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Combatting *Clostridioides difficile*: stress, adaptation and antimicrobial resistance

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Ilse M. Boekhoud

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Colophon

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Combating *Clostridioides difficile*: stress, adaptation and antimicrobial resistance

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1

GENERAL INTRODUCTION

***Clostridioides difficile* infection and disease**

Originally designated as '*Bacillus difficile*' reflecting the difficulty of isolating and culturing this organism in the past, *Clostridioides difficile* (formerly referred to as *Clostridium difficile*) is a Gram-positive and spore-forming obligate anaerobe (1-3). The organism is capable of causing *C. difficile* infection (CDI), a disease with symptoms ranging from mild diarrhea and gastrointestinal discomfort to potentially lethal conditions like pseudomembranous colitis and toxic megacolon (2, 4). *Clostridioides difficile* infection is the leading cause of nosocomial infectious diarrhea in adults and responsible for 10-25% of all antimicrobial-associated diarrhea (AAD) (5-7). Antimicrobial use is the most important risk factor for CDI development, as a healthy and intact intestinal microflora provides colonization resistance against *C. difficile* (8, 9). Although virtually all antimicrobials predispose for CDI, the use of clindamycin, cephalosporins and fluoroquinolones appear to particularly increase this risk (10). Other risk factors for CDI development include prior hospitalization, age and underlying comorbidities (11).

There are several ways to characterize *C. difficile* isolates beyond the species level. This can be challenging, as it potentially leads to multiple typing designations for identical strains (12). One such typing method, PCR ribotyping, is based on the specific banding pattern obtained after amplifying 16S-23S intergenic spacer regions and separating them by (capillary) gel electrophoresis (13). Currently, PCR Ribotyping is the most widely adapted method for classifying *C. difficile* in Europe. Another classification method is multilocus sequence typing (MLST) based on the nucleotide sequence of appointed housekeeping genes, resulting in a sequence type (ST) classification (14). Though there generally is congruence between the methods, strains of one PCR ribotype (RT) can belong to multiple STs, and vice versa (12).

A healthy, stable microbiome will prevent colonization (colonization resistance) and outgrowth of pathogenic organisms such as *C. difficile*. Although it is not entirely clear how colonization resistance occurs, immunological factors as well as the composition of the microbiome are thought to contribute (15-17). The disruption of the gut microbiome can also alter the composition of bile acids present in the gastrointestinal tract, potentially contributing to *C. difficile* colonization (15, 16). As a result, CDI is considered to occur after disruption of the gut microbiota by antimicrobial treatment when spores are ingested through the fecal-oral route, or when *C. difficile* is already present in so-called asymptomatic carriers (18-20). In the first situation, spores then enter the gastrointestinal tract and are able to germinate

into vegetative cells after encountering primary bile acids such as (tauro)cholic acid and chenodeoxycholic acid (21, 22). The toxins produced by vegetative cells are considered the primary cause of disease in CDI (23). Three toxins can be produced by *C. difficile*, which are Toxin A (TcdA), Toxin B (TcdB) and the binary toxin *C. difficile* transferase (CDT). The presence and variability in the toxin encoding genes can be assessed by toxinotyping, and strains can contain all or various combinations of these toxin genes (20, 24, 25). Additionally, strains not carrying any toxin genes (non-toxigenic *C. difficile*, NTCD) have been described (24). TcdA and TcdB-encoding genes are located on the pathogenicity locus (PaLoc) along with three genes encoding proteins that may be involved in their secretion and regulation, TcdR, TcdC and TcdE (26, 27). Once TcdA or TcdB is produced and secreted, these toxins can bind and enter gut epithelium through clathrin-mediated endocytosis (28). Once in the cytosol, certain domains of the toxin interact with- and modify RHO family GTPases and cell division control protein 42 (CDC42), inducing actin cytoskeleton rearrangement, ultimately resulting in cell death (29-31). Apoptosis and subsequent disruption of the gut epithelium and its tight-junctions leads to influx of immune cells and cytokine- and chemokine production, resulting in an inflammatory response (32-34). Certain *C. difficile* strains such as those belonging to RT027- and 078 are also able to produce the binary toxin CDT (35, 36). The binary toxin consists of two components, CDTa and CDTb, located on the binary toxin locus CDTLoc, and is able to depolarize the actin cytoskeleton and increase bacterial adherence by producing microtubule-based membrane protrusions (36, 37). This toxin is also able to suppress a host eosinophilic response (38). Although presence of genes encoding the binary toxin have been reported as associated with increased mortality attributable to CDI, its exact role in *C. difficile* pathogenesis remains debated and unclear, and few TcdA⁺TcdB⁺CDT⁺ strains have been isolated from symptomatic patients (39, 40). Clindamycin-challenged hamsters infected with a TcdA⁺TcdB⁺CDT⁺ *C. difficile* strain demonstrated inflammation, but did not develop CDI (41). Instead it seems the binary toxin increases virulence when TcdA is also present (42).

Outbreaks and economic burden

The incidence and severity of CDI has increased over the last two decades, particularly related to fluoroquinolone use and the emergence of epidemic PCR ribotypes such as RT027 and RT078 (10, 43-45). In North America the CDI incidence increased to 8.6 cases per 1000 hospital discharges in 2008 with a 7.1% mortality rate (46). PCR ribotype 027- and RT078 strains have caused world-wide outbreaks, as illustrated by RT027 in Canada in 2003-2004 and in the

Netherlands in 2005-2006 (47, 48). Two outbreaks of RT027 in the United Kingdom in 2003-2006 ended devastatingly, increasing the annual mortality attributable to CDI by 72% in 2006 (49). Emergence of RT027 and RT078 has been linked to their resistance to fluoroquinolones and its use in the clinic, and their ability to metabolize dietary trehalose, which was used as a food additive prior to their emergence (44, 50, 51). However, CDI is not solely caused by RT027- and 078 strains as Europe and the United States frequently report infections caused by RT001, RT002 and RT014/020 isolates (43, 52). Apart from the ability of *C. difficile* to cause epidemics, patients suffering from (recurrent) CDI have reported a significant reduction in their quality of life compared to the population norm (53, 54). Additionally, a significant economic burden is associated with CDI case management where costs associated with a first- or recurrent CDI in the UK approximated £6294- and £7539 respectively. Annual costs attributable to CDI in the European Union approaches 3 billion euros (45).

Recent epidemiological data on CDI in the Netherlands, obtained from the Netherlands reference laboratory in Leiden University Medical Center (LUMC), indicated that the mean incidence rate from May 2018 to May 2019 was 3.17 CDI cases per 10.000 patients days and was comparable to previous years (55). Mortality of CDI in this time period was 1.1% and the percentage of patients with severe disease decreased compared to results obtained in 2009-2010 (55). Although CDI has primarily been considered to be a nosocomial infection, a substantial amount of hospitalized CDI patients were demonstrated to have an onset in the community (56).

C. difficile and its role in One Health

One Health is a transdisciplinary approach assuming animal health and the environment are closely related to human health (57). The observations regarding reduced quality of life in CDI patients and the enormous resulting economic costs underline the requirement for appropriate preventive measures. However, the metabolically inactive endospores produced by *C. difficile* are highly resistant to stressors such as oxygen, many antimicrobials and disinfectants and other environmental conditions which would be lethal for vegetative cells (58). This complicates eradication of spores in healthcare settings and it is important instilled cleaning protocols are properly executed (59). Additionally, asymptomatic *C. difficile* carriers could potentially contribute to the spread of spores in hospital settings (60). These resistant endospores allow *C. difficile* to be ubiquitous despite that proliferation requires a strict anaerobic environment, and

indeed, they are isolated from soil and rivers, the household environment and food products such as meat and vegetables, most likely from manure of infected animals and contaminated compost (57). The occurrence of *C. difficile* spores in many different environments could explain why this organism is often found in farm animals and household pets, and as a commensal bacterium in neonates (**Figure 1**) (1, 61). This relatively large reservoir may facilitate community acquired CDI, which is becoming a more frequent source of infection (62, 63). Worryingly, common risk factors for hospital-acquired CDI do not apply to the community-acquired variant, as many patients who develop community-acquired CDI are younger, demonstrate less comorbidities, have not been exposed to antimicrobials and were not recently admitted to healthcare facilities (62, 64). Although no solid evidence exists that CDI is acquired through food-borne or environmental transmission, there is some proof for transfer of *C. difficile* between farmers and their farm animals (57, 65). It is unclear if this transfer is zoonotic, from human to animals or if the infection resulted from a shared source. However, these observations do imply the need for a One Health approach in reducing the incidence of both hospital-acquired and community acquired CDI and to better understand the directionality of these infections.

The resistance of C. difficile to antimicrobials

Antibiotics disrupt the microbiota, allowing outgrowth of *C. difficile* which explains why antibiotic use is a risk factor for CDI development (20). Additionally, the intrinsic drug resistant nature of *C. difficile* vegetative cells and its spores could contribute to antimicrobial use as a risk factor for CDI, as this implies the gut microbiota is disrupted but *C. difficile* already present in the gut is not killed, allowing outgrowth. Antimicrobial resistance (AMR) is a devastating and urgent, global problem predicted to lead to 10 million deaths worldwide in 2050 (66). *Clostridioides difficile* is often resistant to antimicrobials not used to treat CDI but commonly used in the clinic to resolve other bacterial infections, such as tetracyclines, aminoglycosides, lincomycin, erythromycin, cephalosporins and fluoroquinolones (51, 67, 68). Multidrug resistance (MDR) is commonly encountered in *C. difficile* strains and almost 60% of 139 clinical isolates were found to be resistant to three or more of these types of antimicrobials in the US (69).

General mechanisms of antimicrobial resistance include inactivation of the drug by altering the targets of the antimicrobial or preventing target access by reducing membrane permeability or increasing drug efflux. Approximately 10% of the *C. difficile* genome is made up mobile genetic elements (MGEs), particularly transposons, which could have been acquired by conjugation,

transformation or transduction (51). Resistance determinants are frequently carried on MGEs and the genome of *C. difficile* harbors many resistance genes on these elements. For instance, the erythromycin ribosomal methylase class B (*ermB*) gene present in many *C. difficile* strains confers resistance to macrolide-lincosamide-streptogramin B (MLSb) antimicrobials such as erythromycin, clindamycin and lincomycin, and is thought to be transferred by transposons Tn5398 (or related), Tn6194 and Tn6215 (70). The gene *ermB* encodes a 23S RNA methylase which methylates an adenine residue in the 23S ribosomal RNA (rRNA), reducing its affinity to MLSb antimicrobials which would otherwise inhibit protein synthesis. The Tn916, Tn916-like and Tn6164 transposons participate in conferring tetracycline resistance in *C. difficile* by harboring various *tet* genes such as *tet*(M) and *tet*(W) (71). Additionally, a Tn916-like transposon is associated with the transfer of the chloramphenicol-florfenicol resistance (*cfr*) gene, an RNA methyltransferase capable of conferring resistance to many different antimicrobials such as phenicols, lincosamides and oxazolidinones (72). A putative plasmid carrying the *cfrC* gene was identified *in silico* in *C. difficile*, but no experiments have been performed to demonstrate this was indeed a plasmid and conferred resistance to MLSb antimicrobials (73). Apart from acquiring antimicrobial resistance through (non-conjugative) transposons, resistance can also be acquired by non-transferable chromosomal determinants. This form of resistance can be induced under antimicrobial pressure by selecting for strains carrying specific chromosomal mutations. This is commonly seen for rifamycin-type of antimicrobials considered for CDI treatment, such as rifampicin and rifamixin, for which mutations in the RNA polymerase β subunit *rpoB* were found to cause resistance (71). Exposure to rifamycins before CDI development was a risk factor for infection with rifampicin resistant *C. difficile* strains (74). Fluoroquinolones are antimicrobials that inhibit type II topoisomerases such as gyrase and topoisomerase IV, which are required for DNA replication. While *C. difficile* does not contain topoisomerase IV, it does possess DNA gyrase subunit A (*gyrA*) and subunit B (*gyrB*) genes which can be inhibited by fluoroquinolones (75). Mutations in the quinolone-resistance determining region (QRDR) of GyrA and/or GyrB are associated with resistance to fluoroquinolones and mutations Thr82Ile in the GyrA subunit is frequently encountered in emerged RT027 strains (76, 77).

