	Temperature (°C)	Time	Description
1	95	15 min	Initial denaturation
30	94	0.5 min	Denaturation
	57	1.5 min	Annealing
	72	1 min	Extension
1	72	7 min	Final extension
1	4	∞	Storage

2.1.7 Pulse Field Gel Electrophoresis (PFGE)

PFGE for molecular subtyping of *C. difficile* was carried as described previously [56]. Briefly, a colony was picked from a BAK agar plate after 48 h growth and suspended in 5 mL BHI broth and incubated for 6-8 h at 37°C. The BHI culture was

taken out of the anaerobic chamber and into the biological safety cabinet (BSC). Then, 400 µL of culture was centrifuged at 14,000 x *g* for 1 min. The supernatant was removed and the pellet resuspended in 150 µL of cell lysis buffer: 6mM Tris-HCl pH 8.0 (Sigma, Oakville, ON), 1.0M NaCl, 100mM EDTA (Sigma, Oakville, ON), 0.2% deoxycholate (Sigma, Oakville, ON), 0.5% N-Laurylsarcosine (Sigma, Oakville, ON), 0.5% Brij 58 (Sigma, Oakville, ON), and ddH_2O.

Next, 150 µL of melted 1% SeaKem Gold PFGE agarose (1.2% SeaKem Gold agarose, Cambrex Bio Science Rockland, Inc., East Rutherford, NJ, 1% SDS, 0.5X TBE, 0.045 M Tris-borate, 0.001 M EDTA, Fisher Scientific, Ottawa, ON) was added to the cell suspension. After mixing, 200 µL of the agarose mixture was dispensed into a plug mold (Bio-Rad, Mississauga, ON). After solidifying for 10 min at room temperature, the "plugs" were transferred into 500 µL of cell lysis buffer with 20 µg/mL RNase, 2 mg/mL lysozyme (Sigma, Oakville, ON), and 1.25 U/mL mutanolysin (Sigma, Oakville, ON). The tubes were then incubated overnight in a 37°C waterbath.

After overnight incubation (approximately 16 h), the tubes were removed from the waterbath and 500 µL of 50 µg/mL of proteinase K in ddH_2O (Sigma, Oakville, ON) was added, and incubated at 55°C for 2 h. The proteinase K-containing solution was then discarded, and the plugs rinsed with 1.5 mL of room temperature 1X TE (10 mM Tris-Cl, 1 mM EDTA, all pH 8.0) three times at 20 min intervals on a shaker. One of two agarose plugs was removed and one-third was excised and allowed to equilibrate in 150 µL of 1X buffer A (Roche #1417159, Mississauga, ON). The *Salmonella enterica* serovar Braenderup H9812 standard (used for all *C. difficile* PGFE) was equilibrated in 150 µL of 1X buffer H (Roche #1417991, Mississauga, ON). After 15 min, buffers were replaced

with 100 µL of fresh buffers (A or H) along with 40 U *Sma*I (Roche, Mississauga, ON) for *C. difficile* samples, and 40 U *Xba*I (Roche, Mississauga, ON) for the standard. The *Sma*I plugs were digested at 25°C overnight, and the *Xba*I plugs were digested in 37°C waterbath overnight (approximately 16 h).

The digested plugs were placed on a casting tray with combs (Bio-Rad, Mississauga, ON), and 100 mL of melted 1.0% SeaKem Gold PFGE agarose in 0.5X TBE (0.045 M Tris-borate, 0.001 M EDTA), held in 55°C waterbath to prevent solidification, was poured.

PFGE electrophoresis was setup with 2.0 L of fresh 0.5X TBE (0.045 M Tris-Borate, 0.001 M EDTA) and 1.0 mL of 100 mM thiourea (Sigma, Oakville, ON), and cooled at 14°C. The run conditions are listed in Table 2-5 (Bio-Rad CHEF DRIII/Bio-Rad CHEF Mapper PFGE systems, Mississauga, ON).

Table 2-5. PFGE conditions.

Description	Value
Initial A switch time	1.0 s
Final A switch time	40.0 s
Voltage	6
Included angle	120
Start ratio	1.0
Run time	22 h

The gel was stained with 0.5 mg/L of EtBr on a gyrating shaker for 30 min, counter-stained in ddH_2O for 30 min,and then visualized and digitized in an UV Multi-Image light cabinet (Alpha Innotech, Santa Clara, CA). Gel images were analyzed using BioNumerics version 4.6 software (Applied Maths, Austin, TX). Dendrograms were

generated using the Dice and UPGMA comparison settings with optimization and band position tolerance set at 1.0%.

2.1.8 *C. difficile* Growth Curves

Microbank™ vials (Pro-Lab Diagnostics, Richmond Hill, ON) containing culture stored in −80°C were thawed on ice. In an anaerobic chamber, one Microbank™ bead was dropped into 5 mL of pre-reduced BHI broth for startup culture and incubated for 24 h at 37°C. Next, 50 µL of this culture was transferred to another 5 mL of BHI broth and incubated for 24 h at 37°C to ensure actively growing cells.

A 20 µL sample of start-up culture (adjusted to 0.5 OD_{600}) was transferred into 20 mL of pre-reduced BHI broth in BD Falcon™ conical tubes (BD Biosciences, Mississauga, ON). Measurements were taken every 1 h including t = 0 h until the OD_{600} value remained stable for 2 successive measurements.

All growth curve assays were performed in triplicate under anaerobic conditions. Readings were made using the BioPhotometer and UVette® (Eppendorf, Mississauga, ON). In an UVette® tube, 100 µL of culture was dispensed every 30 min and OD_{600} measured with the BioPhotometer. All measurements were taken inside the anaerobic chamber to ensure growth consistency (cultures were never exposed to aerobic conditions).

2.1.9 RNA Extraction

RNA extraction was performed with Qiagen RNeasy© Mini Kit (Qiagen, Toronto, ON) following manufacturer's instructions described in protocols 5, 7, 8, and appendix B of RNAprotect© Bacteria Reagent Handbook with modifications for better RNA yield as described below.

For mid-log RNA extractions, 15 mL of culture grown in BHI broth at OD_{600} 0.4 were added to 2 volumes (30 mL) of RNAprotect® Bacteria Reagent (Qiagen, Toronto, ON) inside the anaerobic chamber and mixed immediately, followed by a 5 min incubation at room temperature. The mixture was centrifuged for 10 min at 5,000 x *g*. The supernatant was then removed by decanting and dabbing the inverted tube onto paper towels. Next, the remaining pellet was placed at -20°C for short-term storage for up to 2 weeks, or -80°C up to 4 weeks.

Next, 100 µL of TE buffer (30 mM Tris-Cl, 1 mM EDTA, pH 8.0) containing 15 mg/mL lysozyme (Sigma, Oakville, ON) was added to the thawed pellet, which was resuspended by pipetting the solution up and down. The suspension was then transferred to a microfuge tube, and 10 µL of 5 KU/mL mutanolysin (Sigma, Oakville, ON) and 20 mL of 20 mg/mL proteinase K (Sigma, Oakville, ON) were added. The mixture was then vortexed on high for 5 sec, and incubated on a shaker at 900 RPM for 1 h at room temperature.

Next, 700 µL of Buffer RLT with 10 µL β-mercaptoethanol (Sigma, Oakville, ON) added per 1 mL Buffer RLT was added to the lysis mixture and vortexed vigorously for 10 s. A 50-µL volume of acid-washed beads (212-300 µM, Sigma-Aldrich, Cat. # G1277, Oakville, ON) were added, and vortexed on high for 8 min. The tubes were then centrifuged for 10 s at 15,000 x *g*, and the solution was transferred to a new tube. A590 µL aliquot of 80% ethanol was then added to the supernatant and vortexed vigorously. Next, the 700 µL aliquot of lysate was added to an RNeasy Mini Column placed in a 2 mL collection tube, where the flow-through was discarded after each centrifugation (15 s at 8000 x *g*). Three hundred and fifty µL of Buffer RW1 was then spun through the

column for 15 s at 8000 x *g*. Next, 147 µL of Buffer RDD was added to 21 µL of DNase I stock solution (RNase-Free DNase Set, Qiagen, obtained by dissolving 1,500 Kunitz units (KU) of DNase I in 550 µL of RNase-free water, then fractionated into different tubes for storage) and mixed by gently inverting and tapping the tube; 80 µL of this DNase I incubation mix was then added directly to the column and incubated at room temperature for 30 min. Next, 350 µL of Buffer RW1 was added to the spin column which was allowed to sit for 5 min before being spun through the column for 15 s at 8,000 x *g*. The RNeasy® Mini spin column was then placed in a new 2 mL collection tube, to which 500 µL Buffer RPE was added and centrifuged for 15 s at 8,000 x *g*. The flow-through was discarded, and another 500 µL of Buffer RPE was added, followed by a 2 min centrifugation at 8,000 x *g*. The RNeasy® Mini spin column was placed in a 1.5 mL collection tube, and 50 µL of RNase-free water was added to the column twice, with centrifugation at 8,000 x *g* for 1 min in between the additions. The enriched total RNA was used immediately in the DNA subtraction step (described below).

2.1.10 DNA Subtraction of Total RNA

Following RNA extraction, elimination of DNA was carried out using the TURBO DNA-free™ Kit (Ambion®, Streetsville, ON). Manufacturer's instructions were followed with modifications, described below.

First, 0.1 volume of 10x TURBO DNase Buffer was mixed by vortexing and then added to the RNA extracted using the Qiagen RNeasy© Mini Kit suspended in RNase-free water. A 40-µL volume of TURBO DNase was added to the RNA and incubated at 37°C for 45 min, with periodic gentle shaking by hand to mix. Next, 3 µL TURBO DNase was added to the reaction, and incubated for another 45 min. A 0.2-volume of

resuspended DNase Inactivation Reagent was added to the reaction and incubated for 5 min at room temperature with occasional mixing so as to not let the reagent settle. The mixture was then centrifuged at 10,000 x *g* for 1.5 min, followed by a repeat treatment with 0.1 volume of resuspended DNase Inactivation Reagent to ensure complete inactivation and removal of unwanted particles. The supernatant was then transferred to a new tube and stored at -80°C.

Removal of DNA was checked by PCR amplification of the *tpi* gene (section 2.1.6). Multiplex II was used to check for DNA contamination. PCR amplification products were analyzed by gel electrophoresis with 1% agarose as previously described in section 2.1.6. No band at *tpi* was interpreted as good DNA removal. *C. difficile* sample #11ACD0028 (NAP1) was used as a positive control, and sterile ddH_2O as a negative control.

2.1.11 rRNA Subtraction

MICROB*Express*™ (Ambion®, Streetsville, ON) was used to enrich bacterial mRNA from purified total RNA. rRNA subtraction was carried out following manufacturer's instructions with modifications, as described below.

Prepared total RNA after DNA removal was concentrated to 0.8 µg/µL using the SpeedVac® Plus concentrator (SC210A, Thermo Scientific), and the volume was adjusted with nuclease-free H2O to 0.8 µg/µL, if necessary. A 15-µL volume of purified total RNA was added to 200 µL of Binding Buffer in the 1.5 mL tube provided with the MICROB*Express*™ kit and gently vortexed. A 4-µL aliquot of Capture Oligo Mix was added, and the tube was vortexed again. The mixture was heated to 70°C in a water bath

for 10 min to denature secondary structures, and then placed in a 37°C waterbath for 15 min to allow hybridization to take place.

The Oligo MagBeads were prepared by briefly vortexing. Next, 50 µL per sample was withdrawn and transferred to a 1.5 mL tube. The tubes were then placed on a magnetic stand (Ambion®, Streetsville, ON) for 3 min to capture the beads. The supernatant was removed by aspiration and discarded. The same volume (50 µL) of nuclease-free water was then added to wash the beads by resuspension through vortexing. The beads were recaptured by placing the tubes on the magnetic stand, and the nuclease-free water was discarded. The washed Oligo MagBeads were then washed once with Binding Buffer in the same manner, captured, and the supernatant discarded, Finally, the beads were equilibrated by adding a volume of Binding Buffer equal to that of the original volume and then resuspending by vortexing, and incubating to 37°C.

The wash solution was heated to 37°C in a waterbath before rRNA capture. Next, 50 µL of prepared Oligo MagBeads was added to each RNA/Capture Oligo Mix sample and vortexed to mix, followed by incubation at 37°C for 15 min. The Oligo MagBeads were then captured once again by placing on the Ambion® Magnetic Stand for 3 min. The supernatant, containing the enriched mRNA, was transferred to a new tube on ice. Any remaining mRNA was obtained by addition of 100 µL Wash Solution to the Oligo MagBeads, resuspended and then captured on the Magnetic Stand for 3 min, and the supernatant was pooled with the collection tube on ice. The enriched mRNA was precipitated by first adding 35 µL of 3M sodium acetate and 7 µL glycogen, and vortexed gently to mix. A 1175-µL aliquot of −20°C 100% ethanol was added to the tube which was mixed by vortexing, and then allowed to incubate at -20°C for 1 hr to precipitate then

RNA. The tube was centrifuged at 15,000 x *g* for 30 min. After discarding the supernatant, two consecutive 70% ethanol washes were performed by adding 750 µL ice cold 70% ethanol, vortexing, centrifuging at 15,000 x *g* for 5 min, and then discarding the supernatant. The tube was re-spun briefly, and any remaining supernatant was discarded with a pipet. The pellet was then air-dried for 5 min.

The dried pellet was resuspended in 10 µL of nuclease-free water by rehydrating for 15 min at room temperature. Any residual Oligo MagBeads were removed by placing the tube on the magnetic stand for 3 min and the enriched supernatant was moved to a new tube. Enriched mRNA was either used immediately for cDNA synthesis or stored at -80°C for a maximum of 2 weeks.

2.1.12 cDNA Synthesis

The SuperScript™ Double-Stranded cDNA Synthesis Kit (Invitrogen, Burlington, ON) was used for cDNA synthesis from enriched and purified mRNA. A random 12mer primer was used, which was synthesized by the DNA Core in the National Microbiology Laboratory (Winnipeg, MB). Manufacturer's instructions were followed with modifications as detailed below.

2.1.12.1 First strand synthesis

In a nuclease-free 1.5 mL microfuge tube on ice, 1 µL of primer (100 pmol/µL) and purified mRNA in DEPC-treated water (volumes depend on amount of starting mRNA, up to 5 µg) were combined. Therefore, the combined volume of primer and mRNA in DEPC-treated water varies from 7 to 11 µL (all other reagent volumes were constant). This mixture was heated to 70°C for 10 min then quick-chilled on ice. The contents were collected by brief centrifugation, and the following were added: 4 µL 5X First-Strand

Reaction Buffer, 2 µL 0.1 M DTT, and 1 µL 10 mM dNTP mix (all included with kit). The total volume now varies from 15 to 19 µL, depending on the initial amount of mRNA.

The tube was equilibrated at 45°C for 2 min before addition of SuperScript™ II RT reagent (included with kit) and an equal volume of T4 Gene 32 Protein (10 µg/µL, New England BioLabs® Inc., Pickering, ON). The contents were mixed gently, and incubated at 45°C for 1 h. Next, a second volume of SuperScript™ II RT and an equal volume of T4 Gene 32 Protein were added, and incubated at 45°C for one hour. The tube was then immediately placed on ice to terminate the first strand synthesis.

2.1.12.2 Second strand synthesis

A thermocycler was pre-cooled to 16°C. On ice, the following reagents were added to the first reaction tube (20 µL): 30 µL 5X Second-Strand Reaction Buffer, 3 µL 10 mM dNTP mix, 1 µL *E. coli* DNA Ligase (10 U/µL), 4 µL *E. coli* DNA polymerase, 1 µL *E. coli* RNase H (2U/µL), and DEPC-treated water to a final volume of 150 µL (all included in kit). The contents were gently vortexed, and incubated for 2 h at 16°C. Then, 2 µL (10 U) of T4 DNA polymerase (included with kit) was added and incubated for another 5 min at 16°C.

2.1.12.3 Phenol/chloroform extraction and alcohol precipitation

A 160-µL volume of 25:24:1 phenol:chloroform:isoamyl alcohol (Sigma) was added to the tubes containing the mixture and vortexed thoroughly. The tube was then centrifuged for 5 min at 14,000 x *g*. Next, 140 µL of the top (aqueous) layer was transferred to a fresh new tube, and 160 µL of 24:1 chloroform/isoamyl alcohol (Sigma) was added, vortexed and centrifuged again at 14,000 x *g* for 5 min. The top layer was

removed and transferred to a new tube. Next, a 1/10th volume of 3 M sodium acetate was added and vortexed briefly. Three volumes of ice cold 100% ethanol were to each tubeadded and vortexed thoroughly. The tube was moved to a -20°C freezer for 1 h, centrifuged for 30 min at 15,000 x *g* and the supernatant discarded. Two 70% ethanol washes were then performed by addition of 750 µL ice cold 70% ethanol, vortexing briefly, and centrifuging for 5 min at 15,000 x *g*. The supernatant was removed by aspiration. After the second 70% ethanol wash, the tube was briefly centrifuged to collect any residual liquid, which was removed with a pipet, before allowing the pellet to air dry for 10 min at room temperature. The pellet was resuspended in nuclease-free water (3 µL per starting µg of mRNA). The quality was then checked with Agilent 2100 Bioanalyzer (section 2.1.5).

2.2 Metronidazole Study

2.2.1 Origin of Isolate

In 2009, a *C. difficile* isolate from a stool sample sent to our laboratory from British Columbia, Canada was found to have reduced susceptibility to metronidazole when the stool specimen was directly plated on to CDMN agar containing with 8 µg/mL of metronidazole (MET). This isolate exhibited an MIC to metronidazole of 32 µg/mL by Etest®.

2.2.2 Revertant Protocol

Previous studies have reported that freeze/thawing and repeated passages of *C. difficile* with reduced susceptibility to MET reduced its MIC (a revertant). Thus, we hypothesized that if we could obtain a revertant from our original isolate exhibiting reduced susceptibility to MET, we would then be able to sequence both the original and revertant strains, and compare its genomes to elucidate a mechanism to MET-resistance. This section describes the protocol used to obtain a revertant.

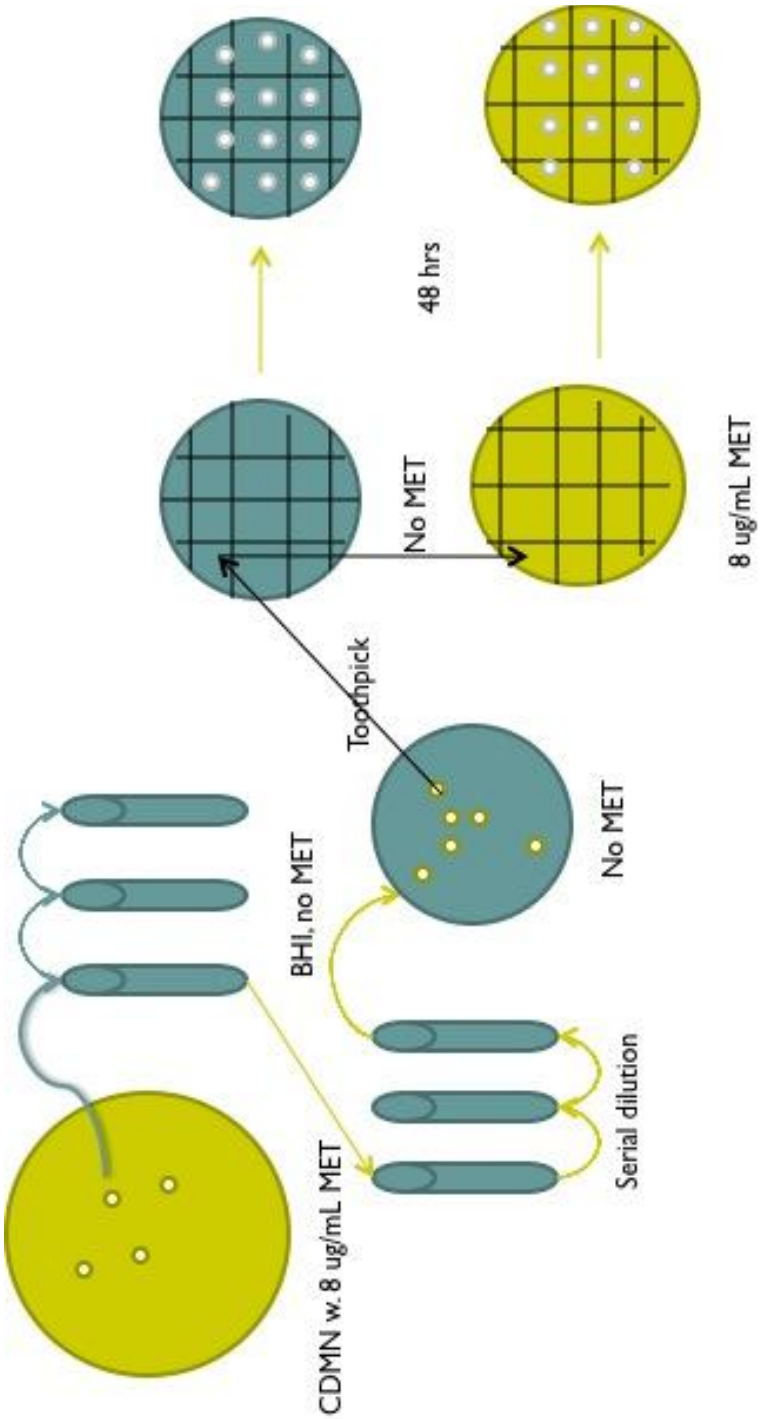
After 48 h growth, one *C. difficile* colony was picked from a BAK plate with 8 µg/mL of metronidazole and suspended in 5 mL of BHI without metronidazole (pre-reduced for 24 h). After 48 h incubation, a 50-µL volume of culture was transferred to a fresh 5 mL BHI broth, and 1 mL of culture was serially diluted (from 10^{-1}) to 10^{-5}. A 100 µL aliquot of each dilution was plated on BAK plates without metronidazole. After 48 h incubation, 32 single colonies from the plate with the best colony separation (usually 10^{-3} to 10^{-4} dilutions with 100-300 colonies) were picked using sterile toothpicks onto two BAK plates without metronidazole and two BAK plates with 8 µg/mL metronidazole (total of 64 colonies picked). The plates were scored into 16 sections (3 lines x 3 lines),

and after a colony was picked with a toothpick, and streaked onto a BAK plate, then the same toothpick was used to streak a BAK plate with 8 µg/mL metronidazole at the same plate coordinate. After another 48 h incubation, the plates with and without metronidazole were compared. Streaks that grew on plates without metronidazole but not plates with metronidazole were subcultured and further tested to confirm any loss of the resistance phenotype.

This process was repeated, using the continuous culture from 5 mL BHI (described above) every 48 h until a revertant was found. The revertant was characterized by a streak of growth on the metronidazole negative plate but exhibited no growth on the plate with metronidazole.

Figure 2-1 provides a schematic of the protocol described above.

Figure 2-1. Schematic of protocol for obtaining a metronidazole (MET) *C. difficile* revertant from a strain that exhibited reduced susceptibility to MET.



2.2.3 MIC Testing

2.2.3.1 Etest®

Etest® antibiotic strips were obtained from BioMérieux (St. Laurent, QC). Manufacturer's protocols were followed.

Recovering isolates from frozen storage. Frozen isolates were first passaged twice on BAK prior to Etest® with 48 h incubation at 37°C in between.

Inoculum preparation. At the time of testing, BioMérieux did not specify a control for the testing of Gram-positive anaerobes with metronidazole. Therefore, *B. fragilis* ATCC® 25285 (ATCC, Burlington, ON) was used as the standard. Isolates were selected from 48 h growth at 37°C on BAK (after two serial transfers). Portions of 5 or more well isolated colonies at least 1 mm in diameter and similar in morphology were picked and suspended into Brucella broth and adjusted to 1.0 McFarland using a visual standard (Remel 1.0 McFarland Equivalence Turbidity Standard and Visual Comparison Card). A sterile cotton swab was dipped into the 1.0 McFarland suspension and pressed against the inside wall of the tube to remove excess fluid. This swab was used to streak the agar surface in three directions for a confluent lawn. Forceps were used to apply the strip, making sure whole length of strip is in complete contact with the agar surface, and movement does not occur after application. Plates were incubated in an inverted position under anaerobic growth conditions for 48 h. MIC values were read such that the edge of the inhibition ellipse intersects the strip. If subpopulations were seen inside the primary zone of inhibition, the edge of the highest growth was used for readings, as recommended by BioMérieux.

2.2.3.2 Agar Dilution

Protocols described by CLSI [160] were used to carry out agar dilution methods for testing of MIC. As per CLSI recommendations, frozen isolates were passaged twice on metronidazole-free BAK media prior to carrying out agar dilution MIC testing. Inocula were prepared under anaerobic conditions. As per CLSI guidelines, *C. difficile* ATCC® 700057 (ATCC, Burlington, ON) was used as the control. Isolates were selected from 48 h growth at 37°C on BAK (after two serial passages). Portions of 3-5 well isolated colonies at least 1 mm in diameter and similar in morphology were picked and s uspended into Brucella broth and adjusted to 0.5 McFarland using a visual standard (Remel 0.5 McFarland Equivalence Turbidity Standard and Visual Comparison Card). A Steers Replicator was used to inoculate plates under anaerobic conditions. A 0.5-mL aliquot of a 0.5 McFarland suspension was transferred to wells of the Steers Replicator. Starting from control plate without antibiotics, plates were manually stamped with the replicator on plates with doubling dilutions from 0.5 to 64 µg/mL of metronidazole. Plates were incubated in an inverted position under anaerobic growth conditions for 48 h at 37°C. MIC endpoints for agar dilution were read according to CLSI guidelines, with the lowest concentration of antibiotic preventing growth as the MIC.

2.2.4 Growth Assay

For growth curves of metronidazole-related strains, the protocol for freezer-stored culture was described previously in section 2.1.8. To ensure growth consistency, cells frozen using Microbank™ beads were taken through two growth cycles (24 h) before being used in a growth curve assay. All instruments and solutions, including the Eppendorf BioPhotometer (Eppendorf, Mississauga, ON) were placed inside the

anaerobic chamber during the growth assay. For culture not stored in the freezer (i.e. persistently passaged on plates), a sterile loop was used to transfer cells from a colony on the plate to 5 mL of pre-reduced BHI broth and incubated for 24 h at 37°C. Next 50 µL of this culture in BHI was transferred to another 5 mL BHI broth and incubated for 24 h to ensure growth consistency. The OD_{600} absorbance readings were made using the BioPhotometer, described in section 2.1.8. Measurements were taken every hour. Again, all growth curve assays were performed in triplicates under anaerobic condition and terminated when OD_{600} remained stable for 2 consecutive readings.

2.2.5 DNA Extraction and Sequencing

Methods for DNA extraction and sequencing were described in section 2.1.4 above.

2.2.6 Comparative Genomic Analyses

2.2.6.1 Genome Sequencing and Assembly

Sequencing was performed by the DNA Core Facility (National Microbiology Laboratory) on the 454 Genome Sequencer (GS) FLX using the GS Titanium Sequencing Kit and GS Rapid Library Kit, and assembled with Newbler software package v2.3 (all by 454 Life Sciences, Roche, Branford, CT). Metrics for the assemblies are described in section 3.2.7.

2.2.6.2 Genome Alignment and Primer Design

Sequenced genomes of *C. difficile* isolates were aligned with Mauve Genome Alignment Software (http://gel.ahabs.wisc.edu/mauve/) by DNA Core (National Microbiology Laboratory), and exported to MEGA 4 (Molecular Evolutionary Genetics

Analysis, http://www.megasoftware.net/) software for viewing of the aligned genomes. All regions of heterogeneity between MetR_stable and MetR_rev, for example SNPs, IN/DELs, unmatched and mismatched regions in the alignment were first recorded (Table 3-4) with respect to their base pair location in the genome. A BLAST search (megablast) against NCBI's nucleotide collection (nr/nt) was performed on all regions where a difference existed between the two sequences. PCR amplification and sequencing of the amplicons were then performed on these putative genomic differences to assess if they were true differences or simply sequencing artifacts. Primers were designed as follows:

1. Approximately 500 bases in both directions of the target sequence/base(s) were copied into the web-based Primer3 primer design software (http://frodo.wi.mit.edu/primer3/).
2. Target sequence and/or bases were closed off with square brackets [ ], primer T_m (melting temperature) were set as min:60 opt:65 max:70, both left and right primers options were selected, and all other options were left to their default settings. This method returned primers most of the time. However, when the web engine did not return any useful results, the parameters were modified until a set of primers was generated.

2.2.6.3 PCR Amplification

PCR amplification was performed on regions where genomic heterogeneity existed between MetR_stable and MetR_rev sequences, as described previously. The default PCR reagents and reaction conditions are listed in Table 2-6 and Table 2-7, respectively.

Table 2-6. Reagents used in PCR amplification.

Volume (µL)	Material
25	2x buffer with $MgCl_2$
2.5	Primers (10 µM, 0.5 µM final)
0.5	AmpliTaq Gold ◆ (5U/µL, 1.25 U final)
5	DNA
17	ddH_2O

◆ AmpliTaq Gold (Applied Biosystems, Burlington, ON)

Table 2-7. PCR reaction conditions.

Cycles	Temperature (°C)	Time	Description
1	95	15 min	Initial denaturation
35	95	30 sec	Denaturation
	60◆	1 min	Annealing
	72	2 min	Extension
1	72	7 min	Final extension
1	4	∞	Storage

◆Annealing temperature varies based on primer.

Viable PCR products were considered to be a single prominent band on an agarose that corresponds to the expected size approximated when designing PCR primers in Primer3. For reactions that did not achieve viable amplicons, the PCR conditions were modified until the reaction returned viable products. For example, if weak, single bands were observed after gel electrophoresis, the number of cycles would be increased from 35 to 40, whereas for amplicons that were expected to be longer than 1 kb, the extension time was increased at approximately 1 min per 1 kb. If amplification failed after many attempts, a new set of primers was designed based on different regions of the gene, with or without different Primer3 parameters.

2.2.6.4 Nucleic Acid Recovery

From the 50 µL volume of amplicon, 10 µL was used in gel electrophoresis to check the integrity of the product (40 µL remaining). Using Millipore™ Microcon® Centrifugal Filter Devices, amplified nucleic acids were concentrated and purified for sequencing as follows: 160 µL of dH_2O was added to 40 µL of amplicon, then loaded to the filter provided in the tubes; the filter tubes were spun at 500 x *g* for 10 min, then placed upside down in a new tube; finally, 20 µL of dH_2O was added to elute the nucleic acid by spinning at 1,000 x *g* for 6 min.

The minimum required concentration for sequencing was 50 ng/µL of DNA, and 1 µM each of the forward and reverse primers. The concentrated and purified amplicons were sent to the DNA Core (NML) for sequencing.

2.2.6.5 Sequence Analysis of PCR Amplicons

Sequence results were edited and analyzed using Lasergene® SeqMan Pro™ (DNASTAR, Madison, WI). The sequences of the PCR amplicons were compared with sequences in MEGA 4 to confirm (or deny) sequence variations between strains. In other cases, sequenced PCR amplicons were used to fill in gaps in the sequences.

3 RESULTS

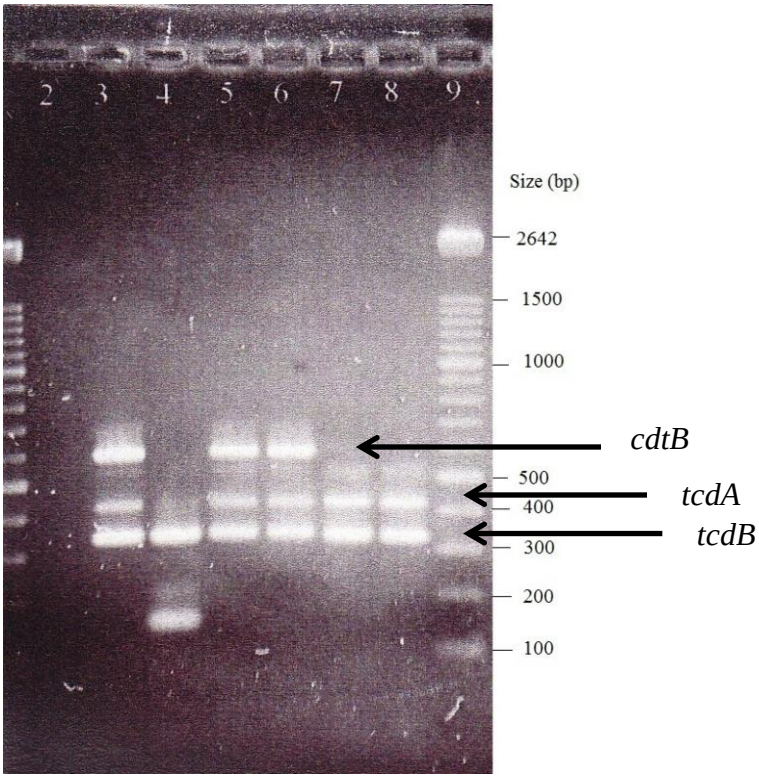
3.1 Transcriptomics Study

3.1.1 Molecular Characterization of *C. difficile* Isolates

Upon receiving VLOO13 and VLOO18 isolates, PCR and PFGE were performed to verify and characterize these strains against other strains in our databases. Figure 3-1 depicts digitized images of gels displaying the toxin profiles of VLOO13 and VLOO18. For PCR, Multiplex I (Figure 3-1A) assessed the presence or absence of binary toxin (*cdtB*), toxin A (*tcdA*), and toxin B (*tcdB*). Multiplex II (Figure 3-1B) on the other hand, tested for presence or absence of *tpi* (triose phosphate isomerase), and toxin C (*tcdC*), including its length and deletions, if any. VLOO13 showed a profile indistinguishable from a reference NAP1 control strain run alongside on the gel: *cdtB*+, *tcdA*+, *tcdB*+, *tpi*+, *tcdC* deletion. VLOO18 showed a profile consistent with NAP2 strains: *cdtB*-, *tcdA*+, *tcdB*+, *tcdC* (highest on the gel on multiplex II). These are depicted in Figure 3-1.

Figure 3-1. Gel electrophoresis results of PCR multiplexes conducted on *C. difficile* strains VLOO13 and VLOO18. (A) Multiplex I: lanes 1 and 9 contain 100bp ladder; lane 2 is the negative control; lane 3 is the positive control (NAP1); lane 4 is the positive control (Tox A-/B+); lanes 5 and 6 are duplicates of VLOO13; lane 7 and 8 are duplicates of VLOO18. (B) Multiplex II: lanes 1 and 13 contain 100bp ladder; lane 2 is the negative control; lanes 3 and 10 are the NAP1 positive controls; lanes 4 and 11 are the Tox A-/B+ positive controls; lanes 5 and 12 are the NAP8 positive controls. Lanes 6 and 7 are duplicates of VLOO13. Lanes 8 and 9 are duplicates of VLOO18.

A


2 3 4 5 6 7 8 9
Size (bp)
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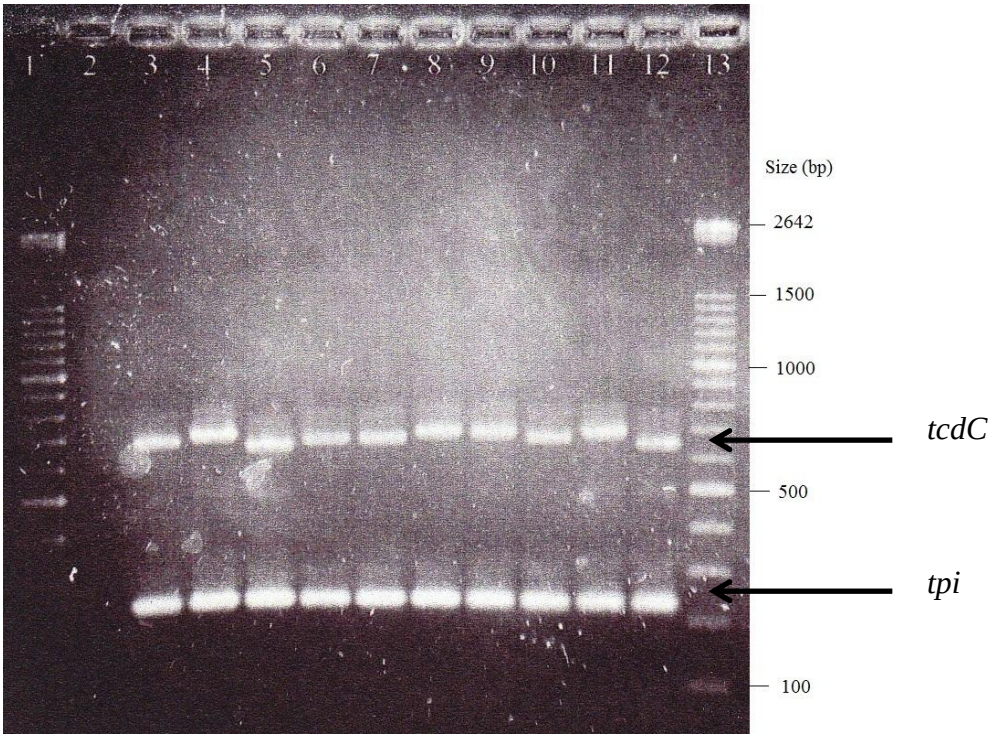
1500
1000
cdtB
500
tcdA
400
tcdB
300
200
100

B


1 2 3 4 5 6 7 8 9 10 11 12 13
Size (bp)
2642
1500
1000
tcdC
500
tpi
100

3.1.2 PFGE

PFGE was performed on *C. difficile* strains VLOO13 and VLOO18, the results were then analyzed with BioNumerics (Applied Maths, Austin, TX). VLOO13 had a digestion pattern consistent with North American Pulse-field (NAP) type 1 (PFGE type # 0001), whereas VLOO18 had a pattern consistent with NAP2 (PFGE type # 0005). The PFGE gel image is shown in Figure 3-2. Additionally, we compared isolates VLOO13 and VLOO18 to the national database of *C. difficile* PFGE fingerprints for NAP1 and NAP2, respectively. The generated dendrograms are presented in Figures 3-4 (VLOO13) and 3-5 (VLOO18) with settings described previously in section 2.1.7.

Figure 3-2. PFGE fingerprinting of *C. difficile* strains VLOO13 and VLOO18. Lanes 1 and 4 contain *Salmonella enterica* serovar Braenderup H9812 standard. Lane 2 contains VLOO13. Lane 3 contains VLOO18.