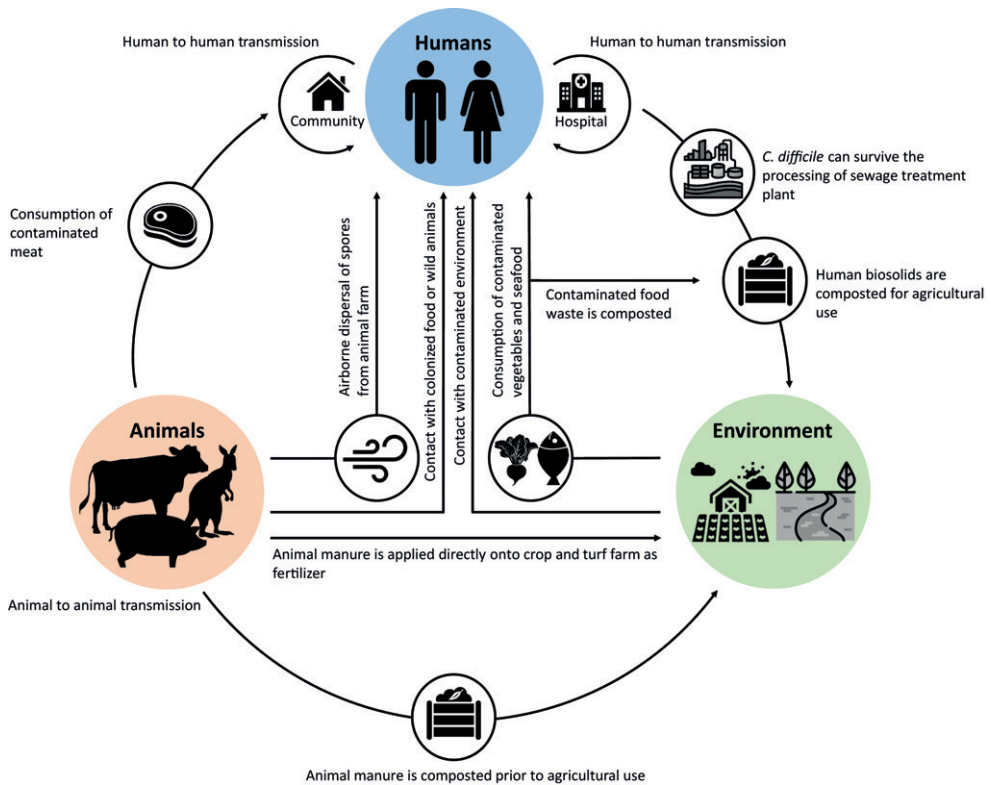


Figure 1. The transmission cycle of *C. difficile* indicating the presence of potential reservoirs.
Reproduced from (57) with permission of the publisher Elsevier.

Although drug efflux by multidrug transporters is most commonly encountered in Gram-negative organisms, a clinical *C. difficile* isolate was reported to contain *cdeA*, a multidrug efflux transporter of the multidrug and toxic compound extrusion (MATE) family (78). This transporter had ethidium bromide efflux activity in *E. coli* and *Clostridium perfringens*, and conferred fluoroquinolone resistance in *E. coli* upon overexpression. However, the authors did not conclusively demonstrate this transporter mediated fluoroquinolone resistance in *C. difficile*. Additionally, laboratory strain 630 Δ *erm* was found to contain the ATP-binding cassette (ABC) transporter CD2068. Inactivation of this transporter led to an increase in ciprofloxacin susceptibility, suggesting this transporter could participate in drug resistance in *C. difficile* (79).

CDI treatment

As *C. difficile* is resistant to many different antimicrobials, the number of drugs used to treat CDI are limited. Currently vancomycin and fidaxomicin are recommended as the first line treatment for CDI according to new guidelines from the Infectious Diseases Society of America (IDSA) (80). Fidaxomicin will be discussed to replace vancomycin as first agent of choice, but its use is limited due to high cost. Formerly metronidazole was the recommended treatment over vancomycin for mild-to moderate CDI as this would prevent outgrowth of organisms such as vancomycin resistant enterococci (VRE), and is widely available due to its low cost (81, 82). The updated US and European guidelines preferred vancomycin over metronidazole treatment, as an overview of randomized-controlled trials demonstrated superiority of effectiveness of vancomycin over metronidazole (80). Despite this guideline update the prescription of metronidazole for CDI treatment decreased by only 3% in the 18 months that followed this change. The use of vancomycin and fidaxomicin did increase significantly during this time (83, 84). The monoclonal antibody bezlotoxumab, directed against TcdB, has gained approval of the U.S. Food & Drug Administration (FDA) in 2016 to be used in CDI treatment (85).

Resistance to vancomycin and metronidazole is relatively rare in *C. difficile*. Encountered resistance rates are also dependent on the method of testing- and breakpoint used, as the EUCAST epidemiological cutoff values in determining resistance are lower than the breakpoints defined by the Clinical and Laboratory Standards Institute (CLSI) (86-88). According to a meta-analysis performed by *Saha et al.* a significant increase in vancomycin resistance from 0.4% to 2.3% occurred after 2012 after exclusion of outlier studies (89). In contrast, the resistance rate of metronidazole in *C. difficile* decreased after 2012 from 2.5% to 1.7%. Individual metronidazole resistance rates varied from 0% to 18.3%. Metronidazole resistance is most frequently encountered in non-toxigenic RT010 isolates and the minimal inhibitory concentration (MIC) of metronidazole is higher in RT001 and RT027 strains (88, 90). Vancomycin and metronidazole resistant *C. difficile* isolates were most frequently encountered in North-, Central- and South-America and Asia (89). Interestingly, no fidaxomicin resistance was reported in the reviewed clinical studies, though preliminary data from the COMBACTE-CDI study show that resistance sporadically occurs in Europe (dr. Kerrie Davies, personal communication). No resistance development during treatment has been reported for fidaxomicin, but there is one mention of a fidaxomicin resistant isolate recovered from a cured CDI patient (91).

Although there is no evidence that resistance to metronidazole, vancomycin or fidaxomicin correlates with CDI treatment failure, broad-spectrum antibiotics such as metronidazole and vancomycin impact the diversity of the gut microbiota and are often unable to eradicate *C. difficile* spores (92). This potentially facilitates recurrent infections which occur frequently. Indeed, there is evidence that more than half of all recurrences are caused by infection with the original strain (93, 94). The chance for a recurrent infection after an initial episode of CDI can be as high as 25% within 30 days of infection clearance, and this risk increases with each subsequent recurrent infection (95, 96). For this reason the development of alternative treatments is urgent. The rationale behind fecal microbiota transplantation (FMT), a procedure in which donor fecal material is transferred to the recipient, is that replenishment of a certain gut microbiota composition would reduce the risk of developing recurrent infection as it facilitates suppression of *C. difficile* outgrowth. This procedure has proven highly effective at treating recurrent CDI compared to vancomycin and should be considered after a patient has had two- or more recurrent infections (97, 98). However, this procedure may not be without risk and long-term effects have not been adequately evaluated yet. Fecal material of one donor transferred extended-spectrum beta-lactamase (ESBL)–producing *Escherichia coli* to two patients receiving FMT (99). Both patients developed bacteremia and one patient died. Additionally, procarcinogenic bacteria such as enterotoxigenic *Bacteroides fragilis*, *Fusobacterium nucleatum*, and *Escherichia coli* expressing the colibactin toxin were demonstrated to be transferable by FMT, harboring the risk of developing colon cancer in the future (100). As a result of these and other outcomes the FDA issued a safety warning regarding the use of FMT and risk of serious adverse events. It is now strongly advised to broaden screening of donor fecal material for these harmful, transferable micro-organisms. Both beneficial aspects and potentially disadvantageous (long term) effects of FMT (including transfer of resistance determinants and potentially harmful members of the microbiota) are studied by longitudinally following donors and recipients in the Netherlands by the Netherlands Donor Feces Bank located at the LUMC (101). However, this current uncertainty advocates for the continued use of antimicrobials in CDI treatment. A recently published review found that a vancomycin taper- and pulse regime was more effective at treating (recurrent) CDI than initially considered due to suboptimal comparisons- and methodology in earlier studies, and that FMT should only be considered when recurrent infection is no longer manageable with antimicrobial use (102). An extended-pulsed fidaxomicin therapy also resulted in higher sustained clinical cure rates than for standard vancomycin therapy (103). As only few antimicrobials are implemented in

CDI treatment it is important to understand how resistance- and adaptation to these clinically relevant antimicrobials in *C. difficile* occurs, in order to develop novel drugs and therapeutics to treat CDI.

Novel drugs in development against CDI

Novel drugs developed to treat CDI should preferentially be narrow-spectrum in order to diminish the disruption of the gut microbiota to reduce the development of recurrence. One such novel drug is the DNA replication inhibitor ibezapolstat (formerly known as ACX-362E). This compound acts as a dGTP analogue and specifically binds and inhibits the DNA Polymerase III α (PolC) of different organisms, including *C. difficile* as was demonstrated *in vitro* (104). As this type of DNA polymerase is absent in Gram-negative organisms the negative impact on the gut microbiota would be less pronounced compared to treatments using broad-spectrum antimicrobials. Ibezapolstat is currently in clinical development and has completed phase I trials, which showed ibezapolstat treatment resulted in a distinct microbiome profile when compared to vancomycin (105). Similarly, the novel drug ridinilazole seemed to primarily inhibit *C. difficile* and was not highly effective against other intestinal micro-organisms (106). Although its method of action is not entirely clear, preliminary data seem to indicate this compound inhibits DNA synthesis (107). It is currently tested in phase 3 clinical trials. Whereas ibezapolstat and ridinilazole seem to inhibit *C. difficile* directly, ribaxamase is a novel drug that inhibits *C. difficile* indirectly. Ribaxamase is a β -lactamase, a type of compound which inhibits β -lactam antibiotics. As β -lactams are considered to predispose patients for CDI by disrupting the gut microbiota, ribaxamase reduced this probability by inhibiting the negative effects of β -lactams on the gut microbiota (108). This drug has completed phase 2 clinical trials.

Although all of the drugs described above are promising candidates, there is no guarantee they will reach the market. Both surotomycin and cadazolid illustrate this, as these compounds were found to be non-superior and inferior to vancomycin, respectively, in phase 3 clinical trials and as a result clinical development was halted (109, 110). Therapies other than small molecule drugs designed to treat or prevent CDI are also in development, but will not be discussed in this thesis (111).

Vancomycin resistance

As mentioned above, vancomycin is the primary drug used for CDI treatment. Vancomycin is a hydrophilic antimicrobial that inhibits cell wall synthesis. It is able to bind to the terminal D-alanyl-D-alanine (D-Ala-D-Ala) moieties of the *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG) peptides of the cell wall backbone, which ultimately inhibits crosslinking of the peptidoglycan layer (112). In VRE vancomycin resistance can be mediated by *vanA* and *vanB* genes which substitute the D-Ala-D-Ala peptides on NAM for D-Alanine-D-Lactate (D-Ala-D-Lac), reducing the affinity of vancomycin to these residues (112). Worryingly, transfer of *vanA* between VRE and *Staphylococcus aureus* is possible by horizontal gene transfer of the Tn1546 transposon (113). A *vanB2* vancomycin resistance operon potentially originating from VRE was also found in a *C. difficile* isolate, although this operon was cryptic (silent) and did not confer vancomycin resistance (114). In contrast, active vancomycin gene clusters altering peptidoglycan composition have been described in *C. difficile* as well. The gene cluster *vanG* in *C. difficile* (*vanG_{cd}*) is involved in the substitution of D-Ala-D-Ala peptides to D-Alanine-D-Serine (D-Ala-D-Ser) and is considered cryptic in different *C. difficile* strains (115). This cluster was expressed in strain 630Δ*erm*, but its activity was not enough to confer vancomycin resistance in this strain. However, this originally cryptic cluster was shown to be activated by mutations in the genes encoding the VanSR two-component system in RT027 strains, increasing vancomycin tolerance in these isolates (116). A different study has generated *C. difficile* isolates with reduced vancomycin susceptibility by serial passaging under selection and analyzed these with whole genome sequencing (WGS) (117). In the isolates with reduced vancomycin susceptibility mutations were found in *murG* and *rpoC*. MurG is involved in peptidoglycan synthesis by converting lipid I to lipid II and the mutated *murG* could reduce vancomycin affinity for the D-Ala-D-Ala portion of lipid II. The mutation in *rpoC* was somewhat surprising as these kind of mutations commonly reduce the effectivity of RNA polymerase (RNAP) inhibitors which are not considered the target of vancomycin. However, *rpoC* mutations have been known to inhibit antimicrobials that do not act on RNAP (118).