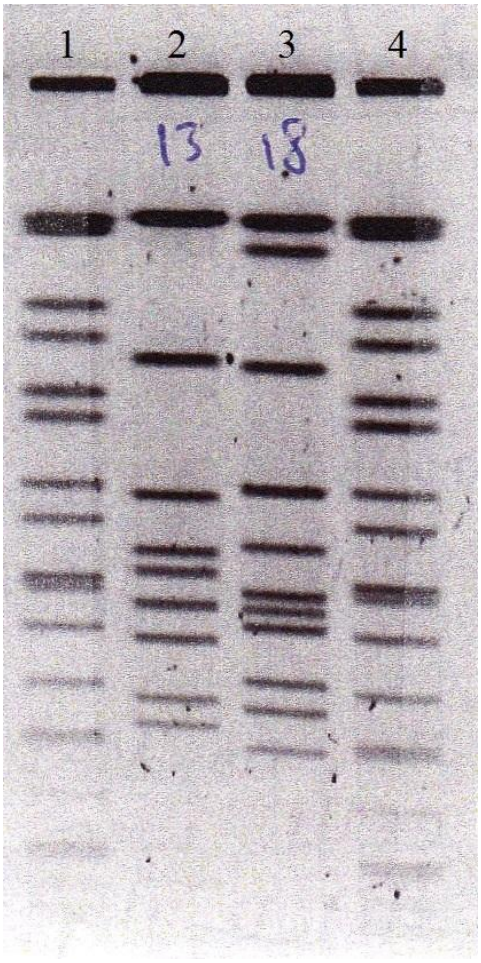


Figure 3-3. PFGE fingerprinting of VLOO13 against other NAP1 strains.

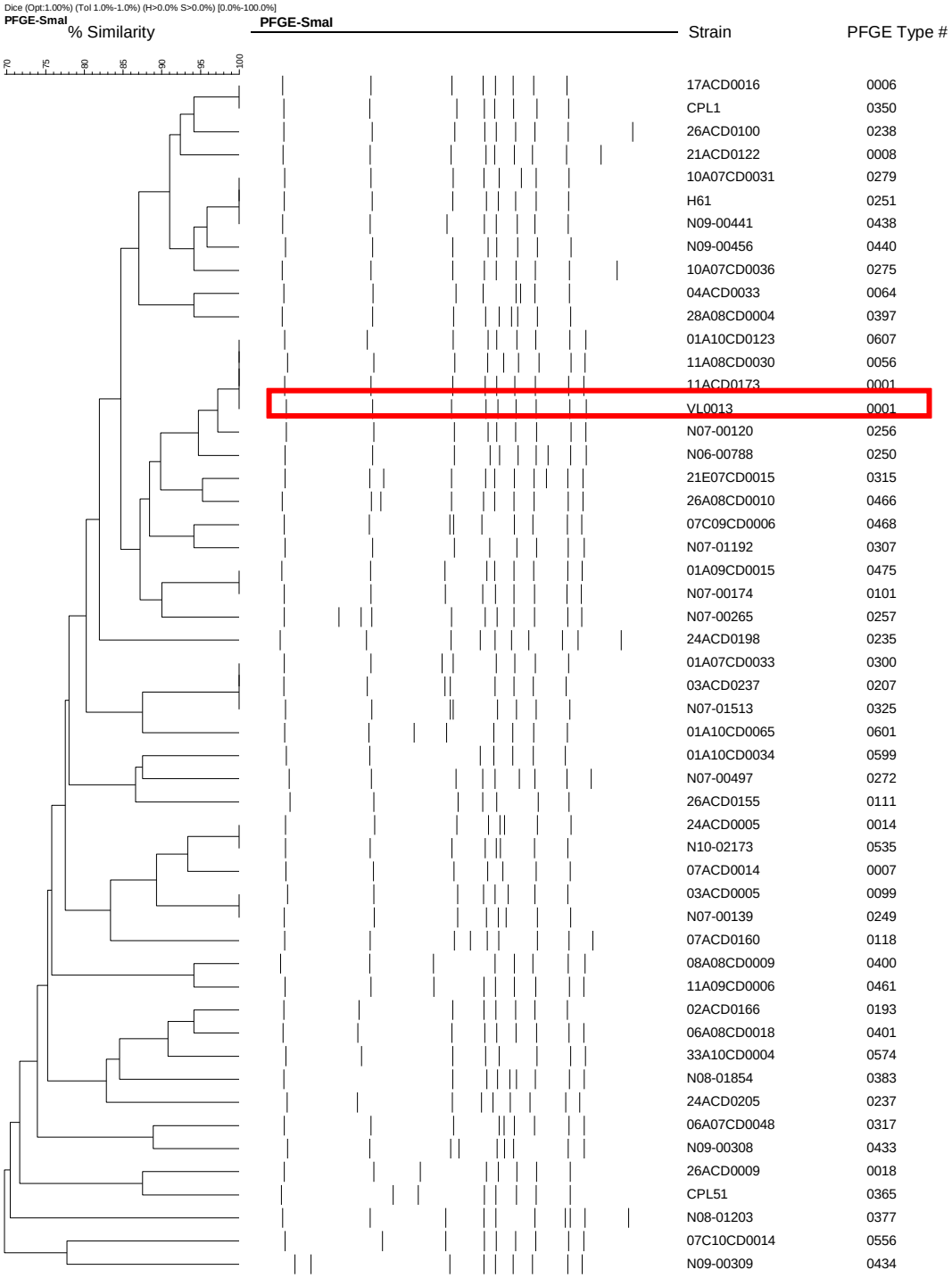
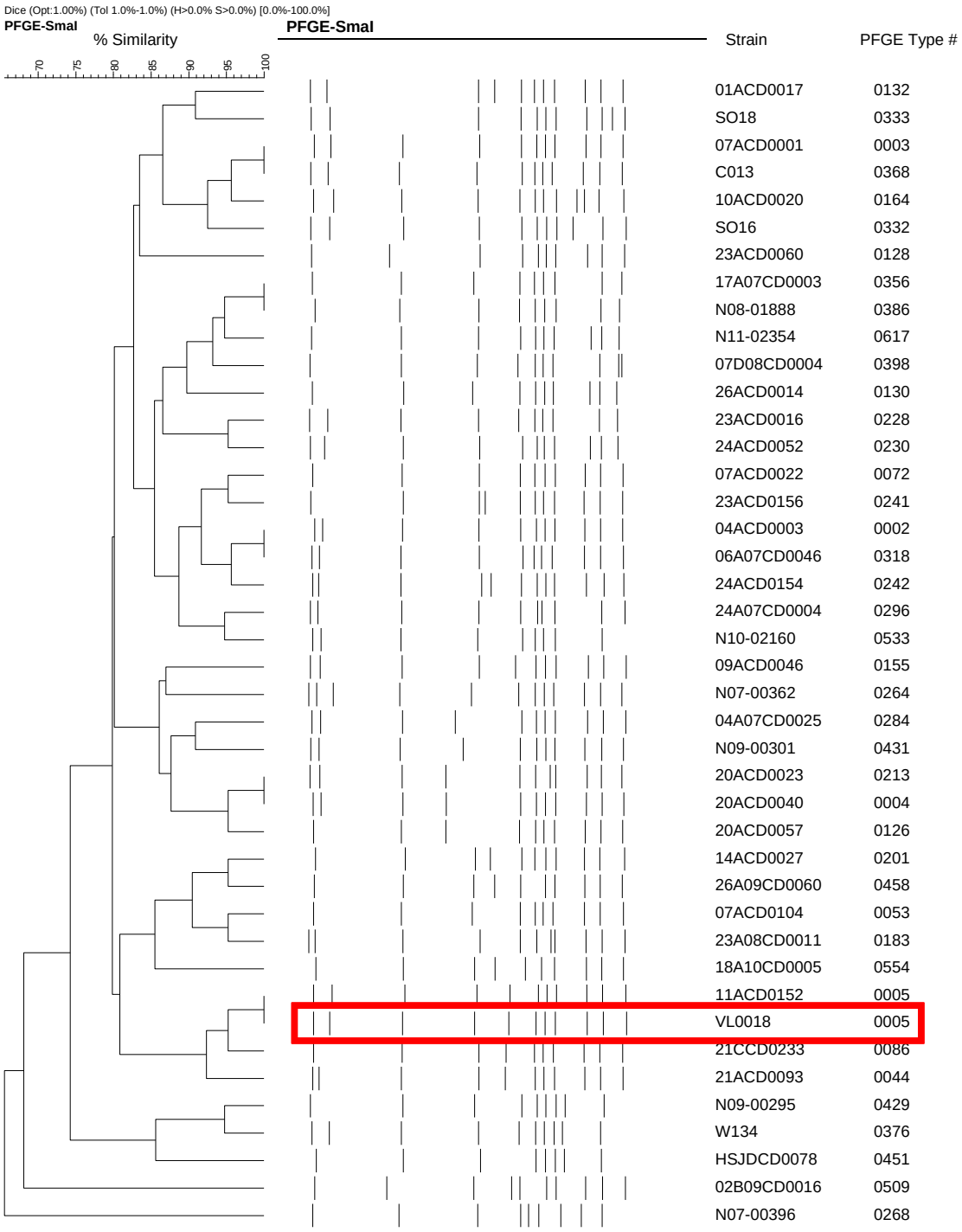


Figure 3-4. PFGE fingerprinting of VLOO13 against other NAP2 strains



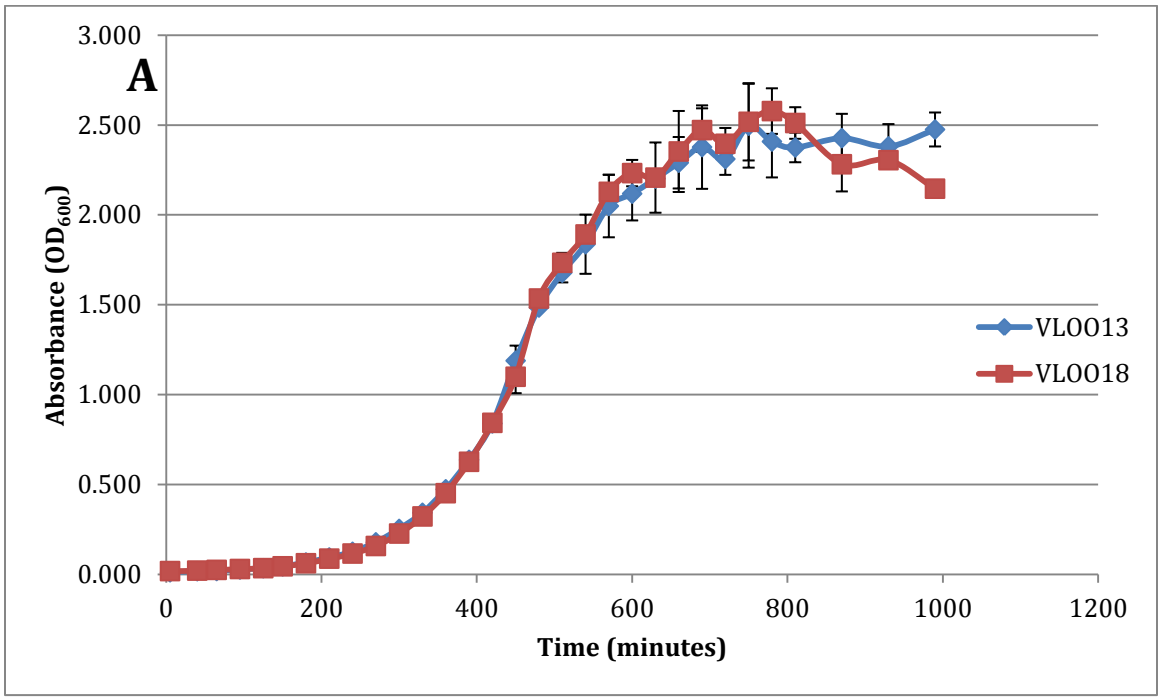
3.1.3 Growth Assay

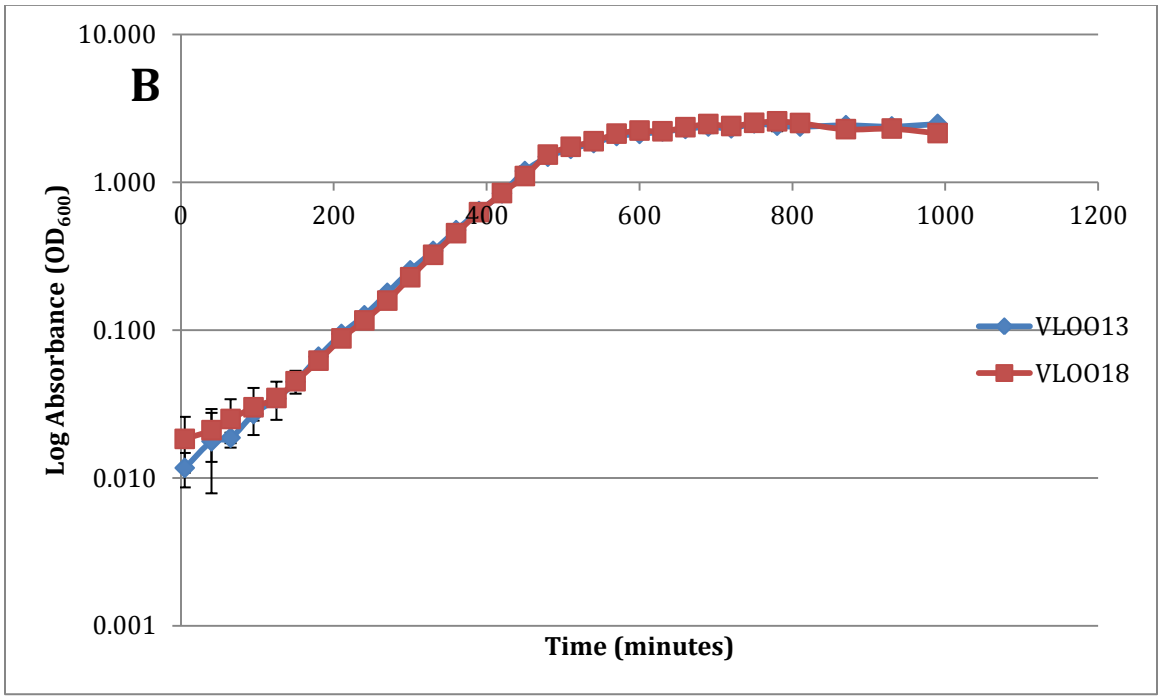
Growth assays were performed for strains VLOO13 and VLOO18 to better understand their growth kinetics, and to evaluate whether there are any differences in growth which may have contributed to differences in their virulence observed as has been previously reported (described previously in section 1.2.1)

Figure 3-5 depicts the growth curves for *C. difficile* strains VLOO13 and VLOO18. The cell density was measured at OD_{600}. Both strains exhibited virtually the same growth pattern. Mid-log was achieved approximately 7 h after the inoculum was introduced into the growth media, at an OD_{600} of approximately 0.4. Both strains reached stationary phase in approximately 10 h.

Figure 3-5. Growth curves of *C. difficile* strains VLOO13 and VLOO18

Standard growth curve with 2 SD error bars depicted as (A) OD_{600} versus time and (B) a semi-logarithmic graph of log (OD_{600}) against time (min) using the same data points.





3.1.4 DNA Extraction

The InstaGene™ Matrix used was to extract DNA for PCR purposes. However, it was not used for DNA extraction for genome sequencing projects due to potential lower quality DNA (presence of unwanted solutes in the InstaGene™ solution). DNA extraction of cells harvested more than 12 h into the *C. difficile* growth cycle yielded DNA that was poor in both quantity and quality when measured using the NanoDrop™ 1000 Spectrophotometer (results not shown). Therefore, cells harvested between 6-8 h of growth were used for DNA extraction. This was the case for both DNA extracted using the InstaGene™ protocol for PCR applications as well as the phenol/chloroform method for sequencing purposes.

3.1.5 RNA Extraction

One particularly challenging and time-consuming aspect of this thesis work was to develop a method for the extraction of both high yield and high quality RNA from the *C. difficile* strains provided. Doing so would eventually allow us to synthesize quality cDNA from the transcriptomes of the VLOO13 and VLOO18 strains for massively parallel sequence analysis for use in transcriptomics studies. The Qiagen RNAprotect® Bacteria Reagent Handbook provided protocols dealing with Gram-positive and Gram-negative bacteria grown in a variety of media types; however, none were specific to *C. difficile*.

Initially, Protocol 4 – Enzymatic Lysis and Proteinase K Digestion of Bacteria (without mechanical disruption) was used. The enzymatic lysis step digested the cell wall with lysozyme, while proteinase K digestion removed proteins to improve purity and yield of RNA. However, after many trials with different enzyme and cell concentrations,

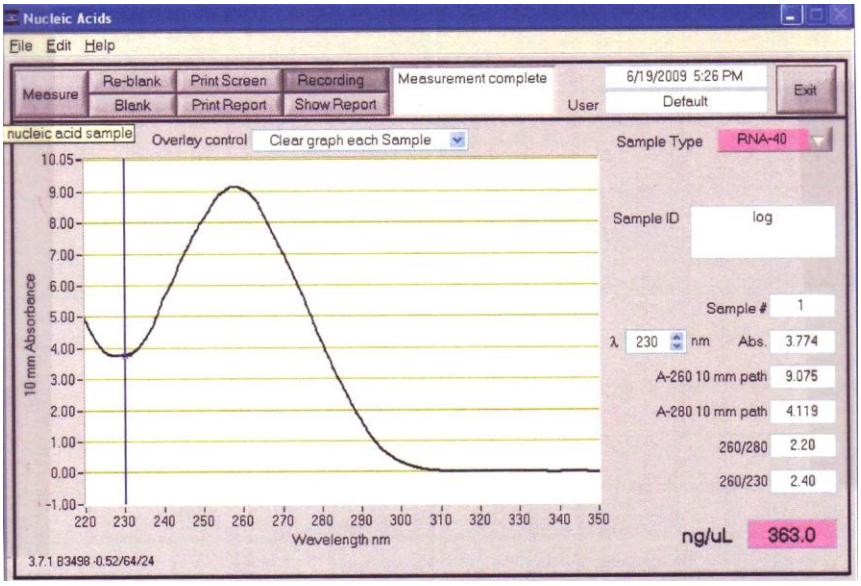
as well as varying digestion times (even overnight digestion), both the yield and purity did not reach satisfactory levels, in that the 260/230 nm ratio was far below 2 (the generally accepted value for a good quality sample). In addition, less than 5 μg of mRNA would have been obtained after the rRNA removal procedure (see Figure 3-6), which is the minimum requirement for massively parallel sequencing. Therefore, Protocol 5 (Enzymatic Lysis, Proteinase K Digestion, and Mechanical Disruption of Bacteria) from the RNAprotect® handbook was partially modified, to incorporate acid-washed beads (Sigma-Aldrich, Mississauga, ON) in addition to enzymatic lysis and proteinase K digestion. The quantity of lysozyme, mutanolysin, proteinase K, glass beads, and digestion time were further adjusted through trial-and-error until the yield did not significantly improve.