Metronidazole resistance

Although vancomycin is recommended as first line treatment for CDI in guidelines in the US, metronidazole use has not decreased severely (83, 84). Metronidazole is considered as a first choice treatment for infections with anaerobic bacteria and is also commonly used as prophylaxis for many surgical procedures (119, 120). Metronidazole is effective in

microaerophilic organisms such as *Helicobacter pylori* and in anaerobes like *B. fragilis* and *C. difficile* (121, 122). It has been widely used against protozoa such as *Giardia lamblia*, *Entamoeba histolytica* and *Trichomonas vaginalis* (123). (119). Metronidazole is a nitroimidazole, which is a prodrug that requires reduction of its nitro-group under low oxygen tension to become activated and therefore cytotoxic (124). It is considered to enter cells by passive diffusion mediated by a concentration gradient of activated and inactivated metronidazole (125). Pyruvate:ferredoxin oxidoreductase (PFOR) is an enzyme involved in fermentation and catalyzes production of acetyl-CoA from pyruvate. The electrons generated in this reaction are transferred to ferredoxins (126). After metronidazole uptake by passive diffusion, the electrons generated in this reaction are transferred to its nitro group instead of to ferredoxin due to the latter's low redox potential (126). Other effectors with sufficiently low redox potential able to reductively activate metronidazole are flavodoxin and hydrogenase, as was seen in *Clostridium pasteurianum* (127, 128). Reductive activation of metronidazole results in formation of nitro-radicals which are postulated to cause DNA damage (129, 130). While wildtype *E. coli* is resistant, strains deficient in DNA repair mechanisms were found to be more susceptible to metronidazole in anaerobic environments, implying metronidazole is indeed bactericidal by damaging DNA (131, 132). Alternatively, the ability of metronidazole to act as an electron sink may disturb the proton motive force and reduce ATP production which may ultimately be toxic to cells (124).

Metronidazole resistance mechanisms reported in other organisms include alterations in PFOR activity or the presence of 5-nitroimidazole reductase (*nim*) genes (124, 133-137). These modifications decrease the reductive activation of metronidazole or reductively inactivate metronidazole to a stable, non-toxic intermediate. Alternatively, adaptations in the oxidative stress response will allow cells to better handle the bactericidal effects caused by activated metronidazole (124). Understanding of metronidazole resistance in *C. difficile* is fairly limited. Experiments conducted with strains which developed reduced metronidazole susceptibility due to evolution under metronidazole pressure in the laboratory, have been informative on the underlying processes associated with metronidazole resistance. For instance, a clinical isolate of RT027 that was serially passaged to become stably metronidazole resistant developed mutations in *hemN* (coproporphyrinogen III oxidase) and *fur* (ferric uptake regulator), involved in electron transport- and iron utilization pathways respectively (138). In line with these findings are the results of a study performed on a mismatch repair deficient mutator strain which was evolved under metronidazole pressure (139). In this strain mutations in the iron transporter

feoB1 increased the MIC to metronidazole and additional mutations in *nifJ* (pyruvate-ferredoxin oxidoreductase, PFOR), *xdh* (xanthine dehydrogenase) or the iron-sulfur cluster regulated *iscR* further decreased its susceptibility to metronidazole. Although the mutations found in *feoB1* were required before the other detected mutations had any effect on metronidazole susceptibility, these results also illustrate the importance of electron transport, iron utilization and fermentation in metronidazole activity. In contrast, a RT027 isolate serially passaged on metronidazole which developed stable metronidazole resistance was analyzed by a shotgun proteomics approach. In the resistant isolate a role was found for DNA repair proteins and putative nitroreductases (140). However, as these studies were performed with laboratory evolved strains, they do not demonstrate how clinical *C. difficile* isolates are resistant to metronidazole. Proteomic analysis on nontoxigenic RT010 isolates with reduced susceptibility- or resistance to metronidazole revealed an increase in RecA after exposure to the antimicrobial, which is involved in DNA repair, but there appeared to be no straightforward relationship between metronidazole resistance and changes in Pfo/NifJ protein levels (141). In summary, it seems iron homeostasis, DNA repair and electron transport are plausible targets for the development of metronidazole resistance in *C. difficile*.

Fidaxomicin resistance

The advantage of fidaxomicin to metronidazole and vancomycin is that fidaxomicin has a smaller spectrum of activity, which reduces disruption of the microbiota and subsequent predisposition for CDI development as explained previously (142). Although the mechanism of action of fidaxomicin is not yet entirely clear, it is thought to inhibit transcription by interaction with the RNA polymerase. After serial passaging, mutations in *rpoB*, *rpoC* and CD2212 have been found in strains with reduced susceptibility to fidaxomicin (117, 143, 144). One clinical isolate with significantly reduced fidaxomicin susceptibility contained a Val1143Asp substitution in *rpoB*, and introducing this mutation in 630 Δ *erm* also reduced fidaxomicin susceptibility in this strain (145). Other mutations in *rpoB* resulting in amino acid substitutions such as Val1143Phe and Val1143Gly can cause reduced susceptibility to fidaxomicin, although these- and Val1143Asp mutations were associated with a fitness cost in *C. difficile* (145, 146). This fitness cost was not observed for *rpoB* mutations conferring resistance to rifamycins. This may explain why fidaxomicin resistance is not encountered frequently in clinical *C. difficile* isolates, even though decreased fidaxomicin susceptibility is developed easily under selective pressure (68).

Adaptation and tolerance in *C. difficile*

Apart from acquiring antimicrobial resistance mechanisms it is known that increased tolerance or persistence can contribute to survival during antimicrobial exposure. This quiescent, dormant state desensitizes cells to antimicrobials without increasing the MIC established during antimicrobial susceptibility testing (147). The ability of bacteria to become more tolerant to antimicrobials results from their necessary ability to adapt to environmental conditions and repair (DNA) damage. Bacteria can induce alterations in replication and transcription in order to become more tolerant to these hostile conditions. For instance, the digestive tract is an environment in which bacteria encounter variable nutrient availability, different oxygen tensions, bile salts, pH- and temperature variations and bacterial competition (149). Additionally, infectious bacteria need to be able to withstand the host inflammatory response and antimicrobial compounds produced by both the host and resident microbiota (150). Although more mechanisms and cellular responses can lead to tolerance or persistence, the SOS response and the general stress response are known to contribute to these tolerant states, and they also play a role in antimicrobial resistance (149).

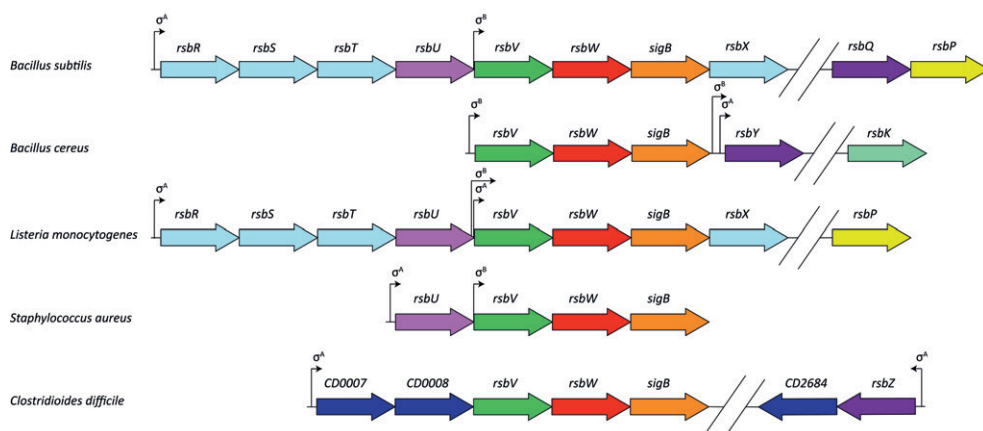


Figure 2. Genomic organization of the σ^B operon and its regulators in different Gram-positive organisms. Bent arrows indicate the location of a promoter and the sigma factor involved in its transcription. Slashes indicate a gap in chromosome location. Adapted from (148).

The SOS response is a regulatory network activated in response to DNA damage and initiates DNA repair (151). It is regulated by the transcriptional repressor LexA and the inducer RecA (152). Other than DNA repair the SOS response is involved in other processes such as pathogenesis, biofilm production and antimicrobial resistance (153). In *C. difficile*, the SOS

response was demonstrated to control biofilm- and toxin production and sporulation in addition to DNA repair (152). Activation of the SOS response was shown to stimulate persister cell formation in *E. coli* and can stimulate spread of antimicrobial resistance genes by horizontal gene transfer (142, 154, 155). Certain antimicrobials such as tetracyclines, (fluoro)quinolones and aminoglycosides are known to induce the SOS response in other organisms (142, 155, 156). In *C. difficile*, the fluoroquinolone levofloxacin was shown to induce the SOS response (152).

In addition to the SOS response, the general stress response is important in regulating adaptation to hostile conditions. This response is regulated by sigma factor B (σ^B ; encoded by the *sigB* gene) in Gram-positive organisms such as *Bacillus cereus* and *C. difficile* (157). Sigma factors are a subunit of the RNA polymerase holoenzyme and confer specificity to the RNA polymerase, initiating transcription of genes belonging to the regulon of the specific sigma factor. It was demonstrated to be important for survival in the presence of reactive oxygen species (ROS), hydrogen peroxide and during acid- and heat stress in organisms like *Listeria monocytogenes* and *Staphylococcus aureus* (158, 159). In *C. difficile* this sigma factor was shown to be involved in sensitivity to mitomycin C, rifampicin and the nitrous oxide donor diethyl-amine NoNoate (DEA/NO) (160). It also proved important for survival during low oxygen tension (161).

Environmental stressors and/or nutrient depletion can lead to σ^B -mediated transcription, which is tightly regulated under unstressed conditions due to its significant energy burden and associated toxicity (162). A conserved partner-switching mechanism regulates σ^B activity (148). In inactive state the anti-sigma factor RsbW is bound to σ^B and consequently inhibits its activity. Upon encountering σ^B -activating (environmental) stressors, the anti-anti sigma factor RsbV is dephosphorylated by a phosphatase upon which it is able to bind to- and sequester RsbW, allowing σ^B to interact with the RNA polymerase. The proteins involved in dephosphorylation of RsbV are stressor- and species specific as the *sigB*-operon of different organisms encompasses different genes (**Figure 2**) (148). For instance, *B. subtilis* and *L. monocytogenes* are able to form a stressosome composed of proteins RsbR, RsbS, RsbT and RsbX, all located in the operon which also encodes σ^B , upstream of *rsbV* and *rsbW* (163). This stressosome is activated upon encountering environmental stress and activates RsbU, which in turn dephosphorylates RsbV. When undergoing energy stress or environmental stress, RsbP and RsbU, respectively, are ultimately responsible for RsbV dephosphorylation in *B. subtilis* and *L. monocytogenes* (148, 164). RsbP is thought to be activated by the hydrolase RsbQ which could act as a cofactor for

RsbP (165). Alternatively, RsbU activity is controlled by the RsbT and RsbS proteins which are a part of the stressosome (148). Energy and environmental stress signals are combined in *B. cereus* by the multi-sensor hybrid kinase RsbK which acts on RsbY, leading to dephosphorylation of RsbV (166). Although the σ^B operon in *S. aureus* does contain *rsbU*, it does not possess *rsbRST* or *rsbX* and RsbU seems to play a different role in *S. aureus* than it does in *B. subtilis* (167, 168). It is possible *S. aureus* RsbU activity is regulated by an as of yet unknown protein encoded elsewhere on the chromosome, as is the case for RsbQ and RsbY in *B. subtilis* and *B. cereus*, respectively.