We initially had hoped to study mid-log and stationary phase transcriptomes of our *C. difficile* isolates to better understand this organisms and its pathogenesis since for example, NAP1 isolates have been shown to produce more toxins throughout its growth cycle compared to NAP2 isolates, where toxins are primarily produced near stationary phase. Due to the relatively unstable nature of RNA compared to DNA and the ubiquitous nature of RNases, both mid-log and stationary phase cells were stabilized with RNAprotect® to maintain the integrity of RNA. The RNAprotect® reagent was added to cells at an OD_{600} of 0.4 (mid-log), and stationary cells at an OD_{600} of 2.0. These values were chosen based on growth curves generated prior (Figure 3-5). The same RNA extraction protocol was carried out on both sets of cells, however, the RNA content in the mid-log sample was approximately four times higher in all repeated extractions; an example is shown in Figure 3-6. In Figure 3-6A (RNA from mid-log growth), both the

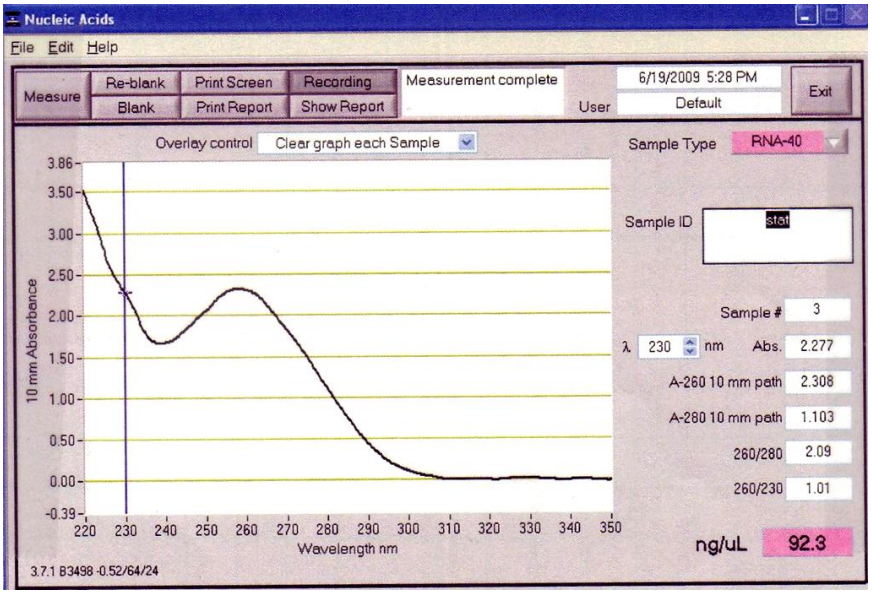
260/280 and 260/230 values were greater than 2, which is generally accepted as pure for RNA according to the manufacturer's manual. In Figure 3-6B (RNA from stationary phase), the 260/230 value was approximately 1, and the yield was fourfold less. The purity of the mid-log RNA was much higher using 260/280 and 260/230 measurements, with values >2.0 for mid-log RNA, which was much higher than those obtained for the stationary phase RNA.

Figure 3-6. Absorption profiles of *C. difficile* RNA extracted at (A) mid-log and (B) stationary phase.

A



B



3.1.6 DNA Subtraction

Since the transcriptome sequencing process is sensitive to DNA contamination, obtaining high quality, pure RNA without any DNA contamination represented another challenge problem for this project. Due to the relative instability of RNA compared to DNA, we wanted to subject our samples to as little processing as possible, both in terms of time and number of procedures. According to the Qiagen RNAprotect® Bacteria Reagent Handbook 2nd ed. (www.qiagen.com/literature/render.aspx?id=179), "DNase digestion is not required when purifying RNA using RNeasy Kits, since RNeasy silica-membrane technology enables efficient removal of most of the DNA without DNase treatment." However, after performing PCR amplification of our samples using multiplex II to check for presence of the *tpi* gene as an indicator of DNA presence (described previously in sections 2.1.6 and 2.1.10), I found DNA contamination in my samples (Figure 3-7A). This occured after using the RNeasy Kits, as well as the TURBO DNA-free™ Kit from Ambion® in its recommended settings. I then used the optional on-column DNase digestion provided in the RNase-free DNase set by Qiagen that complements the RNeasy Kits in combination with the TURBO DNA-free™ kit in effort to remove all of the DNA contamination (methods described previously in 2.1.10). However, PCR amplification of our samples after combining the two methods still showed that the samples contained DNA with distinct *tpi* gene bands on the gel, similar to Figure 3-7A.

This problem was addressed in several ways: first, the on-column digestion time with the RNase-free DNase set by Qiagen was doubled to 30 min. Second, the TURBO DNA-free™ Kit digestion was carried out twice instead of only once as the manufacturer

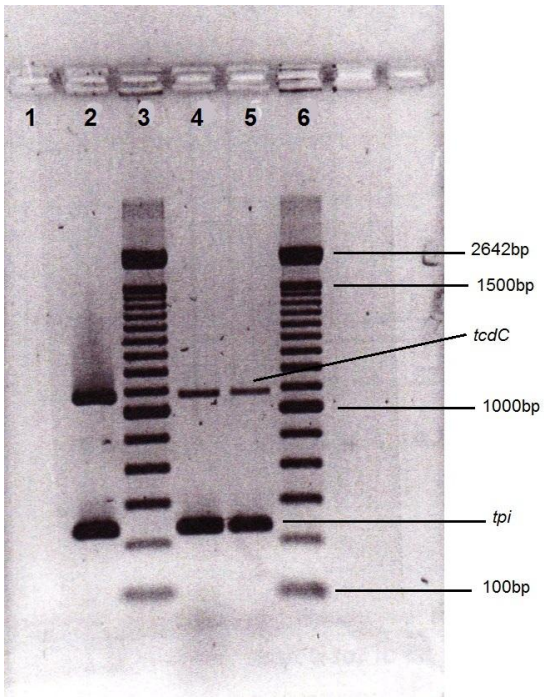
had recommended, with a 45 min incubation instead of only 30 min. Third, the amount of DNase added from the TURBO DNase-free™ kit was quadrupled. Fourth, to insure complete inactivation of enzymes and removal of unwanted reagents in the TURBO DNA-free™ buffer, the sample was treated twice with DNase Inactivation Reagent: once with 0.2 volumes, followed by 0.1 volume (manufacturer had recommended only one treatment with 0.1 volume).

In each instance where I increased either the incubation time, enzyme concentration, or number of digestions, a PCR was run to check for DNA contamination (described previously in sections 2.1.6 and 2.1.10). This was done because of the relatively unstable nature of RNA compared to DNA. Therefore, I wanted to minimize the time our samples remained outside -80°C storage, yet still have complete DNA subtraction. An example of samples with satisfactory DNA subtraction (indicated by the absence of bands at *tpi*, described previously) is shown in Figure 3-7B.

The DNA subtraction steps resulted, on average, of approximately 40-50% loss of nucleic acid from the sample when measured using NanoDrop 1000 Spectrophotometer. An example is shown in Figure 3-8, in which concentrations fell from 690 ng/µL to 370 ng/µL.

Figure 3-7. Gel electrophoresis results of PCR amplicons containing products of RNA extractions on *C. difficile* NAP1 (VLOO13) (A) prior to complete DNA subtraction and (B) after complete DNA subtraction. (A) Lane 1 is a negative control, lane 2 is a NAP1 positive control, lanes 3 and 6 contain 100 bp ladder, and lanes 4 and 5 are duplicate RNA extraction samples. (B) Lanes 1 and 6 contain 100 bp ladder, lane 2 is a negative control, lane 3 is a NAP1 positive control, and lanes 4 and 5 are RNA extraction samples.

A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2
3
4
5
6
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1500bp
tcdC
1000bp
tpi
100bp

B

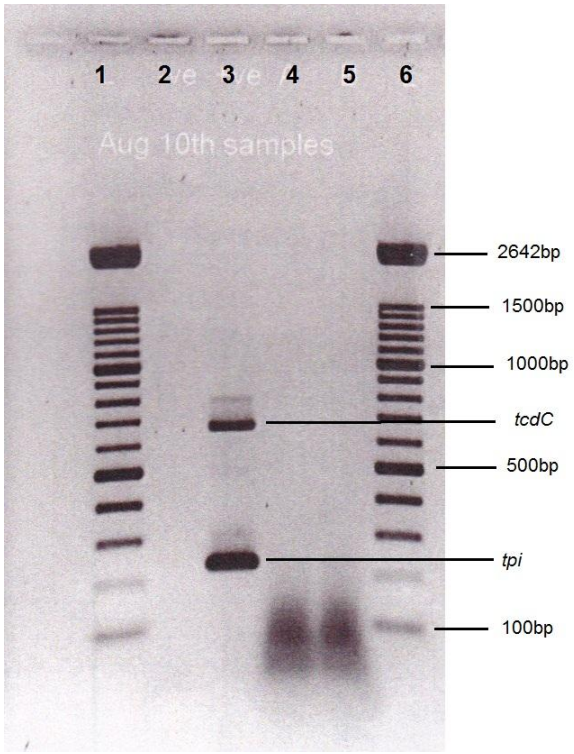
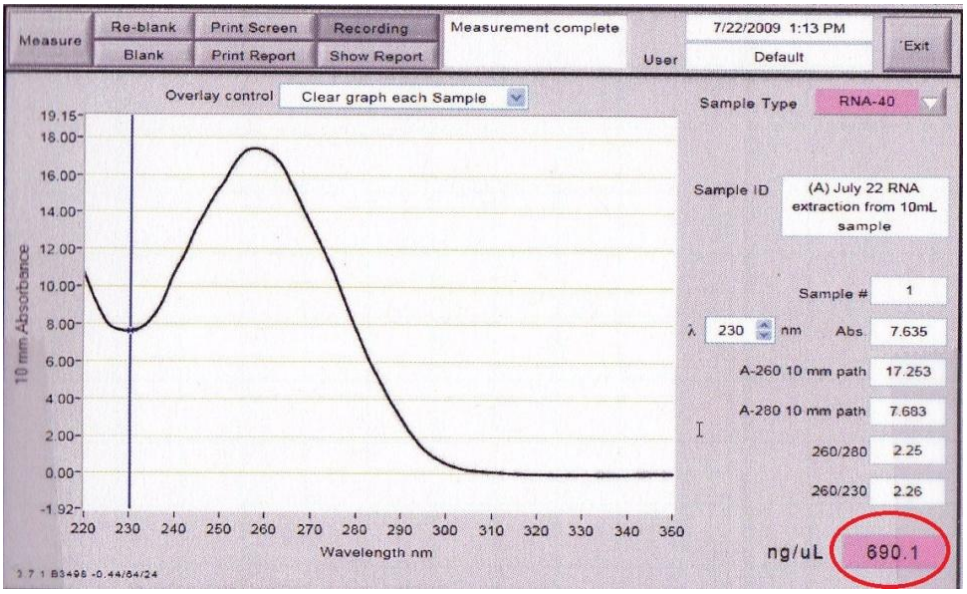
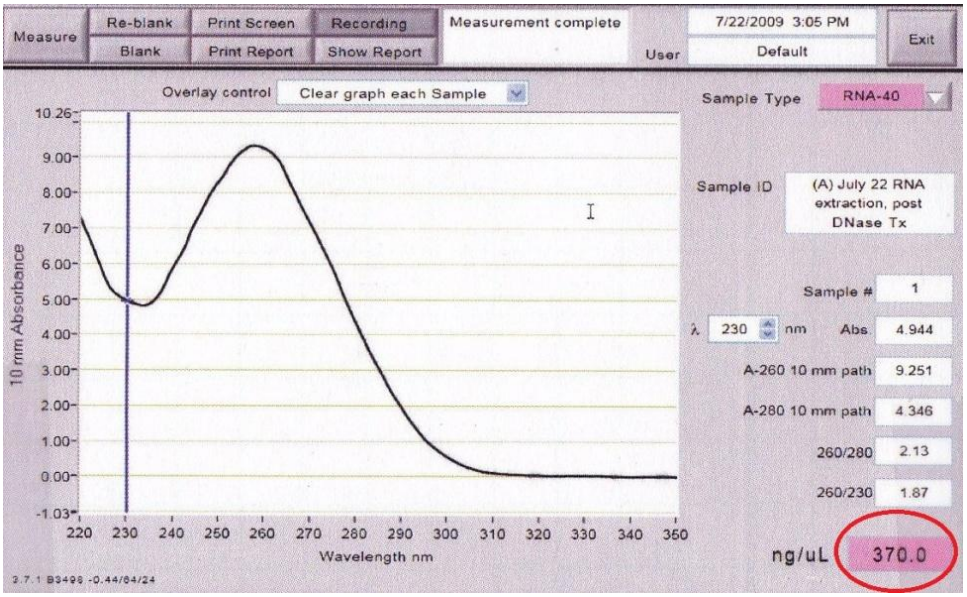

1
2
3
4
5
6
Aug 10th samples
2642bp
1500bp
1000bp
tcdC
500bp
tpi
100bp

Figure 3-8. Effects of DNase treatment on nucleic acid concentration. (A) Prior to complete DNA subtraction. (B) After complete DNA subtraction. Concentrations of nucleic acid measured with the NanoDrop 1000 Spectrophotometer are circled.

A



B



3.1.7 rRNA Subtraction

The predominance of rRNAs in the transcriptome is a major technical challenge in sequence-based analysis of cDNAs from microbial isolates; rRNAs typically comprise more than 90-95% of all RNA in a prokaryotic cell [97,180,181]. The high abundance of rRNAs can make detection of rare transcripts challenging, effectively "drowning-out" mRNA signals. Ways to alleviate this problem include computationally removing high-level transcripts from sequenced data such as rRNA, and rRNA depletion (rRNA subtraction) prior to cDNA synthesis to reduce its representation. For eukaryotic transcriptomic studies, poly(A) selection is very straightforward and effective at enriching mRNAs [99]. However, for prokaryotic mRNA devoid of a binding target, rRNA contamination can instead be removed by hybridization-based depletion to improve mRNA detection sensitivity [181]. The MICROB*Express*™ (Ambion®) Bacterial mRNA Enrichment Kit was used for the *C. difficile* purified total RNA, which has a claimed efficacy of removing >95% of 16S and 23S rRNA. This kit works using oligonucleotides attached to magnetized beads that bind to consensus 16S and 23S rRNA sequences, then selectively removes these rRNA hybrids with magnets.

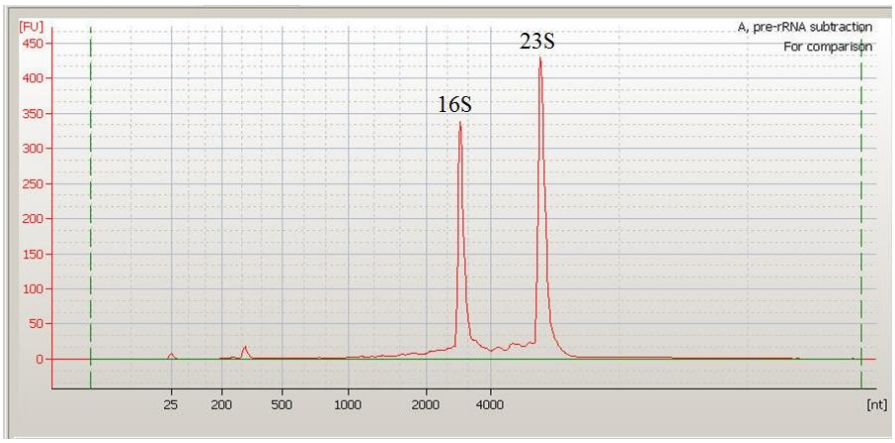
The MICROB*Express*™ Kit from Ambion® was used to isolate mRNA from purified total RNA from *C. difficile* mid-log growth. One disadvantage of this kit was that under recommended settings, only 1-2 μg of mRNA per reaction was achieved, even when loading at the maximal recommended amounts (10 μg total RNA in 15 μL H_2O). Since the minimal accepted amount of RNA required for a massively parallel sequencing run was 5 μg, we decided to run 6 reactions in parallel, then pool these technical replicates to increase the final yield, described previously in section 2.1.11. However,

significant 16S and 23S contamination remained in our samples after one round of rRNA subtraction, as measured with the Agilent 2100 Bioanalyzer (methods described in section 2.1.5), which uses microfluidics to measure the size and quantity of nucleic acids. One example of RNA content assessment is shown in Figure 3-9. In panel A, total RNA was analyzed without any rRNA subtractions; 16S and 23S rRNA peaks clearly predominate and are "drowning-out" the plateau-like base consisting mostly of mRNA. Panel B illustrates RNA profiles after one round of rRNA subtraction, where the 16S and 23S rRNA peaks are still predominating. Therefore, the replicates after one round of rRNA subtraction were combined and taken through the extraction process once more. Figure 3-9C illustrates RNA profiles after two rounds of rRNA subtractions, where the 16S and 23S peaks are no longer dominating, instead a plateau of mRNA of varying sizes is seen.

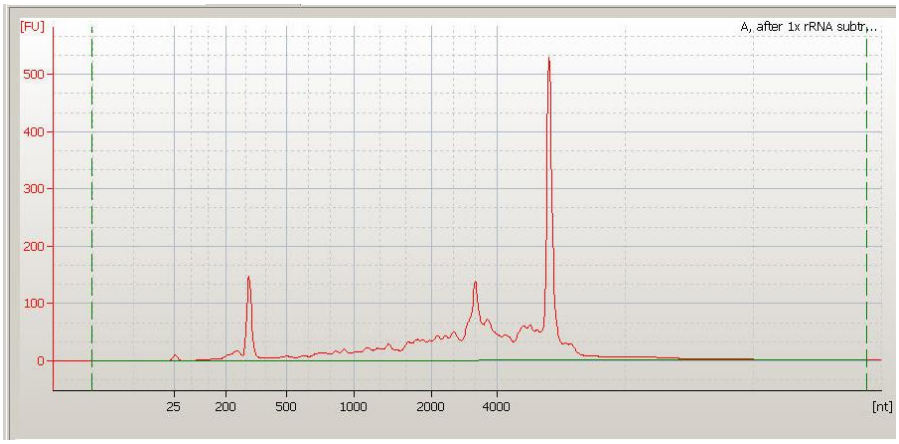
On average, these methods yielded 6-7 µg of purified mRNA as a pooled final product when measured with the Agilent 2100 Bioanalyzer. This purified mRNA was then used in cDNA synthesis (methods discussed in section 2.1.12). A control strain was not used to show that mRNA could be obtained for non-*C. difficile* isolates.

Figure 3-9. *C. difficile* RNA assessment using Agilent 2100 Bioanalyzer. Panel A depicts the RNA before rRNA subtraction. Panels B and C show RNA after one and two rounds of subtraction, respectively. Higher fluorescence (y-axis) indicates a higher concentration. X-axis denotes the size of the nucleotides (nt).

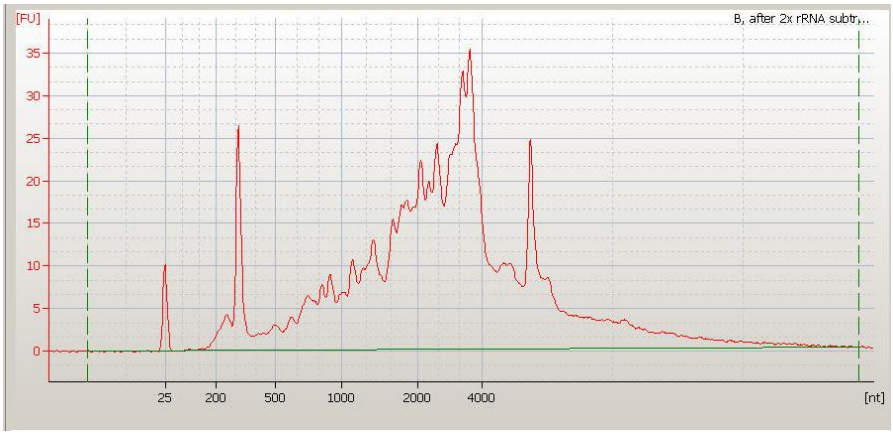
A


[FU]
A, pre-rRNA subtraction
For comparison
23S
16S
450
400
350
300
250
200
150
100
50
0
25
200
500
1000
2000
4000
[nt]

B


[FU]
A, after 1x rRNA subtr,..
500
400
300
200
100
0
25
200
500
1000
2000
4000
[nt]

C


[FU]
B, after 2x rRNA subtr,..
35
30
25
20
15
10
5
0
25
200
500
1000
2000
4000
[nt]

3.2 Metronidazole-Resistance Study

3.2.1 Nomenclature

A *C. difficile* isolate from a fecal sample obtained from Victoria, British Columbia, Canada in 2009 grew on CDMN with 8 μg/mL metronidazole (CDMN+8). However, colony sizes were small and sparse compared to growth on CDMN plates. We termed this isolate **MetR_ori** for "metronidazole resistance_original isolate". PCR and PFGE results of MetR_ori were consistent with a NAP1 strain (gel images not shown). The MIC to metronidazole (MET) was determined using Etest®, which revealed a value of 32 μg/mL.

Previous reports [165,166,168] have documented that *C. difficile* would lose resistance to metronidazole after freezing and passaging on metronidazole-free media. Therefore, MetR_ori was continuously passaged on CDMN+8. A sample of MetR_ori was also stored at -80°C (**MetR_ori(f)** for "frozen") for comparison at a later time (storage of samples was described in section 2.1.3 above). Furthermore, MetR_ori was successively passaged on MET-free CDMN media every five days for comparison at a later date.

Unfortunately, continual passages of MetR_ori on CDMN+8 plates proved to be problematic. First, growth was poor, in that a particularly large amount of inoculant was needed to ensure growth on fresh CDM+8 plates. Second, smaller colonies with slow growth were seen despite the use of large amount of inoculant when compared to CDMN plates without MET. Third, growth on successive passages was inconsistent: some passages did not form new colonies, whereas others grew poorly (small, sparse colonies). Fortunately, switching to BAK agar with 8 μg/mL MET (BAK+8) alleviated these

problems, in that a heavy level of inoculant was no longer needed to sustain growth on new media, and all passages yielded colonies. After it was found that BAK+8 media gave improved growth consistency, MetR_ori was transferred to BAK+8 from CDMN+8 media. To account for this change in media in terms of nomenclature, MetR_ori (grown on CDMN+8 plates) was changed to **MetR_stable** (grown on BAK+8 plates) to reflect that growth had "stabilized". Since MetR_ori(f) had been stored in -80°C without any manipulations, its name did not change as it was isolated from CDMN media.

The strain nomenclatures are summarized in Table 3-1.

3.2.2 Identification of Metronidazole-Susceptible Isolates (Revertants)

Prior to shifting assays from CDMN to BAK media, the protocol for finding revertants was first carried out with CDMN (section 2.2.2). As described above, growth was poor and unpredictable on CDMN+8 plates. Indeed, after only one round of the assay, all streaks grew on the CDMN plates, but no streaks grew on the CDMN+8 plates, exemplified in Figure 3-10 (left and right, respectively). We initially hypothesized that the resistance was extremely unstable and all colonies lost their resistance after only one round of passaging without metronidazole challenge. However, colonies from the CDMN plate were streaked on to new CDMN+8 media and growth was seen after 48 h, suggesting that these colonies did not loose their resistance, and the amount of inoculant was more likely the cause for the lack of growth. This was another major reason for the switch from CDMN+8 to BAK+8 media.

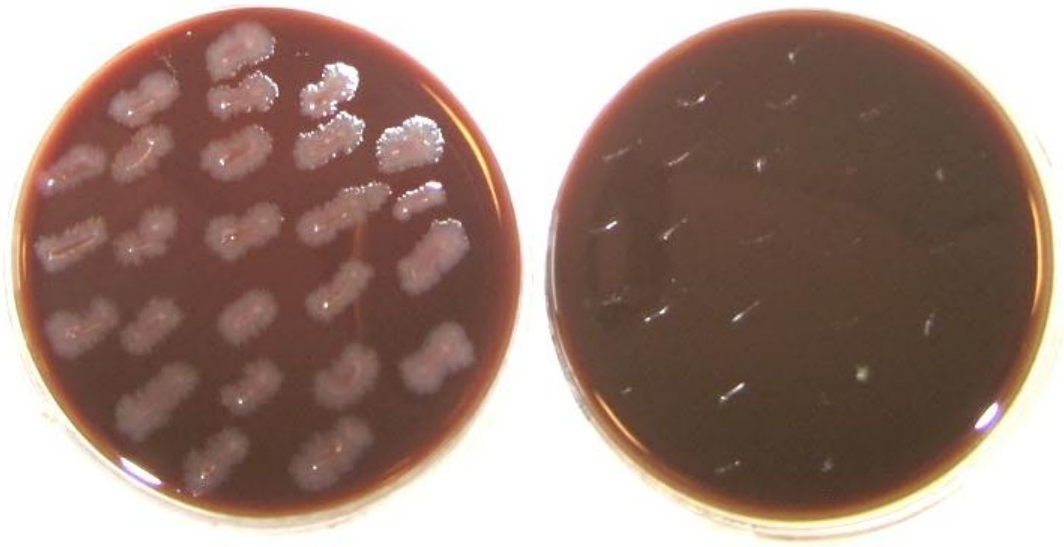
After switching to BAK+8 media, the problem described above did not recur, and all streaks on both MET and MET-free BAK plates formed colonies. After 31 rounds of passaging in MET-free BHI media (described above in section 2.2.2), two possible

revertants were discovered, and were assigned names of MetR_revA and MetR_revB. While both possible revertants were subcultured for collection in Microbank® freezer vials at -80°C, only MetR_revB was subsequently sequenced (chosen arbitrarily, mainly due to the high cost of sequencing), while MetR_revA was frozen in storage. Therefore, MetR_revB is synonymous with MetR_rev. Table 3-1 tabulates the names of *C. difficile* strains.

Table 3-1. Summary of nomenclature of *C. difficile* isolates for the metronidazole-resistance study.

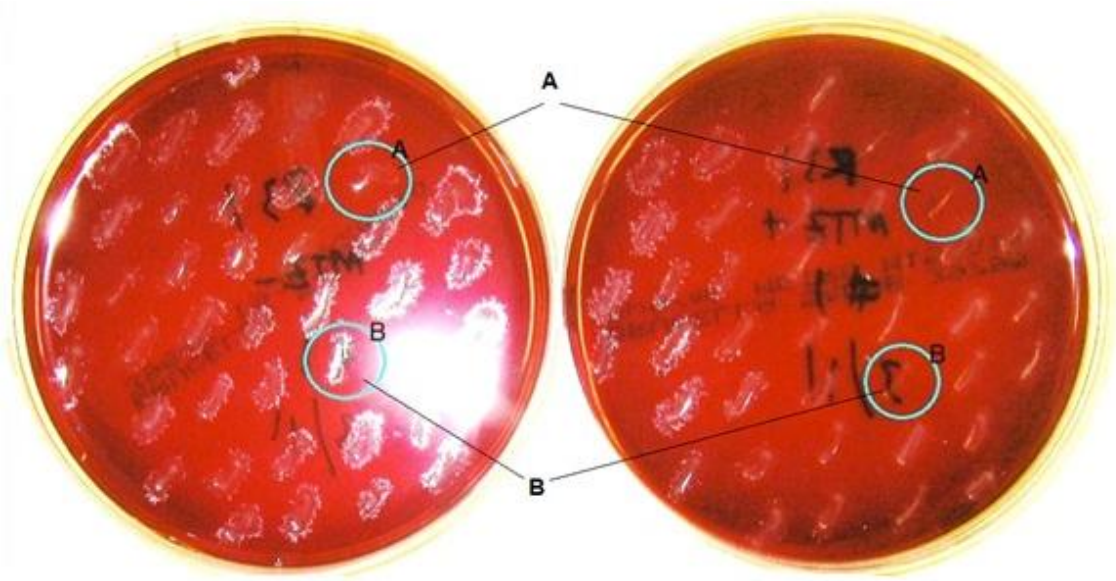
Strain	Description
MetR_ori	Originally discovered in 2009 from Victoria, BC, Canada, exhibiting MIC of 32 µg/mL by Etest®; exhibited inconsistence growth on CDMN+8 media when continuously passaged
MetR_ori(f)	MetR_ori stored at -80°C
Ori	MetR_ori continuously passaged initially on CDMN plates, then BAK plates (see text for explanation)
MetR_stable	MetR_ori after switching from CDMN to BAK media and growth became consistent
MetR_revA	One of the two revertant found after 31 rounds of passage with the revertance protocol. This isolate was not sequenced.
MetR_revB	One of the two revertant found after 31 rounds of passage with the revertance protocol. This isolate was sequenced, and referred to synonymously as MetR_rev.

Figure 3-10. CDMN plates with (B) and without (A) 8 μg/mL of metronidazole after performing one round of the revertance assay.



A B

Figure 3-11. Corresponding BAK plates (BAK on left, BAK+8 on right) of streaks of *C. difficile* colonies after 31 passages of the revertant identification protocol. Two possible revertants were found (A and B), which did not grow on the corresponding streak on the BAK+8 plate.



3.2.3 PCR and PFGE

In order to ensure that the isolated revertants were not accidental contaminants, or possibly another strain of *C. difficile*, PCR and PFGE were performed on the revertants alongside controls (methods described previously in sections 2.1.6 and 2.1.7), the results of which are presented in Figure 3-12 and Figure 3-13.

PCR results between MetR_stable and MetR_rev were indistinguishable for both the *tpi* and *tcdC* genes (*tcdC* gene length is commonly variable between strains). Figure 3-13 shows the PFGE fingerprint comparison between MetR_ori, MetR_stable, MetR_rev and a reference NAP1 strain, which were indistinguishable.

Figure 3-12. PCR-toxinotyping analysis of *C. difficile* strains MetR_stable (lane 3) and MetR_rev (lane 4). Lane 1 contains 100 bp ladder, lane 2 contains negative control.

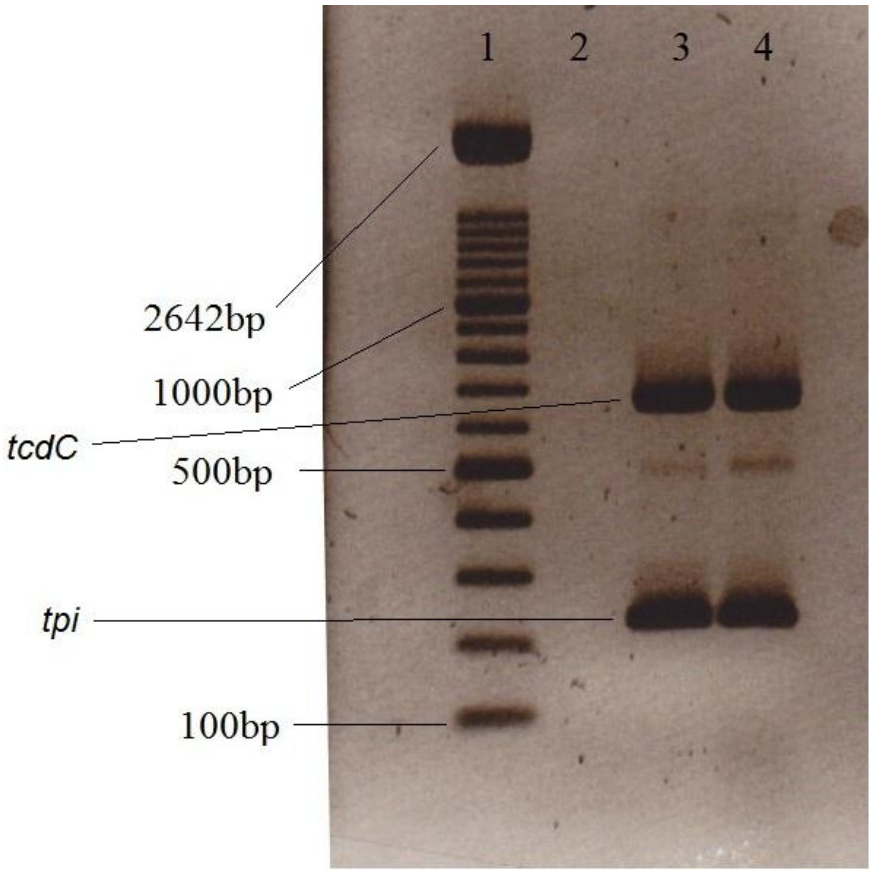
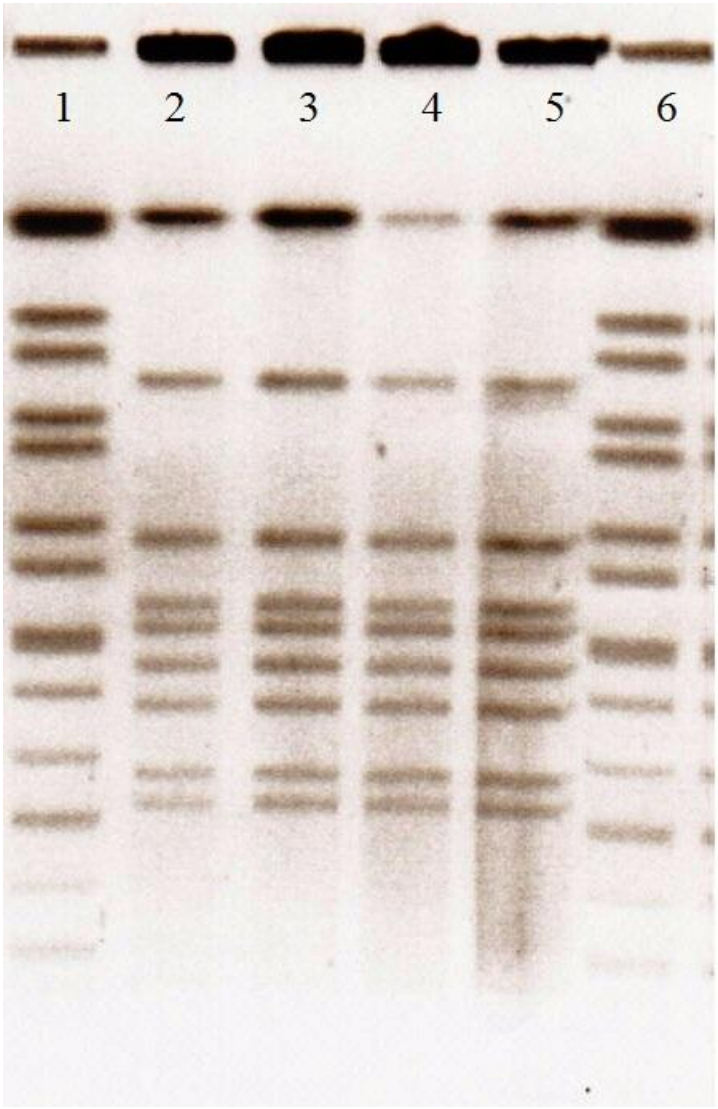


Figure 3-13. PFGE gel image of *C. difficile* strains. Lanes 1 and 6 contain *S. seratia* Braenderup H9812 standards. Lane 2 contains a positive NAP1 control. Lane 3 contains MetR_ori. Lanes 4 and 5 contain duplicates of MetR_rev.



3.2.4 MICs

Minimum inhibitory concentrations (MICs) are important clinically to assess resistance of microorganisms to antimicrobials. Hence, it was important for us to measure the MIC of our strains to metronidazole (MET). As mentioned previously, MetR_ori achieved an MIC of 32 μg/mL by Etest® to MET. It is important to note that this Etest® plate exhibited a zone of microcolony (small colony variants) growth inside the primary zone of inhibition resembling a "haze" (actual plate not shown, as it is hard to visualize by photography). Subsequently, the MIC value was read according to the cut-off point of the microcolonies, following the Etest® reading guide provided by the manufacturer. This peculiar Etest® MIC pattern with two apparent subpopulations is illustrated in Figure 3-14.

After MetR_rev was discovered through the revertance protocol described previously, the MIC to MET was performed using the Etest® method, alongside the MetR_stable and *B. fragilis* ATCC® 25285 control strain (ATCC, Burlington, ON). MetR_rev achieved a MIC of 1.5 μg/mL and MetR_stable at a MIC of 24 μg/mL. The Etest® plates are displayed in Figure 3-15. For MetR_stable, its MIC to MET at 24 μg/mL is interesting in that the initial Etest® (MET) performed on MetR_ori (nomenclature change described earlier in text) achieved a MIC of one dilution higher at 32 μg/mL. This will be discussed further in the results section.

The agar dilution method of determining MICs (MET) was also performed on MetR_stable and MetR_rev, which gave MICs of 16 μg/mL and 2 μg/mL, respectively. Figure 3-16 depicts BAK plates used in MIC testing to MET by the agar dilution method.

C. difficile ATCC® 700057 (ATCC, Burlington, ON) was as a control, per CLSI recommendations [160]. Colony #1 is MetR_stable and #2 is MetR_rev.

As described previously, earlier reports of MET resistant *C. difficile* strains losing their resistance after freeze-thaw cycles or after passaging on MET-free media have been reported [166]. Therefore, we tested the MICs to MET of MetR_ori after being stored at -80°C for 6 months (MetR_ori(f)) and found that its MIC to MET had decreased to 2 μg/mL (both Etest® and agar dilution), from 32 μg/mL by Etest®. In addition, MetR_ori that had been passaged on MET-free CDMN media every 5 days for 6 months had MIC values drop to 1.5 μg/mL by Etest® and 2 μg/mL by agar dilution.

Earlier, it was mentioned that a change in name from MetR_ori to MetR_stable was to signify the more "stable" growth on BAK+8 plates compared to CDMN+8 plates. After a switch to BAK+8 plates from CDMN+8 plates was made and more "stable" growth was observed, a sample of MetR_stable was also stored at -80°C. MICs to MET were determined 3 months later, which yielded values of 16 μg/mL by Etest® (from 24 μg/mL before freezing), and 8 μg/mL by agar dilution (from 16 μg/mL before freezing). It is important to note that MetR_stable had also been continuously passaged on BAK+8 plates, since previous reports indicated that frozen storage may decrease its MIC to MET (we have also found this to be true, as indicated by our data presented here). MIC testing of MetR_stable passaged on BAK+8 plates was repeated approximately every 3-4 weeks by Etest® (MET), and its MIC value has remained steady at approximately 16-24 μg/mL, unlike its frozen counterpart. These values are summarized in Table 3-2

Table 3-2. Effects of repeated passaging and freezing on MIC of *C. difficile* isolates tested by Etest® and agar dilution (AD) methods. MIC values are in μg/mL.

	Initial		After freezing		After passaging	
	Etest®	AD	Etest®	AD	Etest®	AD
MetR_ori◆	32	np	2	2	1.5	2
MetR_stable❖	24	16	16	8		

◆ MetR_ori(f) was frozen for 6 months
❖ MetR_stable(f) was frozen for 3 months
np – not performed

Although the primary interest of this project involves metronidazole resistance, susceptibility of MetR_stable to other antibiotics were also tested by Etest®, the results of which are shown in Table 3-3.

Table 3-3. MIC profile of MetR_stable with five different antibiotics (μg/mL).

Rifampin	Moxifloxacin	Clindamycin	Vancomycin	Tigecycline
<0.002	>32	6	0.75	0.094

Figure 3-14. Schematic diagram showing small colony variants (microcolonies) of *C. difficile* forming a "hazy" pattern inside the primary zone of inhibition on a metronidazole Etest® plate.