The genomic organization of the *sigB*-containing operon in *C. difficile* differs from other organisms as four genes are upstream of *sigB*, including *RsbV*-, *RsbW*-, and two genes of unknown function that do not appear to be involved in regulation of σ^B (160, 169). The PP2C phosphatase responsible for dephosphorylation of RsbV in *C. difficile* is named RsbZ and is located downstream the *sigB* promoter adjacent to CD2684 (169). Although the σ^B regulon in *B. subtilis* and *L. monocytogenes* consists of approximately 150- and 130 genes, respectively, σ^B was proposed to control almost a 1000 genes in *C. difficile* (148, 160).

Apart from having a crucial role in survival in the hostile environment encountered during the bacterial life cycle, σ^B seems to also be involved in antimicrobial resistance. In *S. aureus* σ^B overproduction lead to thickening of the cell wall and increased resistance to beta-lactam antimicrobials (170). Additionally, σ^B was involved in resolving growth-arrest induced by rifampin in *B. subtilis* (171). In *C. difficile*, knowledge about the role of σ^B in *C. difficile* and how it influences antimicrobial resistance is limited. Hydrogen peroxide, rifampicin and mitomycin C were more lethal against a *sigB* mutant strain compared to wildtype, but the mechanism behind this is unclear (160).

In order to develop new therapeutics against *C. difficile* it is important to understand how antimicrobial resistance and adaptation occur in this organism. A more complete understanding of these processes will demonstrate novel pathways and subsequent targets for drug development or alternative treatment strategies.

Outline of this thesis

This introduction provides an oversight of *C. difficile* as a pathogen and why antimicrobial use is such an important risk factor for CDI. Metronidazole, fidaxomicin and vancomycin resistance are discussed, as well as other processes that contributes to the resilience of *C. difficile*. **Chapter 2** describes how a novel drug in clinical development, ibezapolstat (ACX-362E), inhibits the DNA polymerase and is able to cause a gene-dosage shift. This study demonstrated that an indirect effect of this gene-dosage shift is mediated by σ^B due to its origin-proximal location. **Chapter 3** describes that the σ^B regulon is much smaller than previously proposed and that a substantial part is involved in detoxification of oxidative- and nitrosative stresses. Hydrogen peroxide and antimicrobials with a different mechanism of action, including ibezapolstat and metronidazole, activated σ^B -controlled genes in a manner dependent on this sigma factor. **Chapter 4** describes the discovery of the plasmid pCD-METRO which mediates metronidazole resistance in clinical *C. difficile* isolates and is most likely acquired horizontally from an unknown donor organism. **Chapter 5** illustrates that clinical *C. difficile* isolates utilize heme to become less susceptible to metronidazole and that a deletion in *hsmA* correlates with heme-mediated metronidazole resistance in a specific PCR ribotype. **Chapter 6** is the general discussion and provides an outlook of the results described in this thesis.



2

GENOME LOCATION DICTATES THE TRANSCRIPTIONAL RESPONSE TO POLC-INHIBITION IN *CLOSTRIDIoidES DIFFICILE*

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Abstract

Clostridioides difficile is a potentially lethal gut pathogen that causes nosocomial and community acquired infections. Limited treatment options and reports of reduced susceptibility to current treatment emphasize the necessity for novel antimicrobials. The DNA-polymerase of Gram-positive organisms is an attractive target for the development of antimicrobials. ACX-362E (N^2 -(3,4-Dichlorobenzyl)-7-(2-[1-morpholinyl]ethyl)guanine; MorE-DCBG) is a DNA polymerase inhibitor in pre-clinical development as a novel therapeutic against *C. difficile* infection. This synthetic purine shows preferential activity against *C. difficile* PolC over those of other organisms *in vitro* and is effective in an animal model of *C. difficile* infection. In this study we have demonstrated that at concentrations below the minimal inhibitory concentration, the presumed slowing (or stalling) of replication forks due to ACX-362E leads to a growth defect. We have determined the transcriptional response of *C. difficile* to replication inhibition and observed an overrepresentation of up-regulated genes near the origin of replication in the presence of PolC-inhibitors, but not when cells were subjected to sub-inhibitory concentrations of other antibiotics. This phenomenon can be explained by a gene dosage shift, as we observed a concomitant increase in the ratio between origin-proximal versus terminus-proximal gene copy number upon exposure to PolC-inhibitors. Moreover, we show that certain genes differentially regulated under PolC-inhibition are controlled by the origin-proximal general stress response regulator sigma factor B. Together, these data suggest that genome location both directly and indirectly determines the transcriptional response to replication inhibition in *C. difficile*.

Introduction

Clostridioides difficile, formerly known as *Clostridium difficile* (3), is a Gram-positive, anaerobic bacterium that can asymptomatically colonize the intestine of humans and other mammals (2, 20, 172). However, when the normal flora is disturbed *C. difficile* can overgrow and cause fatal disease, as has been dramatically demonstrated in the Stoke Mandeville Hospital outbreaks in 2004 and 2005 (173). The ability to form highly resistant endospores coupled to its extensive antibiotic resistance have contributed to its success as a nosocomial and community acquired pathogen (2, 20, 172). Recent years have seen an increase in the incidence and severity of *C. difficile* infections (CDI), due to the emergence of certain PCR ribotypes (20, 174). Antibiotic use is a well-established risk factor for CDI (175), and the emergence of the epidemic PCR ribotype 027 has been linked to fluoroquinolone resistance (44). At present two antibiotics, metronidazole and vancomycin are commonly used to treat CDI and a third, fidaxomicin, is indicated for the treatment of relapsing CDI (176, 177). Clearly, limited treatment options and reports of reduced susceptibility to current treatment (88, 140, 178) emphasize the necessity for the development of novel antimicrobials and a better understanding of tolerance and resistance towards existing therapeutics.

It is increasingly realized that off-target effects that occur when cells are exposed to antimicrobials can contribute to their efficacy, but also facilitate the emergence of tolerance and/or resistance (179). Antimicrobials may act as signaling molecules which modulate gene expression (179). Additionally, in particular those targeting DNA replication (such as polymerase inhibitors) can cause transcriptional effects as a result of differences in gene dosage (180).

The polymerase of Gram-positive organisms is an attractive target for the development of novel antimicrobials (181). First, these PolC-type polymerases are absent from Gram-negative organisms and humans (182, 183). HPUra, one of the first such compounds, is therefore highly active against a wide range of Gram-positive bacteria, but does not affect Gram-negative bacteria (182, 183). Template-directed elongation is blocked by the inhibitor through simultaneous binding to the cytosine of the DNA strand and near the active site of PolC. Second, compounds can be derived that have an increased specificity towards specific microorganisms. ACX-362E (**Figure 1**) is a compound in pre-clinical development as a novel therapeutic against *C. difficile* as it shows preferential activity against *C. difficile* PolC over those of other organisms *in vitro*

(184, 185), and will progress to clinical trials in the near future (Acurx Pharmaceuticals, personal communication). PolC-inhibitors can cause a stress response and cell death after prolonged exposure. In *Bacillus subtilis*, this stress is characterized by a combination of DNA damage (SOS) response, and an SOS-independent pathway dependent on the DNA replication initiator DnaA (186, 187). In *Streptococcus pneumoniae* cells, devoid of an SOS-response, competence for genetic transformation is induced upon replication stress (188). The response of *C. difficile* to this particular class of compounds is unknown.

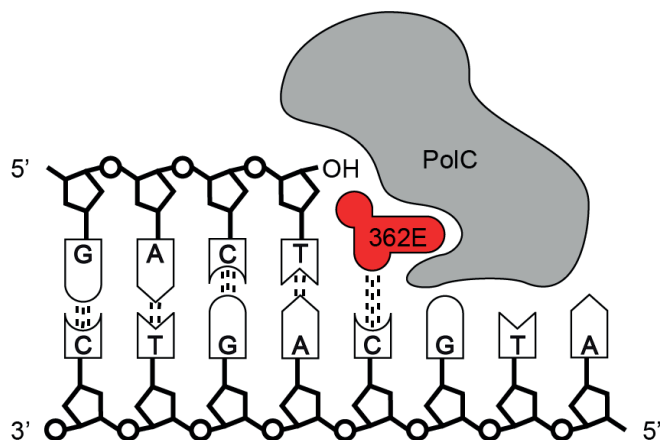
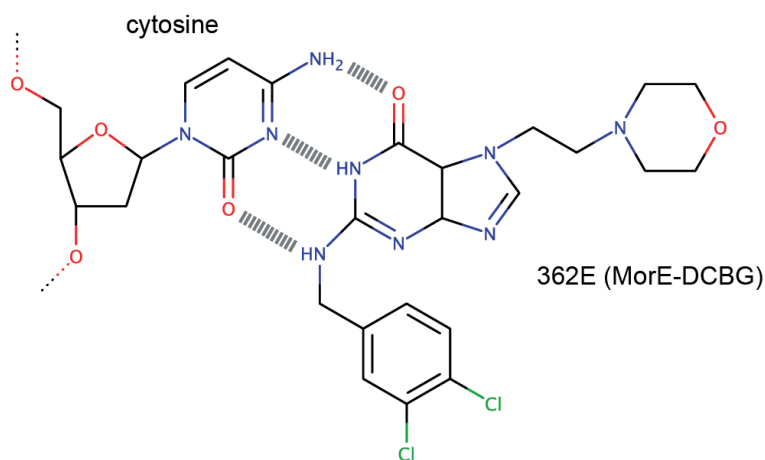
A.**B.**

Figure 1. Mechanism of action of the PolC-inhibitors ACX-362E. **A)** Ternary complex of inhibitor ACX-362E, DNA, and PolC. **B)** H-bonding between inhibitor molecule ACX-362E and a cytosine residue of DNA.

In this study, we characterized aspects of the action of PolC-inhibitors towards *C. difficile*. We investigated the effects of sub-inhibitory levels of PolC-inhibitors on growth of *C. difficile* in liquid medium and performed RNA-seq analyses to determine the transcriptional response to PolC-inhibitors in our laboratory strain 630 Δ erm. Finally, marker frequency analysis and transcriptional reporters were used to provide a mechanistic explanation for the observed up-regulation of origin-proximal genes under conditions of replication inhibition.

Results

Treatment with ACX-362E leads to a pleiotropic transcriptional response

In order to determine the transcriptional response of PolC-inhibitors, we established the optimal concentration of both inhibitors which affected growth of *C. difficile* in liquid medium. The laboratory strain *C. difficile* 630 Δ erm (PCR ribotype 012, MLST Clade 1)(189, 190) was grown in medium with varying amounts of HPURa (10-40 μ g/mL) or ACX-362E (0.25-8 μ g/mL) (**Supplemental Figure 1**). The growth kinetics of *C. difficile* under the influence of varying concentrations of HPURa was marginally affected when using concentrations from 10-40 μ g/mL, at >80 percent of the non-treated culture. Growth kinetics of cultures containing PolC-inhibitor ACX-362E 1-8 μ g/mL were similar and resulted in -30-40 percent reduced growth compared to the untreated culture. For subsequent experiments we used concentrations of PolC-inhibitors that result in a maximum reduction in growth of 30% compared to a non-treated culture (**Supplemental Figure 1**) (HPURa: 35 μ g/mL; ACX-362E: 4 μ g/mL).

Above, we established that growth of *C. difficile* is partially inhibited at certain concentrations of PolC-inhibitors. Slowing down or stalling of replication forks might lead to a stressed state, as observed for other organisms (187, 188). As nothing is known about the effect of replication inhibition on the physiology of *C. difficile*, we determined the transcriptional response to replication inhibition by sub-MIC levels of PolC-inhibitors through strand-specific RNA sequencing (RNA-Seq).

C. difficile 630 Δ erm was grown for 5h in medium with HPURa (35 μ g/mL) or ACX-362E (4 μ g/mL) starting from an OD₆₀₀ of 0.05 after which cells were harvested for RNA isolation. Purified RNA was converted to cDNA and used for RNA-Seq as described in the Materials and Methods.

For ACX-362E, 722 genes were differentially expressed, of which 438 genes were up-regulated and 284 genes were down-regulated. The number of differentially expressed genes in HPURa treated samples was approximately 2-fold lower: 360, of which 124 genes were up-regulated and 236 genes were down-regulated. The full list of differentially regulated genes is available as **Supplemental Table 1** and the top 10 of up- and down-regulated genes are shown in **Table 1** (HPURa) and **Table 2** (ACX-362E). Here, we highlight three aspects of the results.

First, we performed a Gene Set Enrichment Analysis (GSEA)(191) via the Genome2D web server (192) using the locus tags of the differentially regulated genes (**Supplemental Table 1**) as input. Among the genes up-regulated by ACX-362E, there is a strong overrepresentation of those involved in translation, ribosome structure and ribosome biogenesis. Not unexpectedly, also replication, recombination and repair processes are affected. This suggests that genes from these pathways show a coordinated response in the presence of ACX-362E. Among the genes down-regulated in the presence of ACX-362E, the levels of significance for specific processes are generally much lower, suggesting that there is a more heterogeneous response among genes from the same pathway. Nevertheless, metabolic pathways (especially carbon metabolism and coenzyme A transfer) and tellurite resistance were found to be significantly affected. Strikingly, a GSEA analysis on lists of genes that are differentially expressed in the presence of HPURa revealed similar processes to be affected.

The findings from the GSEA analysis prompted us to evaluate the overlap in the lists of differentially regulated genes between the ACX-362E and HPURa datasets in more detail. If both compounds act via a similar mechanism, we expect a conserved response. Indeed, we observe that >90% of the genes that are up-regulated in the presence of HPURa compared to the non-treated condition, are also identified as up-regulated in the presence of ACX-362E (**Figure 2A**). Though the overlap is not as strong for the down-regulated genes, we find that >30% of the genes affected by HPURa are also identified as affected by ACX-362E (**Figure 2B**). Notably, the directionality of the response is conserved, as no genes were found to be up-regulated in one but down-regulated in the other condition. Based on these observations, we believe that the differentially expressed genes identified in this study are representative for a typical response to inhibition of PolC in *C. difficile*.

Finally, we related the changes in transcription to genome location. *C. difficile* has a single circular chromosome and one origin of replication (*oriC*) from which the process of DNA replication occurs bi-directionally towards the terminus (*terC*) (**Figure 3A**). Though neither *oriC* nor *terC* has been definitively defined for *C. difficile*, it is assumed that *oriC* is located at or near *dnaA* (CD0001; CD630DERM_RS00005). The terminus region is generally located at the inflection point of a GC skew ($[G - C]/[G + C]$) plot. Such a plot places the *terC* region around 2.2Mb from CD0001, near the CD1931 (CD630DERM_RS10465) open reading frame (**Figure 3A**) (193). We noted that the differential expression appeared to correlate with genome location (**Table 1**, **Table 2** and **Supplemental Table 1**): many of the up-regulated genes have either low or high gene identifiers (CD numbers) indicative of an origin proximal location and, conversely, many of the down-regulated genes appear to be located away from *oriC*. Though this correlation is not absolute, we observed a clear trend when plotting the mean fold-change against genome location for all genes (**Figure 3B**).

Overall, our data shows that inhibition of DNA replication by PolC-inhibitors causes a consistent and pleiotropic transcriptional response that is at least in part is directly dictated by genome location.

Gene dosage shift occurs at sub-inhibitory concentrations of ACX-362E PolC-inhibitor

A possible explanation for the relative up-regulation of *oriC*-proximal genes and down-regulation of *terC*-proximal genes is a gene dosage shift (195-197), due to the fact that PolC-inhibition slows down replication elongation but does not prevent re-initiation of DNA replication (188, 198). To determine if this in fact occurs in *C. difficile* when replication elongation is inhibited, we performed a marker frequency analysis (MFA) to determine the relative abundance of an origin proximal gene relative to terminus proximal gene on chromosomal DNA isolated from treated and untreated cells.

We designed qPCR probes against the CD0001 and CD1931 regions, representing *oriC* and *terC*, respectively (190, 193). Using these probes, we could show that *C. difficile* demonstrates multi-fork replication in exponential growth phase and that the MFA assay detects the expected decrease in *oriC:terC* ratio when cells enter stationary growth phase (data not shown). Next, we analyzed the effects of PolC-inhibitors on the *oriC:terC* ratio. When cells were treated with HPURa (35 µg/mL), the MFA showed a modest increase in *oriC:terC* ratio of 2,6-fold compared

to untreated cells. However, when cells were treated with ACX-362E (4 µg/ml), the MFA showed a >8-fold increase in the *oriC:terC* ratio compared to untreated cells. In contrast, such an increase was not observed for cells treated with metronidazole (0.25 µg/mL; a DNA damaging agent), fidaxomicin (0.00125 µg/mL; an RNA polymerase inhibitor) or surotomycin (0.625 µg/mL; a cell-wall synthesis inhibitor) (**Figure 4**) or chloramphenicol (2 µg/mL; a protein synthesis inhibitor) (**Supplemental Figure 2**). We conclude that inhibition of PolC-activity, but not the actions of any of the other tested antimicrobials, lead to a gene dosage shift in *C. difficile*.

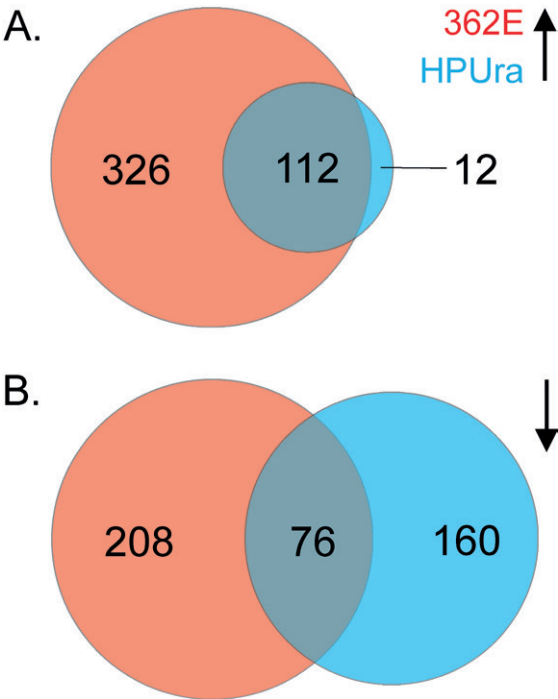


Figure 2. Overlap in the transcriptional response to different PolC-inhibitors. **A)** Venn diagram of the number of genes up-regulated in the presence of ACX-362E (red), in the presence of HPURa (blue) or under both conditions (overlapping region). **B)** Venn diagram of the number of genes down-regulated in the presence of ACX-362E (red), in the presence of HPURa (blue) or under both conditions (overlapping region). The size of the circles is proportional to the number of genes that showed differential expression.

Top 10 significantly changed genes HPURa UP									
RefSeq locus tag ¹	logFC	logCPM	LR	adj_pvalue	Fold	minFDR	Gene name ²	Product	Protein ID
CD630DERM_RS12480	11.16	3.84	9.8	2.4e-02	2291.8	5.37	CD2305B	Hypothetical protein	WP_004454601.1
CD630DERM_RS15730	6.02	12.24	39.2	1.9e-07	64.9	22.3	-	Hypothetical protein	WP_032506906.1
CD630DERM_RS17185	5.12	8.69	48.8	3.8e-09	34.8	27.97	CD3206	Hypothetical protein	WP_003434302.1
CD630DERM_RS17015	4.64	8.61	45.7	1.0e-08	25	26.55	CD3174	Type I glyceraldehyde-3-phosphate dehydrogenase	WP_003421962.1
CD630DERM_RS17020	4.62	8.68	54.7	2.7e-10	24.6	31.77	CD3175	Transcriptional regulator	WP_003429564.1
CD630DERM_RS00700	4.37	10.08	33.9	2.1e-06	20.7	18.85	CD0080A	50S ribosomal protein L29	WP_003421158.1
CD630DERM_RS00655	4.26	8.55	22.7	1.4e-04	19.1	12.77	CD0072	30S ribosomal protein S10	WP_011860633.1
CD630DERM_RS16270	3.96	8.16	34.9	1.4e-06	15.6	19.44	CD3031	Transcriptional antiterminator	WP_003432034.1
CD630DERM_RS19860	3.93	10.72	31.8	4.1e-06	15.2	17.89	CD3663	30S ribosomal protein S6	WP_003420522.1
CD630DERM_RS00775	3.83	6.09	15.1	2.7e-03	14.2	8.53	CD0093	50S ribosomal protein L14	WP_009895197.1
Top 10 significantly changed genes HPURa DOWN									
RefSeq locus tag ¹	logFC	logCPM	LR	adj_pvalue	Fold	minFDR	Gene name ²	Product	Protein ID
CD630DERM_RS09945	-14.85	4.52	17.0	1.2e-03	-29514.0	9.72	CD1794	Hypothetical protein	WP_003430297.1
CD630DERM_RS12730	-14.52	4.33	17.3	1.1e-03	-23428.2	9.87	CD2355	Thiol reductase thioredoxin	WP_003416870.1
CD630DERM_RS00870	-14.39	4.91	21.8	1.9e-04	-21483.7	12.35	-	tRNA-Asn	
CD630DERM_RS00255	-14.35	5.48	45.8	1.0e-08	-20874.2	26.55	-	tRNA-His	
CD630DERM_RS00925	-14.18	4.91	23.1	1.3e-04	-18514.8	12.89	-	tRNA-Ser	
CD630DERM_RS17905	-14.02	5.11	26.3	4.1e-05	-16646.0	14.57	-	Hypothetical protein	WP_042741280.1
CD630DERM_RS05300	-14.01	4.27	12.7	7.3e-03	-16475.8	7.11	CD0923	Hypothetical protein, putative phage protein	WP_021362052.1
CD630DERM_RS00170	-13.98	4.82	13.8	4.5e-03	-16156.5	7.78	-	tRNA-Asn	
CD630DERM_RS03115	-13.96	5.24	56.8	1.9e-10	-15908.4	32.28	CD0498A	Hypothetical protein	WP_011860839.1
CD630DERM_RS05160	-13.92	3.89	15.3	2.5e-03	-15523.8	8.65	CD0896	Hypothetical protein	WP_003418667.1

Table 1. List of the genes most highly up- and down-regulated in the presence of HPURa compared to non-treated cells.