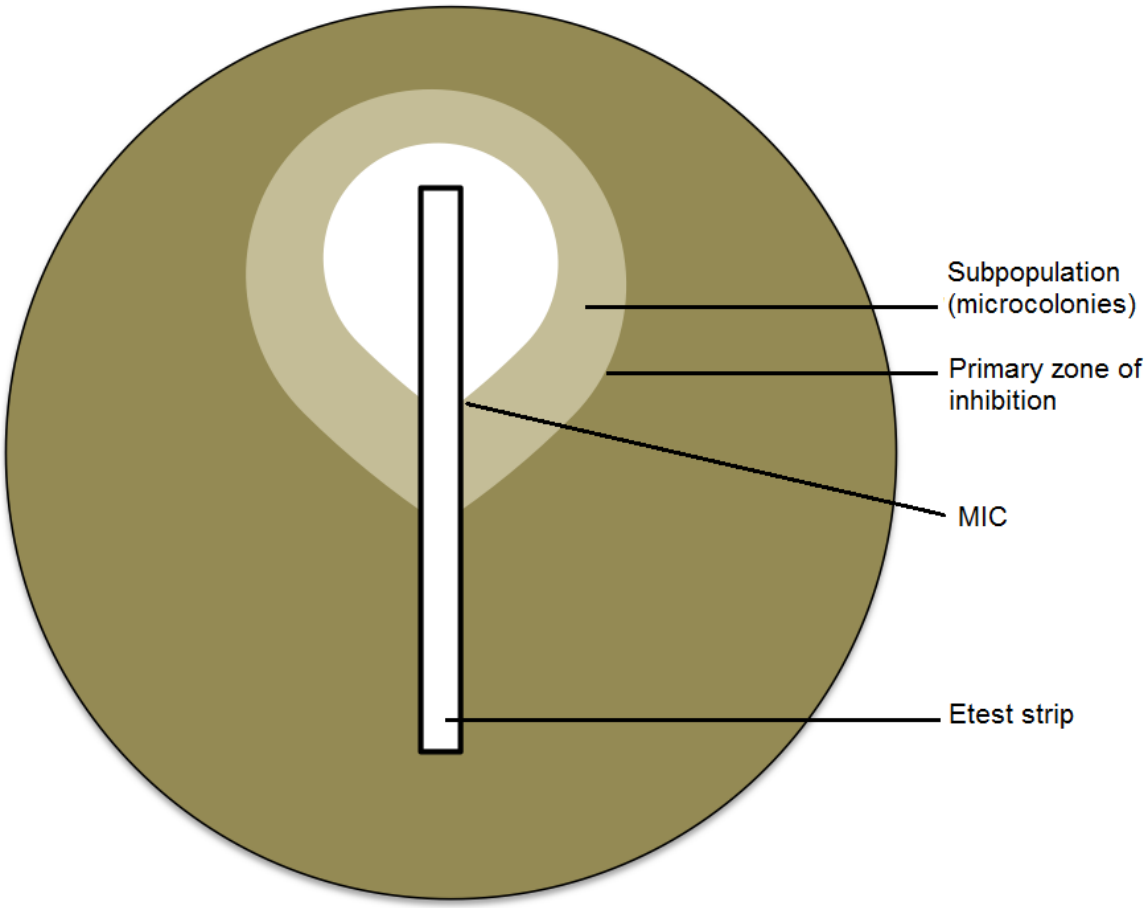
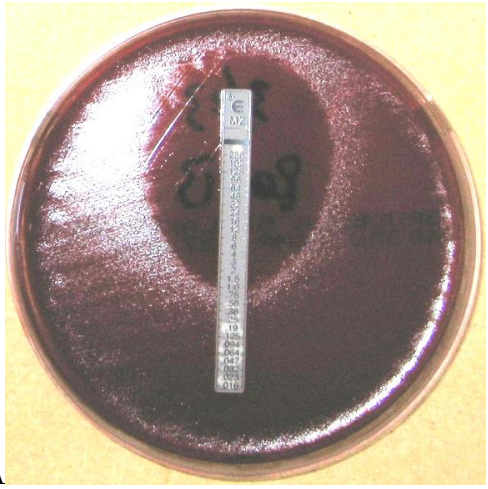
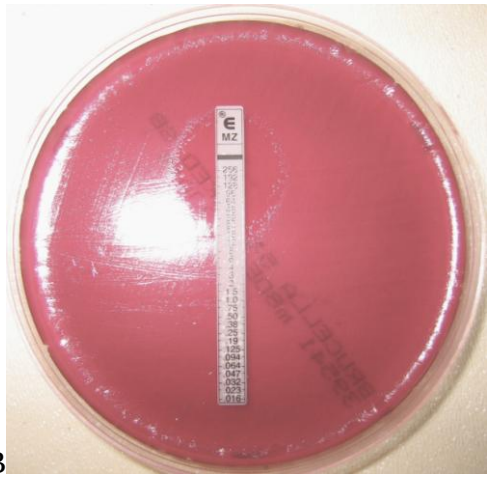


Figure 3-15. MIC of *C. difficile* by Etest® method to metronidazole. (A) MetR_rev; MIC = 1.5 µg/mL. (B) MetR_stable; MIC = 24 µg/mL. (C) *B. fragilis* ATCC® 25285 control.

A 

B 

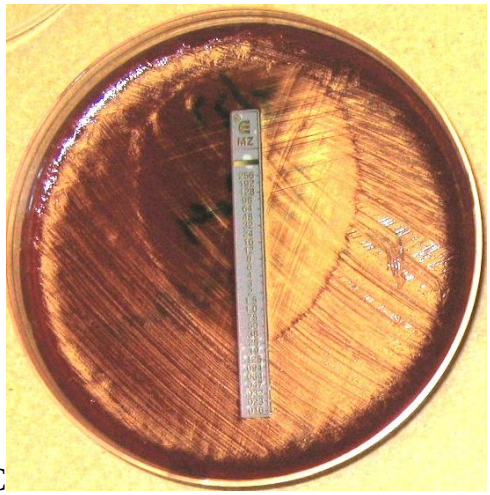
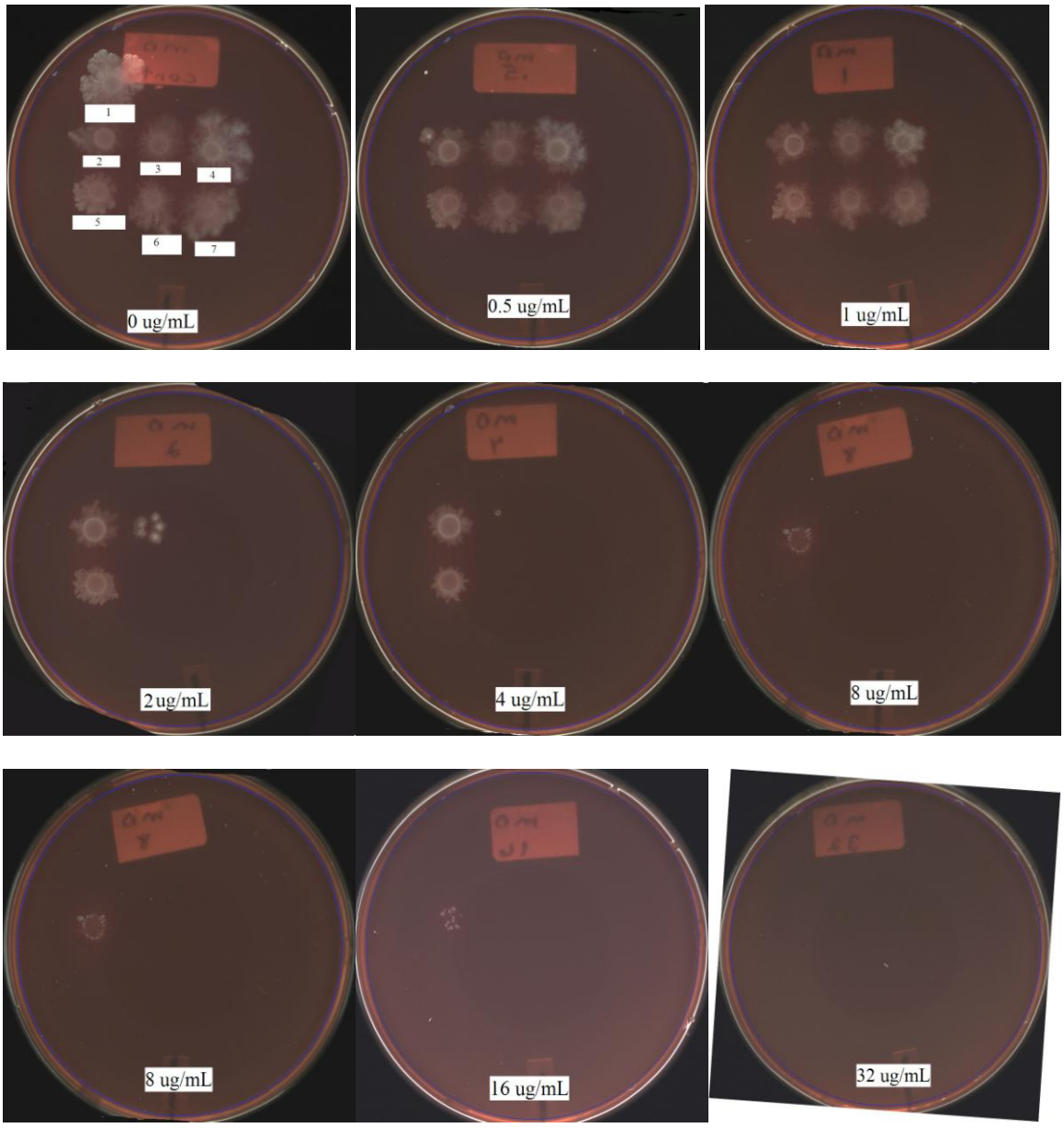
C 

Figure 3-16. Agar dilution method of determining MIC of *C. difficile* to MET. Legend: (1) *C. difficile* ATCC® 700057 control; MIC = 0.5 µg/mL; (2) MetR_stable; MIC = 16 µg/mL. (3) MetR_rev; MIC = 2 µg/mL. (4) MetR_ori sub-cultured on MET-free CDMN plates every 5 days for 6 months; MIC = 2 µg/mL. (5) MetR_stable stored at -80°C for 3 months; MIC = 8 µg/mL. (7) MetR_ori stored at -80°C for 6 months; MIC = 2 µg/mL.



3.2.5 Growth Assay

Growth assays for MetR_stable and MetR_rev were performed to assess its growth kinetics. Figure 3-17 depicts the growth curves for strains MetR_stable and MetR_rev. MetR_rev had a higher rate of OD_{600} increase throughout the exponential phase and attained a higher final OD_{600} value compared to MetR_stable. Both strains exhibited similar timing of lag, exponential, and stationary phases. However, these phases occurred at different OD_{600} readings.

Figure 3-18 illustrates the phenotypic differences between the colonies of MetR_stable and MetR_rev on BAK plates without metronidazole after 24 h incubation. An equal amount of cells for each strain were spread onto BAK plates and allowed to incubate for 24 h. MetR_stable colonies are bigger than MetR_rev colonies after being incubated for the same amount of time.

Figure 3-17. Growth curves of MetR_stable and MetR_rev with 2 SD error bars. (A) Absorbance at OD_{600} against time. (B) Log absorbance OD_{600} value against time.

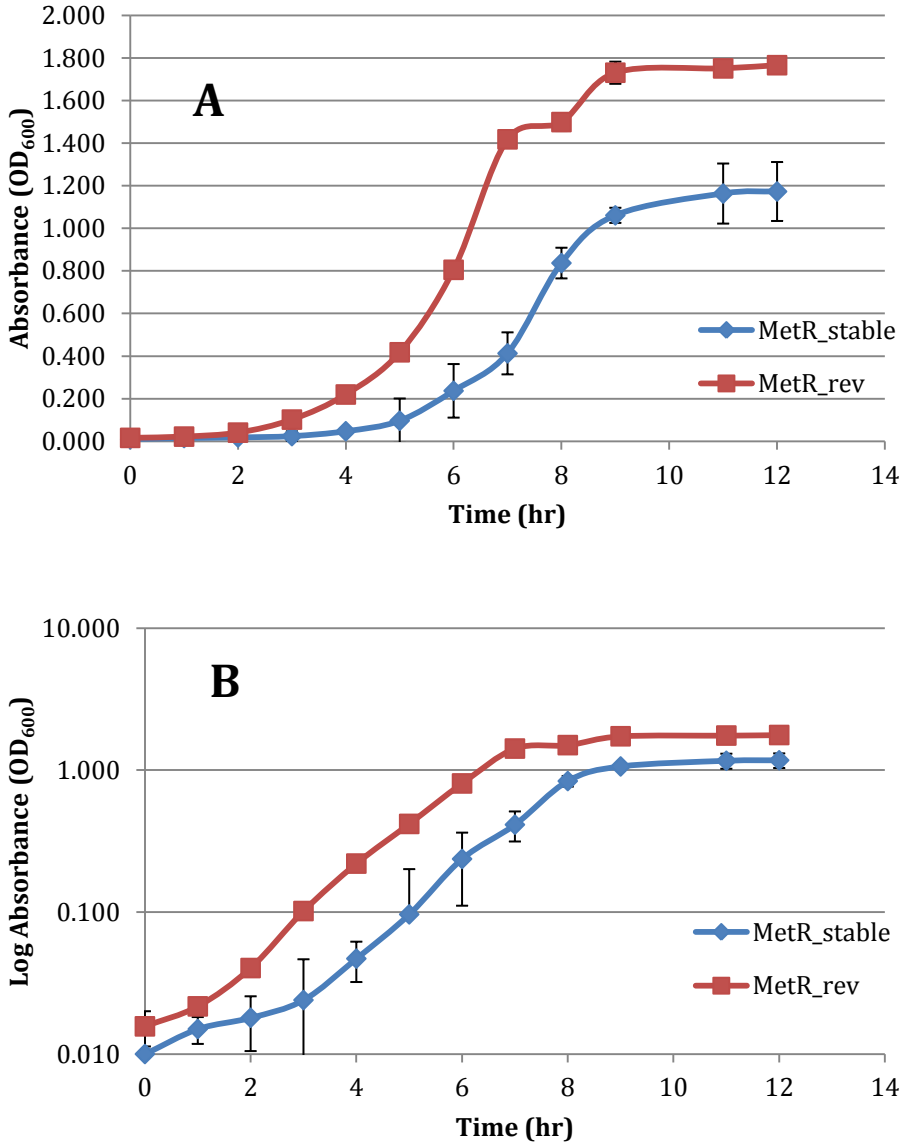


Figure 3-18. Comparison of growth on BAK plates between *C. difficile* strains MetR_rev (left) and MetR_stable (right) after 24 h incubation on MET-free BAK plates.

MetR_rev MetR_stable

3.2.6 Detection of *nim* Genes

A search was performed with GeneQuest™ software (DNASTAR®) on the sequenced genome of MetR_stable using the *nim* sequence [182]. We did not find any homologous sequences to *nim* genes in the MetR_stable genome.

3.2.7 Comparative Genomic Analyses

The genomes of MetR_stable and MetR_rev were sequenced and assembled using the 454/Roche Genome Sequencer (GS) FLX using the GS Titanium Sequencing Kit and GS Rapid Library Kit (all by 454 Sequencing, Roche, Branford, CT). The assembly metrics of MetR_rev and MetR_stable are listed in Table 3-4 and Table 3-5, respectively.

Table 3-4. Metrics for assembly of MetR_rev using Newbler assembler.

Metric	Quantity	Percent
Number of aligned bases	84,050,935	98.58%
Number of contigs	175	-
Number of bases	4,068,628	-
Average contig size (bases)	23,249	-
N50 contig size ◆	42,687	-
Largest contig size (bases)	133,778	-
Q40 plus bases ❖	4,047,869	99.49%
Q39 minus bases §	20,759	0.51%

◆N50 length is defined as the length N for which half of all bases in the sequences are in a sequence of length L < N, i.e. N50 is the contig length such that using equal or longer contigs produces half the bases of the genome.
❖Q denotes quality scores, with higher values corresponding to higher quality, and is linked logarithmically to error probabilities. Q40 has a probability of a wrong base call of 1 in 10,000 (i.e. accuracy of 99.99%), and has been generally regarded as acceptable. As a general consensus, the percentage of Q40 plus bases approximately 99.5% or over is considered to be of high quality.
§Q39 denotes all bases having a Q score of less than 40.

Table 3-5. Metrics for assembly of MetR_stable using Newbler assembler.

Metric	Quantity	Percent
Number of aligned bases	99,368,432	99.39%
Number of contigs	114	-
Number of bases	4,072,518	-
Average contig size (bases)	35,723	-
N50 contig size	63,608	-
Largest contig size (bases)	176,820	-
Q40 plus bases	4,057,260	99.63%
Q39 minus bases	15,258	0.37%

As described in section 2.2.6, sequenced genomes of MetR_stable and MetR_rev were aligned using Mauve computer software and viewed with MEGA 4 software. Briefly, any heterogeneity between the genomic sequences, for example SNPs and IN/DELs, were assigned a number based on their position in the genome reported by MEGA 4. A BLAST search was performed on each location where a difference was found and results were tabulated (Table 3-6). Primer construction and PCR amplification was subsequently carried out on these genomic regions of heterogeneity and the amplicons were sequenced to assess if the differences were true or false. Out of 53 areas, 22 had only a single base pair difference, 17 areas had differences in 2 base pairs, 15 areas had differences in 3-8 base pairs, and 2 areas had differences over 27 base pairs. These regions of interest were divided based on practical PCR amplicon sizes, which were generally kept to less than 1 kb. In other words, if two genomic differences between MetR_stable and MetR_rev were found to be more than 1 kb apart, they would be grouped into two separate regions.

Table 3-6 lists the preliminary primer sequences used to investigate the 53 heterogenous regions between MetR_stable and MetR_rev genomic sequences, along with their T_m and expected size. The location number denotes the position of the first

base in the MEGA 4 alignment file of the sequence disagreement between the two strains. In the case of a single base pair difference, the location number is specific to the base pair. For certain primer sets, the length could not be accurately predicted due to extensive gaps in the alignment file, therefore estimates were made; these were denoted with an asterisk. While all of these primers were used in a PCR amplification reaction, only a few have returned viable amplicons when checked by gel electrophoresis (single band); in these cases, the primers and reaction conditions were adjusted (described in section 2.2.6 above) until viable amplicons were obtained (single band on gel electrophoresis).

Not all of the regions listed in Table 3-7 was verified by PCR at the time of this writing. This was due to the high degree of putative genomic heterogeneity between MetR_stable and MetR_rev, as well as extensive adjustments of the primer design and PCR reaction conditions by trial-and-error were required to obtain amplicons that could be sequenced.

Out of eight single base pair differences that were investigated through sequencing of PCR amplicons, six were found to be false (base pairs are the same), while two were found to be true differences, including #543660 (putative membrane protein), and 4076225 (RNA polymerase sigma-B factor; rRNA-16S ribosomal RNA). These results are displayed in Table 3-7, which also includes BLAST results and the base difference to be investigated at each location. Certain areas of the genomic alignment included a number of unmatched bases; these were not included in Table 3-7, however the primers used to investigate genomic differences were designed to enclose these regions (Table 3-6).

Table 3-6. Primers used for PCR amplification for purposes of investigating areas of sequence disagreement between genomic sequences of MetR_stable and MetR_rev.