¹ At time of analysis https://www.ncbi.nlm.nih.gov/nuccore/NZ_LN614756.1

² <https://www.ncbi.nlm.nih.gov/nuccore/AM180355.1>

Top 10 significantly changed genes ACX-362E UP									
RefSeq locus tag ¹	logFC	logCPM	LR	adj_pvalue	Fold	minFDR	Gene name ²	Product	Protein ID
CD630DERM_RS12480	11.31	3.84	9.9	1.3e-02	2544.2	6.25	CD2305B	Hypothetical protein	WP_004454601.1
CD630DERM_RS17185	7.73	8.69	87.6	3.3e-17	211.9	54.77	CD3206	Hypothetical protein	WP_003434302.1
CD630DERM_RS17955	6.88	7.78	28.5	5.0e-06	117.8	17.62	CD3352	AraC family transcriptional regulator	WP_009891821.1
CD630DERM_RS17950	8.87	9.82	31.9	1.0e-06	117.3	19.88	CD3351	ATP-dependent Clp protease proteolytic subunit	WP_003436212.1
CD630DERM_RS15730	6.67	12.24	45.3	4.0e-09	101.9	27.89	-	Hypothetical protein	WP_032506906.1
CD630DERM_RS00990	5.92	8.75	50.1	5.7e-10	60.4	30.7	CD0109	Anaerobic ribonucleoside-triphosphate reductase activating protein	WP_003432598.1
CD630DERM_RS00655	5.65	8.55	35.0	2.7e-07	50.3	21.8	CD0072	30S ribosomal protein S10	WP_011860633.1
CD630DERM_RS00985	5.5	8.94	66.3	7.6e-13	45.2	40.27	CD0108	Anaerobic ribonucleoside triphosphate reductase	WP_003436320.1
CD630DERM_RS05405	5.49	10.12	43.5	8.0e-09	45.0	26.89	CD0941	Hypothetical protein	WP_009894743.1
CD630DERM_RS15695	5.24	10.08	41.5	2.0e-08	37.9	25.61	CD2925	Hypothetical protein	WP_009894743.1

Top 10 significantly changed genes ACX-362E DOWN									
RefSeq locus tag ¹	logFC	logCPM	LR	adj_pvalue	Fold	minFDR	Gene name ²	Product	Protein ID
CD630DERM_RS05350	-14.17	5.04	40.4	3.1e-08	-18382.7	24.94	CD0391	Hypothetical protein	WP_011861014.1
CD630DERM_RS05160	-13.92	3.89	15.0	1.6e-03	-15523.8	9.33	CD0896	Hypothetical protein	WP_003418667.1
CD630DERM_RS05375	-13.72	4.09	14.3	2.0e-03	-13454.4	8.95	CD0936	Endonuclease	WP_011861020.1
CD630DERM_RS15535	-13.57	4.3	23.5	4.4e-05	-12181.6	14.47	CD2895	Membrane protein	WP_011861046.1
CD630DERM_RS13040	-13.55	3.91	11.4	7.1e-03	-11987.2	7.15	CD2414	PTS sorbose transporter subunit IIB	WP_003430855.1
CD630DERM_RS05345	-13.55	4.41	16.5	8.2e-04	-11969.7	10.25	CD0930A	Hypothetical protein	WP_011861013.1
CD630DERM_RS11925	-13.53	4.10	14.0	2.3e-03	-11799.9	8.76	CD2199A	4Fe-4S ferredoxin	WP_003423406.1
CD630DERM_RS01060	-13.52	4.51	14.9	1.6e-03	-11718.7	9.33	-	tRNA-Glu	-
CD630DERM_RS15350	-13.44	3.89	15.3	1.4e-03	-11076.8	9.53	-	Membrane protein	WP_003426729.1
CD630DERM_RS06360	-13.43	4.12	23.9	3.7e-05	-11012.2	14.72	CD1116	Transposase	WP_011861118.1

Table 2. List of the genes most highly up- and down-regulated in the presence of ACX-362E compared to non-treated cells.

¹ At time of analysis https://www.ncbi.nlm.nih.gov/nuccore/NZ_LN614756.1

² <https://www.ncbi.nlm.nih.gov/nuccore/AM180355.1>

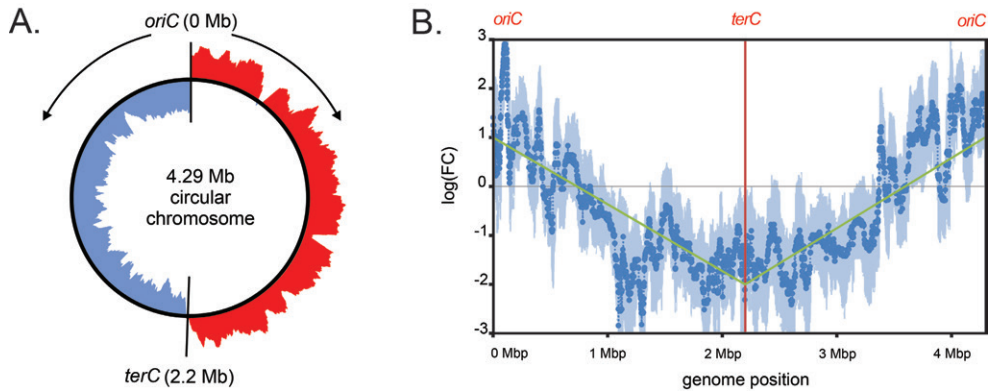


Figure 3. Genome location correlates with differential expression upon PolC inhibition. **A)** Schematic representation of the chromosome of *C. difficile*. Higher than average GC skew ($[(G - C)/(G + C)]$) (red) and lower than average GC skew (blue) were calculated with DNAPlotter (194). Vertical lines indicate the position of the predicted origin (*oriC*) and terminus (*terC*) of replication. Arrows indicate the direction of replication. **B)** Sliding window analysis (bins of 51 loci, step size 1) of the median log fold change (FC) projected on a linear genome map. The *oriC* of the circular chromosome is located on either side of the linear graph (0/4.29Mb), whereas *terC* is indicated with a vertical red line. The trend in $\log(\text{FC})$ is highlighted using a green line. Light blue shading indicates the median absolute deviation of the mean (188).

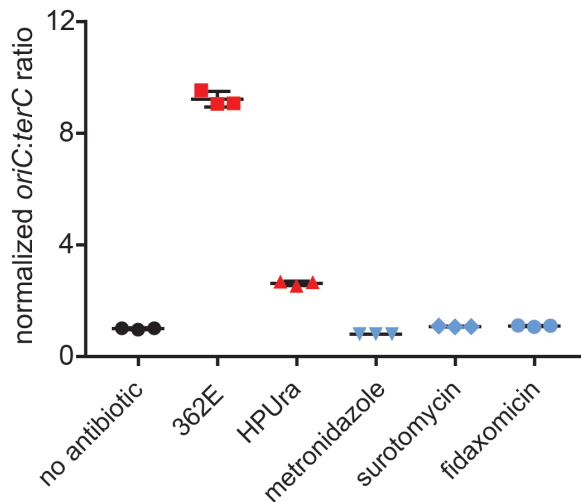


Figure 4. Polymerase inhibitors lead to an increase in *oriC:terC* ratio. A marker frequency analysis of the effects of sub-inhibitory amounts of polymerase inhibitors (red; HPURa: 35 $\mu\text{g}/\text{mL}$; ACX-362E: 4 $\mu\text{g}/\text{mL}$) and three antibiotics with different modes of action (blue; metronidazole: 0.25 $\mu\text{g}/\text{mL}$; fidaxomicin: 0.00125 $\mu\text{g}/\text{mL}$; surotomycin: 0.625 $\mu\text{g}/\text{mL}$) compared to non-treated cells (black). Datapoints are averages of technical replicates (N=3). Black lines behind the datapoints indicate the average of the biological replicates (N=3) and whiskers indicate the standard deviation of the mean. Data have been normalized compared to the non-treated control. The mean of HPURa and ACX-362E treated samples is statistically different from the other samples ($p < 0.0001$).

Origin proximal sigB contributes to the transcriptional response

Elegant work in *S. pneumoniae* has shown that the transcriptional response to replication inhibition can also be affected by origin-proximal regulators that respond to the gene dosage effect (188). In *C. difficile* the gene encoding the general stress response sigma factor σ^B (*sigB*, CD0011) is located close to the origin of replication (199). We wondered whether this regulator contributes to the transcriptional effects observed in our studies.

First, we compared the list of differentially regulated genes from our study (**Supplemental Table 1**) to those under the control of σ^B (160). In contrast to most anaerobic gram-positive organisms, *C. difficile* encodes a homolog of the general stress response sigma factor, σ^B (199, 200). A transcriptome analysis comparing *sigB* mutant versus wild type cells was recently published (160). Strikingly, we find ~40% of the genes (21/58) identified as involved in stress response under the control of σ^B to be differentially expressed in our ACX-362E dataset (**Supplemental Table 2**). Similarly, we observed that 7/20 (~35%) of the genes containing a transcriptional start site with a σ^B consensus sequence are differentially expressed in our ACX-362E dataset (**Supplemental Table 2**). These data suggest that the response to DNA replication inhibition is at least partially dependent on σ^B .

To demonstrate that exposure to ACX-362E causes a transient up-regulation of *sigB*, we constructed a reporter fusion of the secreted luciferase reporter *sLuc^{opt}* with the predicted promoter of the *sigB* operon (P_{CD0007}). We monitored luciferase activity of a strain harboring a plasmid containing this reporter fusion (WKS2003) after dilution of an overnight culture into fresh medium with or without ACX-362E (**Figure 5**). In non-treated cells, expression from the *sigB* promoter is relatively stable over the course of 5.5h. By contrast, luciferase activity strongly increases from 1.25h to 3h after inoculation into medium with ACX-362E. These data show that exposure to ACX-362E transiently induces transcription of the *sigB* operon.

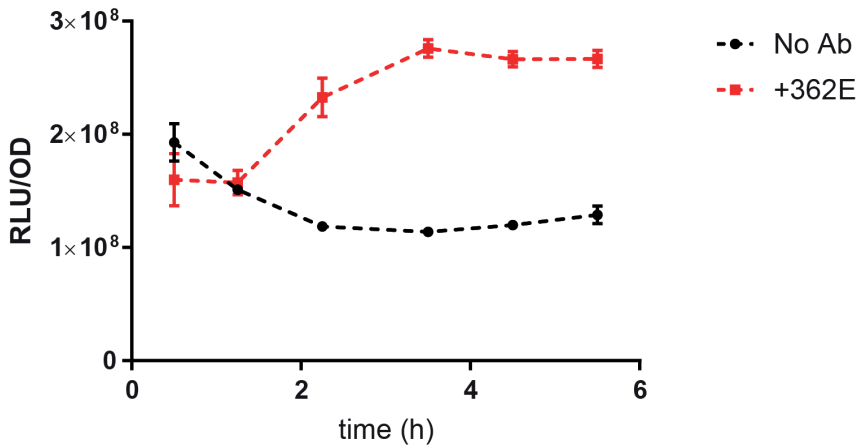


Figure 5. The *sigB* operon is transiently induced upon exposure to ACX-362E. The putative promoter of the *sigB* operon (160) was fused transcriptionally to a plasmid-based luciferase reporter (201). Luciferase activity was measured regularly between 30 mins and 5.5h of growth in liquid medium in the presence (+362E) or absence (no Ab) of polymerase inhibitor ACX-362E.

Next, a *sigB* mutant was constructed using Allele-Coupled Exchange (202) as described in the Materials and Methods. The chromosomal deletion of *sigB* and absence of σ^B protein was verified by PCR and Western Blot, respectively (**Supplemental Figure 3**). To directly demonstrate a role for *sigB* in the regulation of genes with altered transcription upon PolC-inhibition, we fused the predicted promoter regions of selected genes to a secreted luciferase reporter (201) and evaluated luminescence in wild type and *sigB* mutant backgrounds, after 5-hour growth in the presence and absence of 4 μ g/mL ACX-362E. All genes tested demonstrated a significant increase in promoter activity in a wild type background in the presence of ACX-362E compared to the non-treated control, validating the results from the RNA-Seq analysis (**Figure 6**). Three distinct patterns were observed. For CD0350 (encoding a hypothetical protein) and CD3963 (encoding a putative peptidoglycan-binding exported protein) there was virtually no expression in a *sigB* mutant background (**Figure 6A and B**). We conclude that these genes are strictly dependent on σ^B for their expression under the conditions tested. CD3614 (encoding a hypothetical protein) shows a basal level of expression, but no significant increase in transcription levels in the presence of ACX-362E in a *sigB* mutant background compared to the non-treated control (**Figure 6C**). This suggests that the transcriptional up-regulation under these conditions is σ^B dependent, and indicates that the basal level of transcription observed is likely independent of σ^B . Finally, CD3412 (*uvrB*, encoding a subunit of an excinuclease) shows reduced transcriptional up-regulation in ACX-362E treated cells compared to non-treated

controls (**Figure 6D**). Thus, the transcription of this particular gene under conditions of ACX-362E exposure is brought about by both a σ^B -dependent and a σ^B -independent regulatory pathway.

Together, these results demonstrate that *sigB* controls the expression of at least a subset of genes that are up-regulated under PolC-inhibition.

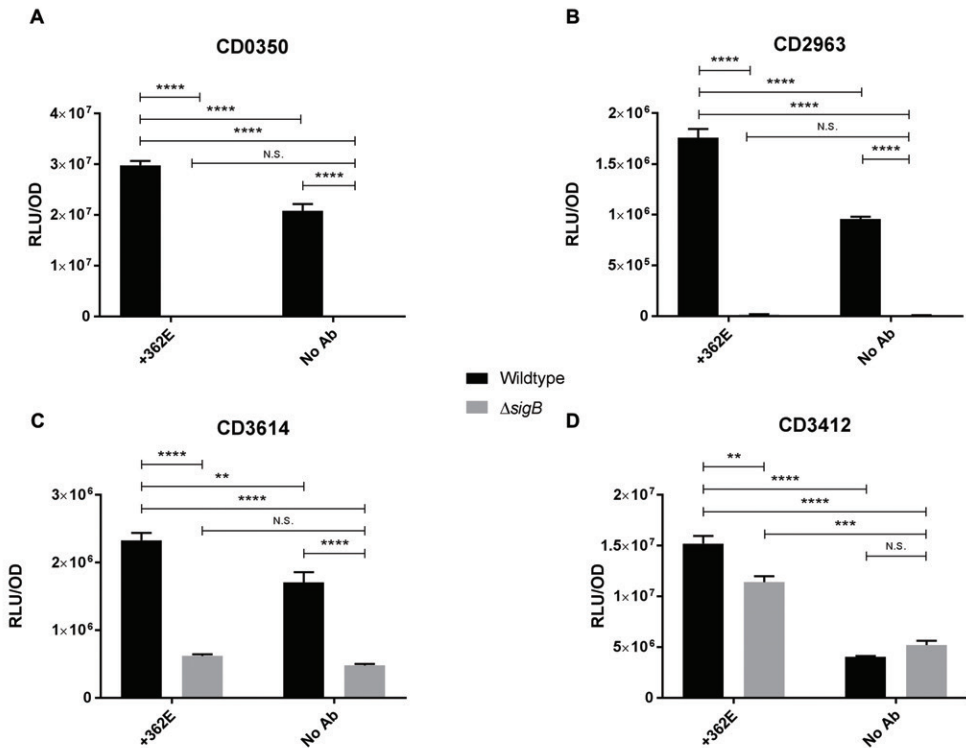


Figure 6. Genes differentially expressed due to polymerase inhibitors are regulated by σ^B . The putative promoters of the indicated genes were fused transcriptionally to a plasmid-based luciferase reporter (201). Luciferase activity was measured after 5 h of growth in liquid medium in the presence (+362E) or absence (no Ab) of polymerase inhibitor ACX-362E. N.S. = non-significant, *= $p < 0.05$, **= $p < 0.005$, ***= $p < 0.0005$, ****= $p < 0.00005$. **A)** P_{CD0350} -*sluc*^{opt}. **B)** P_{CD2963} -*sluc*^{opt}. **C)** P_{CD3614} -*sluc*^{opt}. **D)** P_{CD3412} -*sluc*^{opt}.

Discussion

Activity and specificity of ACX-362E

Limited treatment options and reports of reduced susceptibility to current treatment (140, 178, 203) emphasize the necessity for the development of novel antimicrobials. As CDI can be induced by use of broad spectrum antibiotics (175), new antimicrobials ideally should only target *C. difficile*, thereby maintaining integrity of the colonic microbiota. In this study, we have tested inhibitors HPURa and ACX-362E which specifically target the PolC enzyme, essential for DNA replication. The majority of PolC-inhibitors target Gram-positive bacteria with low G+C content, but ACX-362E has been reported to demonstrate increased specificity towards *C. difficile* PolC *in vitro* and showed promising results for the efficacy *in vivo* based on a limited set of *C. difficile* strains (184, 185). The compound will progress to clinical trials in the near future (Acurx Pharmaceuticals, personal communication). Mutations that abolish binding of HPURa or ACX-362E are likely to affect the essential enzymatic activity of the polymerase and for that reason unlikely to occur *in vivo*. A single mutation (*azp-12*) has been described in *B. subtilis* that confers resistance to HPURa (204). This T>G transversion results in the replacement of a serine with an alanine in the highly conserved PFAM07733 domain of the polymerase (205). To our knowledge, it is unknown whether this mutation prevents binding of HPURa to PolC of *B. subtilis*. Few other mutations have been described that confer resistance against other PolC-inhibitors (206, 207). It will be of interest to see if similar mutations in *C. difficile* result in resistance to HPURa and/or ACX-362E and what the effect on binding of these compounds to *C. difficile* PolC is.

ACX362 may have off-target effects unrelated to its replication-inhibitory activity. For *C. difficile*, it has not been established that PolC inhibition is the sole mode of action of the inhibitor. PolIII-inhibitors can affect PolC, DnaE or both (207), though *in vivo* activity appears to correlate with PolC-inhibition. *C. difficile* possesses PolC and DnaE polymerase (208). To verify the mode of action the activity of ACX-362E towards purified DnaE and PolC should be determined.

Though there is an indication that ACX-362E inhibits *C. difficile* efficiently and shows limited activity towards certain other anaerobes (184), these findings highlight the necessity to perform additional (microbiome) studies to more clearly define the antimicrobial spectrum of this compound. It also shows that ACX-362E may have therapeutic potential outside treatment of CDI.

Regulators of the transcriptional response to PolC-inhibitors

The present study is the first to describe the transcriptional response of *C. difficile* to inhibition of DNA replication. We find that ~200 genes show differential expression under conditions of PolC-inhibition by both HPUra and ACX-362E, when compared to non-treated cells. When considering only ACX-362E, approximately 13% of all genes in the genome show statistically significant altered transcription. We demonstrate that this large reprogramming of transcription is likely to be caused directly by a gene dosage shift.

In addition to direct effects, it is conceivable that at least part of the transcriptional response is indirect. Our list of differentially regulated genes includes several putative regulators; sigma factors (including *sigE*, *sigG* and *sigH*), transcription factors and anti-terminators. The relatively long time (5h) at sub-MIC levels of antimicrobials may have contributed to secondary effects, through one or more of these regulators. Though shorter induction times are thought to provoke more compound-specific responses (209), we did observe a highly consistent transcriptional signature with both HPUra and ACX-362E.

Major stress response pathways are poorly characterized in *C. difficile*. On the basis of experiments in other organisms (186-188, 210-212), we expect that inhibition of DNA replication inhibition might possibly induce an SOS response (LexA)(213), a DnaA-dependent transcriptional response (186), and possibly a heat shock response (HrcA/CtsR)(214) and/or a general stress response (σ^B)(200). Of these, the best characterized stress response pathway in *C. difficile* is the one governed by σ^B (160). We noted a significant overlap in putatively σ^B -dependent genes and those differentially expressed upon exposure to PolC-inhibitors. In addition, our luciferase reporter fusions directly implicate *sigB* in the expression and/or up-regulation of some of these. It should be noted that the *sigB*-operon itself was not differentially expressed in our RNA-seq analysis. A *sigB*-reporter fusion suggests that *sigB* is transiently up-regulated prior to the time point of sampling for the RNA-Seq analysis. Similar transient up-regulation of *sigB* followed by a persistent response of σ^B -dependent gene expression has been observed in other organisms (214-216). To our knowledge, this is the first indication that *sigB*- and σ^B -dependent gene expression could be subject to a gene-dosage effect.

To date, no genes have been identified that are regulated by DnaA in *C. difficile* and direct regulation of genes through the other stress response pathways has not been demonstrated.

Many parameters (such as the medium used, cell density, concentration of antibiotics, and protocol used to arrest transcription between cell harvest and lysis) can influence overall transcription signatures (209), and can also govern an incomplete overlap between our data and the stress regulons proposed by others (215-217).

Genome location contributes to the transcriptional response to PolC-inhibition

Our analysis of differential regulation in relation to genome location revealed a striking pattern of relative up-regulation for *oriC*-proximal genes, and down-regulation for *terC*-proximal genes under conditions of PolC inhibition. Antimicrobials directed at DNA replication in bacteria have a profound negative effect on the processivity of replication forks, though initiation of DNA replication is not or only marginally affected (188, 198). As a consequence, the presence of multiple replication forks simultaneously increases the copy numbers of genes located in close proximity of the origin of replication and such a gene dosage differences can result in functionally relevant transcriptional differences, either directly or indirectly (180). We found an increase of *oriC:terC* ratio when performing MFA on chromosomal DNA of cells subjected to a sub-inhibitory concentration of ACX-362E (and HPUra, albeit less pronounced), consistent with findings in other organisms (188). This is the first demonstration of gene-dosage dependent transcriptional regulation in *C. difficile*. Our experiments suggest that at least part of the transcriptional response to PolC-inhibition can be explained by an indirect gene dosage effect, as also observed for *S. pneumoniae* (188). The positioning of stress-response regulators close to *oriC* may therefore be a conserved strategy in bacteria to respond to DNA replication insults that is independent of the nature of the regulator.

Though it is likely that an increase in gene copy number leads to an increase in transcription of these genes, it is less clear whether this is the case for the observed down-regulation. Most methods of normalization for transcriptome analyses are based on the assumption that there is no overall change in transcription or that the number of transcripts per cells is the same for all conditions and this may not be the case when a global copy number shift occurs (180). Absolute transcript levels for down-regulated genes might therefore be similar under both conditions (but lower relative to *oriC*-proximal transcripts).

It is interesting that certain processes are highly enriched in the list of genes up-regulated under conditions of PolC-inhibition (most notably ribosome function and DNA-related functions),

whereas this is less so for the down-regulated genes. This suggests that pathways susceptible to replication dependent gene-dosage effects demonstrate a functional clustering of genes near *oriC*, whereas clustering of genes from specific pathways in the *terC*-proximal region is less pronounced. Indeed, most ribosomal gene clusters in *C. difficile* are located close to the origin of replication (190, 199) and also many genes involved in DNA replication and repair are located in these regions. Consistent with this, positioning of genes involved in transcription and translation close to the origin appears to be under strong selection as such genomes tend to be more stable (218).

In conclusion, both direct and indirect effects of gene dosage shifts are likely to contribute to the transcriptional response of *C. difficile* to replication inhibition.

Materials and Methods

Bacterial strains and culture conditions

Plasmids and bacterial strains used in this study can be found in **Table 3**. *E. coli* was cultured aerobically at 37°C (shaking at 200 rpm) in Luria-Bertani (LB) broth supplemented with 20 µg/ml chloramphenicol and 50 µg/ml kanamycin when appropriate. *C. difficile* strains were cultured in Brain Heart Infusion (BHI) broth (Oxoid) supplemented with 0,5% yeast extract (Sigma-Aldrich), *Clostridium difficile* Selective Supplement (CDSS, Oxoid) and 20µg/ml thiamphenicol when appropriate. *C. difficile* was grown anaerobically in a Don Whitley VA-1000 workstation in an atmosphere of 10% CO₂, 10% H₂ and 80% N₂. Liquid cultures were grown under gentle agitation (120 rpm).