Location	BLAST result	Left primer	Right primer	T_m (R/L) °C	Size (bp)
4076225	RNA polymerase sigma-B factor; rRNA-16S ribosomal RNA	cagctttgcgacaccaacta	ccataagaagcctccatagcc	60.1/60.1	225
543660	Putative membrane protein	ggggcttgtcttagggtttt	tccacatgcatgatagatatgact	59.5/58.5	446
3304049	PTS system, IIabc component	ctacggctgcaaccattaca	tatgctggtgccaatagctg	59.8/59.9	197
4058935	Putative stage 0 sporulation protein; methyltransferase	ttctgcatctactttaataattccttt	aaaaaggtgatggaaacacca	57.5/59.3	183
167442	Putative membrane protein	tggtcaaagaactcctgatgg	ttacaacaagcttgggcaga	60.1/59.5	N/A*
191193	Putative membrane protein precursor	caattcggaaaagtgtatgttga	aagtatgaaaagattgtcctagcag	59.0/57.4	263
263329	Putative uncharacterized protein	aaaaatgacttctttattcatccaaa	tcacactcactcctcctcaaaa	59.7/59.9	247
408750	putative arginine utilization sigma-54 dependent regulation	gctgcatcaattttaggtatgaa	aagcataaatcgtgccaacttt	58.3/60.3	286
547846	Putative protein (pseudogene)	tcagcaggtaagctccgaat	tcacagggttcctgcttcat	60.0/60.7	213
717046	Conserved hypothetical protein	cagggttaaataatgatgcaaaaa	agacatctgactcttgcttgtca	59.3/59.2	400*
783792	None found	gagctcggggtaagctcttt	ggttttgtagttgcgctattgtc	60.0/60.1	559*
788893	None found	aaaaagtaaacccttagttgtaaaagg	cgtacatagtttccatttgttgtaaa	58.1/58.6	240
914631	ABC transporter	ttcttctgtgttcagctaaattagaca	tggcttattcgtgtttttacca	59.9/59.5	250
971113	None found	caattcacttttttagcccttgtttt	caatatacatttaccttcttcctgcat	61.0/60.1	683
989272	Putative uncharacterized protein	tggtgttatgcgtgcagtaggc	cgagctcttggagtgcttatgaa	65.4/63.1	576
1066013	Hypothetical protein	aggagcgaaaagctcaacaaaa	tgcctttcatcttcatcctcctt	62.5/63.4	720
1357092	Putative regulatory protein; putative petidyl-prolyl isomerase	tcctgtttccatcaacgcaga	cggtagaggacaaatggttcctg	64.0/64.1	661
1377252	Putative phage-related protein	tctggaggtgagagtggaatgg	tgcccccagactactcaattcg	63.8/65.6	439
1406799	Putative nonribosomal peptide synthetase	ttccactaaacccccctatgagca	ccatccaataattcatagctcgttc	63.9/62.1	407

1512104	Putative D-methionine ABC transporter	agctgtgccaaatgatgcaact	ccatattccccaccacttcttca	64.2/64.3	557
1516251	None found	tggcgaaatgagctataaaaa	ttgttcatgtagtcttcttctggt	57.6/57.1	480
1658373	Putative membrane pro; transpose-like prot B; PTS system; putative lysophospholipase; chloride ion channel protein	gacacagcatgggttcatttgc	tctccacaggctatccccgta	64.9/64.0	498
1704862	Putative amidohyrolase, hypothetical protein	tgctgctacaaaaacagcatctga	tgtaactgcgattgtgatgattgc	64.3/64.3	527
1751522	Putative signaling protein	agatgatgcttcaaaagcggtaaa	cgttggctagctccacaaaca	63.2/63.5	679
1818640	Putative C4-dicarboxylate anaerobic carrier	gcctatcttgttgattttgacagc	tggtgacattgtcctcaacatatc	61.3/61.0	659
1875239	Glyceradehyde-3-phosphate dehydrogenase 1	catggaaagaactgggggttga	gccgctgctcttgctcttct	65.4/64.6	360
1900304	MerR-family transcriptional regulator	ccaaataaaattacagatgcagga	tcaattcatccagtctctttcgt	64.4/64.0	205
1925150	Putative two-component sensor histidine kinase	aattaatctatggctgaaaacca	tccaaattgcttactgttgct	62.3/62.7	841
1977589	None found	tatcattgcagcgccttcta	acgcggatattctcgatttt	65.0/64.9	351
2065189	Conserved hypothetical protein; NUDIX-family protein	ttttgctatttcaagcatatttgg	tggaaaagaaatttcgctagg	64.0/64.6	5kb*
2095794	None found	accacctctcatcaaattaccatt	cgtgacaagctagcctcagtt	64.4/64.4	268
2142128	Hypothetical protein	tgaagcaaaaactcataccctgt	tgaactctgttgaaaaatgggtta	64.6/64.2	500
2157836	Conserved hypothetical protein	cctcgtccttaattccattca	tggtgatgcaagtgcaagag	64.2/64.9	668
2221485	Putative multiprotein-complex assembly protein	aaggagggcacccttagtatg	aaagcttagatgtgcagtggtca	65.2/65.2	400
2267700	None found	tgagggttaaaatggctatcct	ggtccaaaagaacttggaggt	64.7/64.7	520
2296375	Maltose phosphorylase	cacagcaacaatggaagctaca	ttgccaaaagagtggaaaaa	65.2/64.1	591
2315396	Putative sodium/solute symporter	ttgaaatgtcattgaaatgcgta	ggaaatcggatgctgttttt	64.7/64.9	500
2339548	Putative membrane protein; putative nitrite transporter	tttccaagagtgttagcatagctg	ggaaatattgttggaggagctt	64.4/64.1	487
2383491	Putative membrane protein	gcccaacatgacatcaaactt	tggtagatgctttaataggcacaa	64.3/64.8	433
2429465	Two component response regulator; two-component sensor histidine kinase	attttctcttcccgtcgaaa	tgtaaccaatgccccacac	64.6/65.2	158
2751791	Lipoprotein signal peptidase	agcaatgatttttccccccta	ttgtttgaagattagtggtgcttg	64.7/64.7	309
2827021	None found	tggctgctgctaaagaaaaa	aggttgtgatatggcccaag	64.7/65.1	663
2949379	Putative signaling protein	gcatgtaatccacttgcattttt	tggtgaggtgcttggattg	65.2/65.2	500

3038187	Putative amino acid racemase	ttcgtgaaaattcaaaacaaatca	tttgatgcaatagtggggatg	65.2/65.5	679
3088587	Putative D-aminoacylase	cccgcataaagtattggttctt	tttcaaagccacaattactagcc	65.3/65.0	587
3128608	Putative signaling protein; ABC transporter, permease protein	atcgtcgtgcagattctcaaa	cacaggcagcagcactagc	65.1/65.7	249
3228911	Putative signaling protein; ABC transporter, permease protein	ggaacgcttgcgtatcatct	cttatgggagtatggacatttgct	65.6/65.3	606
3323292	Putative PTS system, IIa component	cgaaggcatttcttatttctatcc	agaaggaaagccgtttgatg	65.1/65.0	672
3519155	Dihydrodipicolinate reductase; exosporium glycoprotein	ccctgaacataatatcacctccaa	ggcgaaataaagagagttaatgga	65.6/65.3	602
3635017	Conserved hypothetical protein	aaacaaaaagtatgcaccttcctt	ggcaaggagaagcaaaagtc	64.8/64.6	241
3913762	Putative membrane protein; putative peptidase	ggaataaatcccaatggagtca	tagagcgggcatcctacaat	64.9/65.4	227
4004418	Putative Na^+/H^+ exchanger; transposase-like protein b; putative iron ABC transporter permease protein; putative flavodoxin	gtttgttccaaaccctccaa	tgaaatggagttgatgctatgaca	65.1/65.0	372
3519155	Dihydrodipicolinate reductase; exosporium glycoprotein	ttgcaaacggttctatagttgc	gccaacaggtgctactggtc	64.6/65.4	1.5kb*

Table 3-7. Investigations of genomic heterogeneity between MetR_stable and MetR_rev.

Location	MetR_stable	MetR_rev	true/false§	BLAST results (features in part of subject genome)
167442	c	t		Hypothetical protein; putative membrane protein
191193	t	a		Putative membrane protein precursor
191283	a	t		
263329	a	t		Putative uncharacterized protein; flagellar basal-body rod protein
408750	g	a		Putative arginine utilization sigma-54 dependent regulation
408751	a	g		
543660	t (t)❖	a (c)	TRUE	Putative membrane protein
547846	g	a		Putative protein (pseudogene)
547847	g	a		
717046	a	g		Conserved hypothetical protein
717048	g	a		
717049	t	a		
783792	a	g		None found
788893	t	a		None found
788894	a	t		
914631	c	t		ABC transporter
971113	t	g		None found
971168	a	g		
971170	a	g		
971174	g	t		
971175	t	a		
971178	t	c		
971181	t	a		
971187	g	a		
971189	a	g		

971192	g	t	
971198	t	g	
971199	a	g	
971200	t	g	
971209	a	t	
971212	a	t	
971213	t	g	
971218	a	c	
971222	g	a	
971228	a	t	
971229	a	t	
971230	a	g	
971231	t	c	
971235	t	c	
971256	t	c	
971258	a	t	
971268	c	a	
971269	t	a	
971283	a	g	
971284	g	a	
971285	a	g	
989272	t	c	Putative uncharacterized protein
989273	c	t	
1066013	t	a	Hypothetical protein
1066014	a	t	
1357092	g	a	Putative regulatory protein; putative petidyl-prolyl isomerase
1357093	a	g	
1357102	c	t	

1357103	t	c		
1377252	t	a		Putative phage-related protein
1377253	a	t		
1406799	g (a)	g (g)	FALSE	Putative nonribosomal peptide synthetase
1406800	g	a		
1406804	a	t		
1406805	t	a		
1512104	a (a)	a (t)	FALSE	Putative D-methionine ABC transporter
1512105	t	a		
1512113	a	t		
1512114	t	a		
1512130	a	t		
1512131	t	a		
1512136	g	a		
1512137	c	g		
1516251	g	a		None
1658373	t	c		Putative membrane protein; transpose-like prot B; PTS system; putative lysophospholipase; chloride ion channel protein
1658374	g	t		
1658375	g	t		
1658376	t	g		
1658377	c	t		
1658378	a	g		
1658379	g	a		
1658380	a	c		
1704862	t (t)	t (a)	FALSE	Putative amidohyrolase, hypothetical protein
1704865	a	t		
1751522	t (t)	t (a)	FALSE	Putative signaling protein
1818640	a (a)	a (g)	FALSE	Putative C4-dicarboxylate anaerobic carrier

1818641	g	t		
1875239	c	t		Glyceradehyde-3-phosphate dehydrogenase 1
1900304	c	t		MerR-family transcriptional regulator
1900305	a	c		
1925150	a (a)	a (g)	FALSE	Putative two-component sensor histidine kinase
1925151	t	c		
1925317	g	c		
1977589	t	a		None found
1977590	a	g		
1977591	g	t		
2065189	t	a		Conserved hypothetical protein; NUDIX-family protein
2065217	a	t		
2065285	t	c		
2069187	g	a		
2069188	g	a		
2069191	a	g		
2069192	g	c		
2069195	t	g		
2069196	g	a		
2069199	g	t		
2069206	t	a		
2069207	c	a		
2069208	t	g		
2069210	g	a		
2069211	t	a		
2069214	g	a		
2069215	c	t		
2069216	a	t		

2069220	a	g	
2069221	g	a	
2069223	g	a	
2069226	g	a	
2069227	g	a	
2069228	t	g	
2069229	a	c	
2069230	t	c	
2069231	t	a	
2069233	g	t	
2069239	c	t	
2069241	g	a	
2069243	t	a	
2069250	a	g	
2069251	a	g	
2069253	g	a	
2069256	a	g	
2069527	t	c	
2069259	t	g	
2069262	t	a	
2070081	g	a	
2095794	a	t	None
2142128	a	g	Hypothetical protein
2142129	a	t	
2142130	c	t	
2142131	t	c	
2142132	a	c	
2412133	a	t	

2142138	g	a	
2142139	t	a	
2157836	t	c	Conserved hypothetical protein
2221485	t	a	Putative multiprotein-complex assembly protein
2221486	a	t	
2267700	a	c	None found
2296375	a	t	Maltose phosphorylase
2296377	t	a	
2315396	a	t	Putative sodium/solute symporter
2339548	a	c	Putative membrane protein; putative nitrite transporter
2339549	t	c	
2383491	a	t	Putative membrane protein
2429465	a	t	Two component response regulator; two-component sensor histidine kinase
2429466	t	a	
2751791	a	t	Lipoprotein signal peptidase
2751792	t	a	
2827021	g	a	None found
2949379	g	t	Putative signaling protein
3038187	t	c	Putative amino acid racemase
3038188	c	t	
3088587	g	a	Putative D-aminoacylase
3128608	t	a	Putative signaling protein; ABC transporter, permease protein
3128609	a	c	
3128610	c	t	
3228911	g	t	Conserved hypothetical protein
3304049	t	c	PTS system, IIabc component
3323292	c	t	Putative PTS system, IIa component
3519155	t	a	Dihydrodipicolinate reductase; exosporium glycoprotein

3519156	a	t		
3519157	t	c		
3519159	g	t		
3519162	t	c		
3519165	t	c		
3547421	g	a		Valyl-tRNA synthetase
3547422	t	g		
3547423	a	g		
3547531	a	c		
3547532	t	a		
3635017	a	t		Conserved hypothetical protein
3635020	g	a		
3635021	t	g		
3913762	t	a		Putative membrane protein; putative peptidase
3913764	a	t		
4004418	t	c		Putative Na^{+}/H^{+} exchanger; transposase-like protein B; putative iron ABC transporter permease protein; putative flavodoxin
4058935	g	t		Putative stage 0 sporulation protein; methyltransferase
4076225	g	a	TRUE	RNA polymerase sigma-B factor; rRNA-16S ribosomal RNA

§Only some genomic differences were investigated at this writing; reasons stated in text.

❖Bases in brackets indicate the results prior to PCR sequencing analysis.

4 DISCUSSION

4.1 Transcriptomics Study

Since the findings of Louie *et al*. were specific to NAP1 strains and not NAP2, I attempted to develop a method of transcriptomic sequencing and comparative analysis of both strains, which may be used to identify any differences on a transcriptomic level and elucidate the mechanism of increased toxin production by NAP1 *C. difficile* related to PPI use. To the best of my knowledge, at the time of this study no prior publications have used next-generation sequencing (NGS) technologies to study the transcriptome of the hypervirulent *C. difficile* NAP1 strain. As described in section 1.2.3, the goals for this project were four-fold: (1) carry out PCR, PFGE, and growth studies to characterize two *C. difficile* isolates received from Louie *et al.* (NAP1 and non-NAP1) used in their toxin assays described previously; (2) develop methods for the efficient extraction and cleanup of *C. difficile* NAP1 and non-NAP1 DNA and RNA based on current protocols; (3) sequence genomes of both NAP1 and non-NAP1 *C. difficile* received from Louie *et al.* using the 454 Genome Sequencer FLX, and submit to DNA Core for contig-closure using reference strains; and (4) develop methods for the efficient extraction and concentration of mRNA and its conversion to cDNA in quantities acceptable for RNA-Seq, again using the 454 Genome Sequencer FLX sequencing system. I believe that these goals have been accomplished. First, through PCR, it was determined that "VLOO13" received from Louie *et al.* was *cdtB*+, *tcdA*+, *tcdB*+, with a deletion in *tcdC*, consistent with a NAP1 control, and "VLOO18" was *cdtB*-, *tcdA*+, *tcdB*+, without a deletion in *tcdC*, which is consistent with a NAP2 control. PFGE demonstrated that VLOO13 exhibited a pattern

consistent with NAP1, and VLOO13 exhibited a pattern consistent with NAP2. Second, through growth assays (section 3.1.3), it was found that the NAP1 and NAP2 strains from Louie *et al.* exhibited similar growth patterns. Although an *in vitro* model was used in this study, whereas Louie *et al.* used an *in vivo* model, this study demonstrated that there was no difference in growth under these conditions, laying the groundwork for future transcriptomics studies. In addition, data from the growth assays were used to determine the time points of mid-log and stationary phases to aid us in collection of RNA samples for transcriptomic analysis.

Interestingly, few publications report *C. difficile* growth rate data, and none exist for the hypervirulent NAP1 strain, and some did not include detailed methods for carrying out the assays. Carter *et al.* used *C. difficile* 630 [184] grown in BHI medium, which reached mid-log phase at approximately 4 h and stationary phase at 6 h. Govind *et al.* [185] used *C. difficile* VPI 10463 grown in tryptone-yeast extract-glucose medium, which also reached mid-log phase at 4 h, but stationary phase at 8 h. This was significantly different than results in this study, as the NAP1 strain used here entered mid-log approximately 2-3 h later than both the 630 and VPI 10463 strains, and entered stationary phase approximately 4 h later than the 630 strain and 2 h later than the VPI 10463 strain. This difference could be due to the difference in strains, but differences in growth media and handling of materials (which were not specified in either publication) cannot be ruled out. In the growth assays carried out by Gerber *et al.* [186], OD_{550} was used, where as OD_{600} was used in this study. Gerber *et al.* measured CFU at 4 h intervals, where as this study measured OD_{600} as a function of time, and terminated the assays at 16 h when growth clearly plateaued. Furthermore, Gerber *et al.* used *C. difficile* 630 instead

of the hypervirulent NAP1 strain. The differences in strain and wavelength used for measurement makes a direct comparison difficult.

The second objective of this project was to develop a method for obtaining NAP1 and non-NAP1 *C. difficile* DNA and RNA in a quality appropriate for sequencing and reverse transcription purposes. It was observed that the RNeasy® protocols for RNA extraction far underestimates the degree of DNA contamination after *C. difficile* RNA extraction. To overcome problems with low RNA yield and DNA contamination, manufacturer's recommendations were modified by adding additional time for cell lysis and enzymes for DNA digestion.

Next, the RNA yield between mid-log and stationary phase *C. difficile* was compared. It is generally accepted that the RNA profile of an organism can quickly fluctuate with the slightest change in conditions; therefore, it was important for us to determine at what time point in the growth phase the RNA should be extracted. Initially, I wanted to explore the possibility of extracting and sequencing RNA from both mid-log and stationary phases. However, the yield from stationary phase cultures were consistently three- to fourfold less than the mid-log phase cultures and of significantly lower purity. These findings suggested that there may be significant RNA degradation near stationary phase, which may be secondary to increased RNase activity due to spore formation, or spontaneous degradation at 37°C, or a combination of both. Therefore, the methods developed in this study for RNA extraction were based on cells during mid-log phase.

The third objective of this project was to sequence both of the strains obtained from Louie *et al.* Instead of using NCBI reference sequences for *C. difficile* 630, the genomes

the NAP1 and NAP2 strains were sequenced and assembled (by DNA Core) so that I can use the same strain as a reference to which transcripts can be mapped.

The fourth objective was to develop a method for extraction and concentration of mRNA and its conversion to cDNA in quantities acceptable for RNA-Seq using the 454/Roche system. At the time of our experiments, I was not able to find any publications detailing mRNA purification and cDNA synthesis from *C. difficile* transcripts. Since mRNAs rarely make up more than 4% of the total RNA in bacteria and are short-lived [88,97-99,187], the major challenge of *de novo* sequencing of transcriptomes is the detection of low relative abundance of mRNAs in total cellular RNA. To meet the quantity of purified mRNA required for cDNA synthesis and sequencing (5 μg), a commercially available RNA isolation kit produced by Qiagen was chosen. Modifications of the manufacturer's protocols were required to increase the purity of the mRNA. This included increasing the number of DNA subtraction reactions and combining technical replicates. Through these modifications, I achieved the minimum requirement of 5 μg RNA at the appropriate purity. For future studies, I would recommend multiple rRNA depletion reactions in parallel (with the maximum amount of total RNA recommended), then combining the resultant samples followed by a final depletion reaction.

There currently exist two methods of cDNA synthesis from bacterial mRNA. Since bacterial mRNA lacks a poly(A) tail, one method of cDNA synthesis would be to first enzymatically attach poly(A) tails to purified mRNA, and then use poly(T) primers and reverse transcriptase to convert it to cDNA. Another method would be to use random primers. After experimenting with both options, I found that the poly(A) tail method did

not give any appreciable amount of cDNA when measured with the Bioanalyzer, whereas the random 12mer primers did. Modifications to the manufacturer's protocol were made to improve yield, namely the use of T4 Gene 32 protein (New England Biolabs®, Inc.), which functions to stabilize transiently formed regions of ssDNA [188]. The

T4 Gene 32 protein has been shown to improve the yield and efficiency of reverse transcription reactions [189-191]. To further improve cDNA yield, an additional volume of SuperScript™ II reverse transcriptase and T4 Gene 32 protein were added again for the second hour of incubation. The cDNA extraction process was also slightly modified to increase yield. In the alcohol precipitation step, sodium acetate instead of ammonium acetate was used, and glycogen was not added to the precipitation mixture. Through these modifications, it was found that cDNA yield improved by approximately 1-2 μg. Therefore, for future studies, I recommend the use of T4 Gene 32 protein and extra reverse transcriptase to improve yield in cDNA synthesis from *C. difficile* mRNA.

Recently, alternative next generation sequencing (NGS) technologies such as the Helicos Biosciences platform have been used in applications that demand quantitative information in RNA–Seq [192] or direct RNA sequencing (single-molecule direct RNA sequencing technology), as it sequences RNA templates directly without the need to convert them into cDNAs [193,194]. This new technology has the potential advantage of avoiding the cDNA synthesis step, which may introduce biases and artifacts [121,195], and eliminates this tedious step all together. For future projects, it may be prudent to examine this approach.

4.2 Metronidazole Study

Although resistance to metronidazole is uncommon among anaerobic bacteria [137], resistance in *Bacteroides* and *Helicobacter pylori* have been well documented and the underlying mechanisms mostly elucidated. The transferrable *nim* genes, which encode a special nitroimidazole reductase, endows *Bacteroides* with the ability to convert metronidazole into nontoxic substances [151], whereas *H. pylori* owes its resistance to mutations in the *rdxA* gene encoding NADPH nitroreductase [158]. *C. difficile* have been reported to be resistant to many antibiotics, including penicillins and fluoroquinolones (which are strongly associated with CDI) [56]; most of the resistance mechanisms are similar to other Gram-positive bacteria, such as mutation and selection and acquisition of the genetic information that encodes resistance [196]. Despite several reports of *C. difficile* resistance to metronidazole described earlier, the basis for this resistance still remains elusive, as mechanisms in other organisms have not been found in *C. difficile*. Metronidazole resistance in *C. difficile* can have drastic complications: oral metronidazole remains the treatment of choice for initial therapy in mild to moderate disease [28] due to its lower cost and inability to select for vancomycin-resistant enterococci, unlike vancomycin [31,32]. Therefore, the emergence of metronidazole resistance will directly impact treatment outcomes. Recently, metronidazole has seen increased rates of failure and relapse [21,29], perhaps owing to reduced susceptibility by *C. difficile*.

The issue and impact of metronidazole resistance in *C. difficile* is complex. Indication for testing of patients for *C. difficile* in Canadian healthcare facilities is based on symptomatology, and patients infected with *C. difficile* but presenting atypically may

not even be tested. Furthermore, Canadian healthcare facilities currently use different criteria to determine when symptomatic patients should be investigated for *C. difficile*, as well as how the samples are tested: the majority of institutions only perform tests for the presence of toxins using EIA or latex agglutination assays, with relatively few incorporating culturing as a method of detection [42]. To my knowledge, no healthcare institution routinely tests for antibiotic resistance to metronidazole or vancomycin—the drugs of choice for CDI management—perhaps because reports of resistance have been limited. To further complicate things, samples collected from hospitals are sent to labs in frozen storage; our group, as well as previous authors [166], have found that freezing *C. difficile* cultures reduces the MIC of metronidazole, discussed in detail below.

Since 2009, the Antimicrobial Resistance and Nosocomial Infectious group at the NML, as part of the Canadian Nosocomial Infection Surveillance Program (CNISP), routinely tests all samples received for resistance by planting the stool specimen directly onto a CDMN plate containing 8 μg/mL of metronidazole, as well as a CDMN plate without metronidazole. CNISP is a collaboration between the Canadian Hospital Epidemiology Committee, 29 participating members in 9 provinces that includes 50 hospitals, the Nosocomial and Occupational Infections Section, and the NML (PHAC) [56]. In 2009, our group discovered a *C. difficile* isolate originating in Victoria (British Columbia, Canada) that formed colonies on CDMN media with 8 μg/mL of metronidazole. This isolate was confirmed to be a NAP1 strain by PCR and PFGE

fingerprinting against databases, and had a MIC value of 32 μg/mL by Etest®, which is considered to be resistant by CLSI guidelines[4].

Initially, CDMN with 8 μg/mL of metronidazole was chosen as the growth media for propagation, since it is the same media used when screening for resistant isolates. However, the isolate experienced poor growth on this media: a great amount of inoculant was needed to produce new colonies after incubation, and isolated colonies transferred to a new plate with metronidazole would either not grow, or grow very small and sparse colonies (i.e. resistance would be apparently lost). This observation may have been due to the selective nature of CDMN media, which contains the antibiotics norfloxacin and moxalactam, used for isolating *C. difficile* directly from feces [197]. The specific functions of these reagents are as follows: norfloxacin inhibits the growth of *Enterobacteriaceae* and fecal *Streptococci,* and moxalactam inhibits the growth of *Bacteroides spp.* and other *Clostridia* [197]. It is possible that these two antibiotics, together with metronidazole, may have increased stress, or have had synergistic effects that hindered the growth of our isolate. In support of this notion, growth problems associated with CDMN disappeared when BAK plates with 8 μg/mL of metronidazole was used instead: single colonies could be grown successfully without the need for a huge inoculant volume, and when colonies were transferred to a new BAK plate with metronidazole the growth continued. It is worth noting that for both Etest® and agar dilution MIC testing, bioMérieux and CLSI recommend using BAK plates. Thus, the choice of using BAK media was consistent with protocols used for MIC testing. It

[4] CLSI breakpoints for metronidazole are <16 μg/mL = susceptible, 16 μg/mL = intermediate, and ≥16 μg/mL = resistant (CLSI 2007)

appears that although CDMN media is effective for isolation of *C. difficile* directly from fecal matter, it does not suite antimicrobial susceptibility applications as well as BAK plates (especially for long term growth). For future metronidazole resistance studies, I recommend that once colonies are discovered on CDMN plates with metronidazole, these colonies be transfered to BAK plates with metronidazole immediately for propagation and molecular testing with PCR and pulsefield to confirm identity.

Although *C. difficile* with reduced susceptibility to metronidazole have been reported prior to 2002 [161-164], all were non-NAP1 isolates. However, recently Peláez *et al.* and Martin *et al.* have both recently reported on hypervirulent NAP1 *C. difficile* isolates with reduced susceptibility to metronidazole in Spain and Canada, respectively [165,166,168]. Furthermore, both groups found that reduced susceptibility to metronidazole were unstable, where it can be lost upon successive passaging on metronidazole-free media or by freezing in storage. Similar phenomena have been described in *H. pylori*, where metronidazole resistance decreased after frozen storage [198]. Interestingly, the stability of reduced susceptibility to metronidazole in *H. pylori* was significantly associated with treatment outcome: isolates retaining resistance were found in patients who had higher treatment failure rates compared to isolates that lost resistance easily [198]. Since previous studies have demonstrated loss of resistance upon freezing, I chose not to freeze a working sample of our isolate; instead, I continually passaged it under metronidazole pressure (BAK with 8 μg/mL metronidazole). A replicate sample was also frozen and continually passaged on metronidazole-free media so that I could measure its MICs at later point. My findings are similar to those of previous authors: after three months of storage at -80°C, resistance of MetR_stable

decreased approximately by one-fold by both agar-dilution and MIC testing methods. After six months, MICs decreased by fourfold. Continual passaging on metronidazole-free BAK plates over a six-month period also resulted in a fourfold decrease in MIC. Currently, it is unclear why freezing and passaging on metronidazole-free media would result in decreased MIC. Mechanisms of resistance have been relatively well described in other organisms such as *Bacteroides* spp. and *H. pylori* (section 1.3.2). Similar to previous authors [166], *nim* genes were not found in my isolates. To my knowledge no *nim* genes have been described in *C. difficile*, suggesting that resistance is unlikely due to *nim* genes, and other factors may be involved. Indeed, there is growing evidence that metronidazole resistance may be multifactorial, where studies of metronidazole resistance in *Giardia lamblia* suggests that pyruvate:ferredoxin oxidoreductase (PFOR) reduction of the ferredoxin pathway may not be the only mechanism involved (highly resistant isolates with fully functioning PFOR were found) [153]. In terms of the current practice of freezing hospital stool samples during shipment to labs, it is difficult to make a recommendation, as there are very few practical and safe alternatives. Since we now know that freezing may reduce resistance to metronidazole, one possible suggestion would be to transport fecal samples immediately after collection in an anaerobic container (however this may be impractical).

Interestingly, Peláez *et al*. found that Etest® MIC values were up to fourfold less at higher antibiotic concentrations than agar dilution methods [199]. In addition, Poilane *et al.* compared Etest® to agar dilution MIC testing for *C. difficile* with a variety of antibiotics, including metronidazole, and also found Etest® to report lower MICs than the agar dilution method, especially at higher antibiotic concentrations. Martin *et al.* in 2008

reported on metronidazole-resistant NAP1 *C. difficile* isolates, but only provided Etest® data. Both Peláez *et al.* and Poilane *et al.* found the two testing methods reporting similar MIC values at low antibiotic concentrations, but were extremely discordant at higher concentrations with Etest® values being several dilutions less than the agar dilution method. In contrast to these studies, I found both methods of determining MIC values yielded results that were within one dilution. At present, the reason for this difference is not known, but may be due to a difference in materials or isolates. It should be noted that the Etest® method of MIC testing is simpler and faster for single isolates and is conducive to detecting heteroresistance where as the agar dilution method is more conducive to multiple isolates, but not ideal for detection of heteroresistance, described below.

Peláez *et al.* [166] described finding heteroresistance (one of two subpopulations exhibiting elevated MIC), which they attributed to sub-inhibitory antibiotic concentrations inside the zone of inhibition. Heteroresistance is defined as resistance to certain antibiotics expressed by a subset of a microbial population that is generally considered to be susceptible to these antibiotics [205]. This phenomenon was also observed in my study, where on Etest® a subpopulation of smaller microcolonies with higher MIC was observed inside of another subpopulation. Heteroresistance has been reported in other organisms, such as *Staphylococcus aureus*, *Bacteroides* spp., *H. pylori*, *Mycobacterium tuberculosis*, *Streptococcus pneumoniae*, and *Cryptococcus neoformans* [151,165,198,206-209], which could have evolutionary functions in drug resistance, where drug-resistant subpopulations would thrive once antimicrobial challenge is applied [208]. According to the mutant selection window hypothesis [210], an antimicrobial

concentration range exists in which selective amplification of a single-step, drug-resistant mutant occurs, thus an anti-mutant dosing strategy should target the upper boundary of the selection window—the mutant prevention concentration. If selection of mutants occurs with sub-optimal concentrations of antibiotics, the mutants may proliferate in the absence of competition. Although heteroresistance has been demonstrated *in vitro*, its role in clinical treatment failures remains contentious, as evidence both supporting and denying it exist [205]. What has been observed in this study appeared similar to the heteroresistance phenomenon observed in previous publications. If resistance was indeed heterogeneous, it would seem as if freezing and passaging selects for the metronidazole-susceptible subpopulation, possibly due to the resistant population being less tolerant of freeze-thawing and slower growing, so that it would essentially be overwhelmed by susceptible populations after continuous passages on metronidazole-free media. Indeed, this study has demonstrated that the resistant isolates exhibited a slower rate of growth and a lower OD_{600} in stationary phase than that of the revertant *in vitro*, as well as having phenotypically smaller colonies after an equal incubation time, lending support to the notion that slower growth may play a role in gain and loss of metronidazole resistance upon freeze-thawing and passaging. However, this does not rule out other possibilities, such as plasmids that are lost on freeze-thawing, highly unstable mutations that are rapidly lost upon withdrawal of metronidazole pressure, or host factors (such as stool content, microflora, and immune response) that are required for resistance to persist that cannot yet be replicated *in vitro*.

The observations described above could have many implications. Currently, most clinical laboratories do not perform metronidazole susceptibility testing of primary fresh

isolates, and isolates are tested after a freeze-thaw cycle and after a number of passages *in vitro* [165]. Indeed, CLSI recommends frozen isolates be subcultured by at least two serial transfers on metronidazole-free BAK media before being tested for antimicrobial resistance. As described previously, it was found that serial passages on metronidazole-free media reduced the MIC value of our isolate. This is important because following currently accepted methods for MIC testing may result in its values being underestimated, hence resistant isolates may be missed, especially since heteroresistance may be correlated with poor clinical outcome [166]. Previous authors have postulated that exposure to sub-inhibitory concentrations of metronidazole inside the primary zone of inhibition may elevated MICs [151,165]. This may be clinically significant in patients who are on prolonged metronidazole therapy (whether for recurrence or primary infection) who fail to reach an inhibitory concentration of metronidazole in their gut. As described in section 1.3.3, metronidazole is highly absorbed in the small bowel, and colon penetration is poor [167] and highly dependent on the inflammatory condition of the colon [175-177]. In addition, the CLSI breakpoint of 16 μg/mL for metronidazole is based on serum levels and not luminal concentration [165]. Given that *C. difficile* is a toxin-mediated disease and the organism itself does not invade intestinal epithelium [123], serum metronidazole levels may not correlate well with concentrations in colonic lumen, and isolates with MICs of $\geq$ 4 μg/mL may be unaffected [6]. In other words, even a modest increase in the MIC of metronidazole may result in insufficient fecal antibiotic concentrations to inhibit vegetative bacteria [177,200]. Therefore, certain patients undergoing metronidazole therapy may sustain only sub-inhibitory metronidazole concentrations in their colons, which may in turn foster resistance and/or suboptimal

response to treatment. As discussed previously, protocols for testing of CDI are disparate among Canadian healthcare institutions. The majority only test for the presence of *C. difficile* toxins in the stool for evidence of CDI [42], and most laboratories receiving the fecal sample sent from hospitals do not routinely test for metronidazole resistance. This has several implications: (1) strains with reduced susceptibility may frequently go undetected (even if retrospective MIC testing of stored strains were to be performed, freeze-thawing has been shown to reduce MIC values); (2) routine laboratory handling of samples may result in a decrease in MIC if tested; (3) any association between isolates with reduced susceptibility to metronidazole and poor clinical outcome may be missed and (4) there may be insufficient data to correlate the recent increase in metronidazole treatment failure and *C. difficile* MIC profiles. Until the clinical significance of heteroresistant strains has been investigated further, infection control measures and antimicrobial stewardship should be practiced. I recommend taking another look at current protocols and perhaps revising them in light of what this study has found, particularly in terms of freeze-thawing practices, which may reduce MIC values. In addition, current metronidazole dosing guidelines and clinical breakpoints may also be inadequate, as sub-optimal concentrations of metronidazole may select for heteroresistant subpopulations.

This study represents the first investigation of genomic differences between susceptible and resistant isolates of *C. difficile*. The revertant-induction method in this study aimed to obtain a susceptible isolate directly from a progeny of MetR_stable, so that any differences in the genome would more likely to be related to the change in susceptibility. Through my experiments, a metronidazole susceptible revertant was only

observed after 32 generations in liquid BHI media, suggesting that the resistance was quite stable. Although MetR_ori, MetR_stable, and MetR_rev were all sequenced with Roche/454 system, only MetR_stable and MetR_rev sequences were compared due to time limitations. Since there were a total of 53 areas that required confirmation by PCR amplification and sequencing, primer design was done crudely (i.e. primer sequences were not checked for repeats in the genome) and PCR was performed in groups based on similar T_m and expected product sizes. Out of eight regions examined as of this writing, six SNPs were found to be false, and two were confirmed: (1) putative membrane protein, and (2) RNA polymerase σ B factor, which is a transcription factor required for initiation of transcription by binding RNA polymerase to promoter regions [201]. However, sequencing errors that resulted in SNPs that masked a true difference could not be taken into account. For example, a sequencing error may change a true SNP into the same nucleotide as the comparison isolate; hence this position would be overlooked. The Roche/454 system uses DNA templates prepared by emulsion PCR (emPCR) coupled to 1-2 million beads deposited into wells on a picotitre plate, together with pyrosequencing technologies. Advantages of the Roche/454 include longer average read lengths of approximately 330 bases, which improve mapping in repetitive regions, and fast run times [102,202]. Disadvantages include high reagent cost and high error rates in homopolymer repeats [102,202]. Indeed, during genome analysis, many homopolymer repeats were discovered in our *C. difficile* isolates, which may explain the high error rate in SNPs. Furthermore, coverage was low, contributing to the error rate. It stands to reason that a strategy for improving alignment and assembly quality is to increase the read coverage. Since each next-generation sequencing (NGS) platforms produce a unique

pattern of variable sequence coverage, combining different NGS read types for alignment and assembly may remedy this shortcoming [102]. Recent reports have demonstrated that mixing of Roche/454 and Illumina/Solexa data improved *de novo* assemblies of microbial genomes compared with individual data sets alone [203,204]. The Illumina/Solexa system also utilizes genome fragmentation, but uses solid-phase template preparation technologies. It is currently the most widely used platform in the field and is capable of providing greater coverage, but has problems with low multiplexing capabilities of samples [102]. Since only the Roche/454 system was used for this part of the project, the Illumina/Solexa system will not be discussed further. It is worth noting that technical advancements in NGS are being made at an extremely fast pace, thus many problems might be overcome if these experiments were repeated in the near future.

Further transcriptomic, proteomic, and knockout studies may be warranted in order to determine the relevance of these SNPs. Additional studies are required to determine whether this is an *in vitro* or potentially relevant *in vivo* effect.

5 CONCLUSIONS

C. difficile infections are increasingly becoming a concern in terms of health and the economy in Canada and rest of the world. Proton pump inhibitors (PPIs) are one of the most prescribed drugs today, and there has been a growing amount of research into the effect of PPI therapy on the development of CDIs. Louie *et al.* recently found significantly increased toxin production by *C. difficile* NAP1 in rats exposed to both PPI and antimicrobials (personal communication). One way to gain insight into the pathogenesis of *C. difficile* is by studying its transcriptome. Emerson *et al.* in 2008 carried out microarray analyses of *C. difficile* in response to environmental and antibiotic stress [94], in which they found altered expression profiles. Since then, RNA sequencing (RNA-Seq) with next generation sequencing (NGS) technologies has quickly emerged as a powerful new way to study gene expression of an organism. Unencumbered by the shortcomings of traditional methods such as microarrays, RNA-Seq provides a tremendously promising alternative to the study of transcriptional profiles.

Methods for the extraction and purification of nucleic acid from the hypervirulent and epidemic *C. difficile* NAP1 strain were successfully developed. In addition to characterization of *C. difficile* strains with PCR and PFGE used in the Louie *et al.* study, both isolates were sequenced with the 454 Genome Sequencer FLX system. Furthermore, methods for DNA subtraction, rRNA depletion, and cDNA synthesis of *C. difficile* NAP1 transcriptome were refined. This, in turn, provides the groundwork for future studies of the *C. difficile* NAP1 transcriptome challenged by antimicrobials and PPIs.

In the second study, a hypervirulent NAP1 *C. difficile* isolate with reduced susceptibility to metronidazole *in vitro* was characterized. Using observations made in

this study and those of previous authors, a novel protocol to obtain a revertant of this initial isolate was developed. In addition, the genomes of both isolates were sequenced in hopes of finding mutations, which may be associated with reduced metronidazole susceptibility. Through growth assays the MetR_rev isolate was found to be slow growing; MIC testing revealed that freezing and passaging on metronidazole-free media reduced the MIC of our resistant isolate, consistent with previous reports. Recommendations have been made in light of these findings.

The recent emergence of the hypervirulent NAP1 *C. difficile* strain and the epidemics associated with it have changed the landscape of CDIs in Canada and the rest of the world. Compounding to this problem are the findings that efficacy of metronidazole therapy may be declining for severe disease. I hope to draw attention to the problem of metronidazole resistance in *C. difficile* NAP1 strain. Current guidelines and protocols for hospitals and laboratories may be ineffective at capturing these resistant isolates. Re-evaluation of current practices may be warranted, and further studies are necessary to characterize and elucidate the mechanism of metronidazole-resistance in *C. difficile.*

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