	Name	Relevant features	Source/reference
Plasmids	pAP24	<i>tetR</i> P _{tet} - <i>sluc</i> ^{opt} <i>catP</i> ; derived from pRPF185	(44)
	pIB27	P _{CD3412} - <i>sluc</i> ^{opt} <i>catP</i> ; derived from pAP24	This study
	pIB54	pMTL-SC7315 containing the up- and downstream area of the 630Δ <i>erm sigB</i> CDS (950 bp each)	This study
	pIB68	P _{CD0350} - <i>sluc</i> ^{opt} <i>catP</i> ; derived from pAP24	This study
	pIB69	P _{CD2963} - <i>sluc</i> ^{opt} <i>catP</i> ; derived from pAP24	This study
	pIB74	P _{CD3614} - <i>sluc</i> ^{opt} <i>catP</i> ; derived from pAP24	This study
	pMTL-SC7315	pMTL83151 with <i>codA catP</i>	(43)
	pH28	P _{CD0007} - <i>sluc</i> ^{opt} <i>catP</i> ; derived from pAP24	This study
Strains	DH5α	<i>E. coli</i> F– <i>endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20 φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(rK–mk+), λ–</i>	Laboratory stock
	CA434	<i>E. coli</i> HB101 [F– <i>mcrB mrr hsdS20(rB–mB–) recA14 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rslL20(SmR)glnV44λ–</i>] R702	(76)
	<i>C. difficile</i> 630Δ <i>erm</i>	MLS susceptible derivative of strain 630	(30, 31)
	IB37	630Δ <i>erm</i> pIB27; <i>thia</i> ^R	This study
	IB56	630Δ <i>erm</i> Δ <i>sigB</i>	This study
	IB95	630Δ <i>erm</i> pIB68; <i>thia</i> ^R	This study
	IB96	630Δ <i>erm</i> pIB69; <i>thia</i> ^R	This study
	IB98	IB56 pIB27; <i>thia</i> ^R	This study
	IB99	IB56 pIB68; <i>thia</i> ^R	This study
	IB100	IB56 pIB69; <i>thia</i> ^R	This study
	IB108	630Δ <i>erm</i> pIB74; <i>thia</i> ^R	This study
	IB111	IB54 pIB74; <i>thia</i> ^R	This study
	WKS2003	630Δ <i>erm</i> pH28; <i>thia</i> ^R	This study

Table 3. Plasmids and strains used in this study.

Sub-MIC determination

C. difficile 630Δ*erm* (189, 190) was grown in 20 mL Brain Heart Infusion (Oxoid) supplemented with 0.5% yeast extract (Sigma-Aldrich) (BHI/YE) starting from an optical density measured at 600nm (OD₆₀₀) of 0.05 using an exponentially growing starter culture (3 biological replicates per concentration). To determine the effects on growth at sub-inhibitory concentrations of ACX-362E, cells were cultured in the presence of the following concentrations; 0.25, 0.5, 1, 2, 4, and 8 μg/mL ACX-362E and compared to an untreated culture. To determine the effects on growth at sub-inhibitory concentrations of HPUra, cells were cultured in the presence of the following concentrations: 10, 20, 40 μg/mL HPUra and compared to an untreated culture. The OD₆₀₀ was monitored every hour until stationary phase was reached.

Name	Sequence (5' → 3') *	Description
CD-0001- F	GAGACAAGAATTGCTACTTA	Forward primer CD0001 MFA (<i>oriC</i>)
CD-0001- R	CAACCACTCTAGTTAATGC	Reverse primer CD0001 MFA (<i>oriC</i>)
CD-0001- probe-FAM	CTCAACTAGAACGTATAGATGTGCCAA	Probe CD0001 MFA (<i>oriC</i>)
CD-1931- F	GCAGGAATTTTAGATGAAGA	Forward primer CD1931 MFA (<i>terC</i>)
CD-1931- R	GGCTGAAGTCTTATTAAATTC	Reverse primer CD1931 MFA (<i>terC</i>)
CD-1931- probe-TXR	CCTCTTAAGTGTAGCAGATTCACCAT	Probe CD1931 MFA (<i>terC</i>)
oIB-26	GGAAGGTACCGTTGAATAAAGTATTATTTCCATG	Forward primer for P _{CD3412} containing a <i>KpnI</i> restriction site
oIB-27	GGTAGAGCTCAGTATCACTCCTTTTTTCGAAC	Reverse primer for P _{CD3412} containing a <i>SacI</i> restriction site
oIB-44	CGTAGAAATACGGTGTTTTTGTTACCCTACTTATAG TAGCAATTTATTAGCTAAAAC	Forward primer for region 950 bp upstream of <i>sigB</i> CDS
oIB-45	TACTTTTTTATATTTTTTAAATATCAACTCCTAAATTTTAGTC	Reverse primer for region 950 bp upstream of <i>sigB</i> CDS
oIB-46	GGAGTTGATATTTAAAAAATATAAAAAAGTATTGACCTACTG	Forward primer for region 950 bp downstream of <i>sigB</i> CDS
oIB-47	GGGATTTTGGTCATGAGATTATCAAAAAGG GGACTACCAGGGTATCTAATC	Reverse primer for region 950 bp downstream of <i>sigB</i> CDS
oIB-53	CTTTAAACAGTAGGTCAATACTTTTTTTATAT	Reverse primer to verify <i>sigB</i> CDS deletion
oIB-76	AAAATATAAAAAAGTATTGACCTACTGTTTTAAA GATGGTATAGTATTAC	Forward primer to verify <i>sigB</i> deletion
oIB-78	GGAGATGTTAACTAATGAATGCATGG	Forward primer to verify <i>sigB</i> CDS deletion
oIB-79	CTAATGCACAGCGCCAGCAAACAAAC	Reverse primer to verify <i>sigB</i> CDS deletion
oIB-80	ctagcataaaaataagaagcctgcatttgcAAAT TTACGAAAAGCTTGC	Forward primer for P _{CD0350}
oIB-82	ctagcataaaaataagaagcctgcatttgcTTG TGTTTAAGGGATTTTGAAAG	Forward primer for P _{CD2963}
oIB-92	ctagcataaaaataagaagcctgcatttgcGAAT AAAAAAGGTGGTGTC	Forward primer for P _{CD3614}
oIB-94	agctattaataatttttacttggtctcatTTTACCT CCATGTAACATTATTG	Reverse primer for P _{CD0350}
oIB-95	agctattaataatttttacttggtctcatAATTAAATC CTTCCTACATTGTAATTAC	Reverse primer for P _{CD2963}
oIB-100	agctattaataatttttacttggtctcatATAAACACCCTCCTATTCTTG	Reverse primer for P _{CD3614}
oPH-19	ctagcataaaaataagaagcctgcatttgcGATTGCCAAAAT AAATATTGAAG	Forward primer for P _{CD0007}
oPH-20	agctattaataatttttacttggtctcatAACAACTACTCCTTT CAATTTTAAATTTTATC	Reverse primer for P _{CD0007}
oWKS-1240	CACCTCCTTTTGTACTTTAAGCCTACGAATACC	Forward primer on pAP24 backbone to amplify insert

Name	Sequence (5' → 3') *	Description
oWKS-1241	CACCGACGAGCAAGGCAAGACCG	Reverse primer on pAP24 backbone to amplify insert
oWKS-1537	TAGGGTAACAAAAACACCG	Reverse primer to amplify pMTL-SC7315 backbone
oWKS-1538	CCTTTTGTATAATCTCATGACC	Forward primer to amplify pMTL-SC7315 backbone
oWKS-1580	ATGAGACCAAGTAAAAATTATTAATAGC	Forward primer to amplify pAP24 backbone
oWKS-1582	GCAATGCAGGCTTCTATTTTTATG	Reverse primer to amplify pAP24 backbone

Table 4. Oligonucleotides and probes used in this study.

* Restriction sites are underlined. Overlap with the vector backbone is indicated in lowercase letters.

Marker Frequency analysis

C. difficile 630Δ*erm* (189, 190) was grown in 20 mL BHI supplemented with 0.5% yeast extract with sub-MIC amounts of antimicrobials (HPUra: 35 μg/mL; ACX-362E: 4 μg/mL; metronidazole: 0.25 μg/mL; fidaxomicin: 0.00125 μg/mL, surotomycin: 0.625 μg/mL), starting from an OD₆₀₀ of 0.05 using an exponentially growing starter culture. These samples were taken in the course of an independent, but simultaneously performed, set of experiments for which we obtained surotomycin and fidaxomicin from Cubist Pharmaceuticals. Metronidazole was commercially obtained (Sigma-Aldrich). We confirmed that these concentrations did not lead to a >30% reduction in growth compared to non-treated cultures (**Supplemental Figure 1** and data not shown). In parallel, cultures were grown without inhibitors from the same starter culture. All conditions were performed in biological triplicates. Previous experiments have shown that *oriC:terC* differences are reliably detected >3h after dilution into fresh medium (188). Therefore, after 5 hours, 1mL cells was harvested (OD₆₀₀ ~ 0.5), and stored at -20°C. Isolation of chromosomal DNA was performed the next day with the QIAamp DNA Blood Mini kit (Qiagen) according to the instructions of the manufacturer. Marker frequency analysis (MFA) was performed to assess the relative abundance of origin proximal genes relative to terminus proximal genes. As a proxy for *oriC*, a probe was designed that targets the CD0001 region (CD0001-probe-FAM). By using plots of the GC skew ($[G - C]/[G + C]$) generated using DNAPlotter (194), the approximate location of the terminus region for the *C. difficile* chromosome was determined and a probe targeting this region (CD1931) was designed (CD-1931-probe-TXR). Probe design was performed with Beacon Designer™ (Premier Biosoft, Palo Alto CA, USA). Real-time PCR reactions were performed on a Biorad CFX96™ real-time PCR detection system (95°C 15 m, 39 × (94°C 30 s + 55°C 30 s + 72°C 30 s). Sequences for primers and probes are listed in

Table 4. For each biological replicate, three technical replicates were performed. Amplification efficiency was determined using standard curves obtained from DNA late stationary phase cells of strain 630 Δ erm, for which an *oriC:terC* ratio of 1 was assumed. RT-PCR results from antibiotic treated cells were normalized to the *oriC:terC* ratio of DNA samples (3 biological replicates) from non-treated cells. Calculations were performed in Microsoft Office Excel 2010, plotted using Prism 7 (GraphPad) and prepared for publication in Corel Draw Suite X8. Significance was determined using a One-way ANOVA and a Tukey's test for multiple comparisons (GraphPad).

Growth and RNA isolation for RNA-Seq

For RNA-Seq analysis, *C. difficile* 630 Δ erm (189, 190) was grown for 5h in BHI medium with HPURa (35 μ g/mL) or ACX-362E (4 μ g/mL) starting from an OD₆₀₀ of 0.05 using an exponentially growing starter culture, after which cells (3mL) were harvested for RNA isolation. These concentrations were some of the highest concentrations that resulted in a modest effects on growth (<30% reduction of growth compared to wild type cells, **Supplemental Figure 1**). RNA isolation was performed with NucleoSpin[®] RNA kit (Macherey-Nagel). Although the kit includes on column rDNase digestion, a second treatment was performed in solution and RNA was precipitated and recovered by NaAc precipitation to remove residual DNA. Concentration determination and quality control (16S/23S ratio and RNA integrity number [RIN]) was performed with a fragment analyzer (Agilent bio-analyzer), according to the instructions of the manufacturer. Samples with a RIN>9 and 16S/23S ratio >1.4 were submitted for analysis by RNA-Seq.

RNA-Seq

RNA-Seq was performed at a commercial provider (GenomeScan, Leiden, The Netherlands). In short, the NEBNext Ultra Directional RNA Library Prep Kit for Illumina was used to process the samples. Sample preparation was performed according to the protocol "NEBNext Ultra Directional RNA Library Prep Kit for Illumina" (NEB #E7420S/L). Briefly, after selective removal of rRNA (Ribo-Zero rRNA Removal Kit for gram-positive Bacteria) and fragmentation of the mRNA, cDNA synthesis was performed. cDNA was ligated to the sequencing adapters and the resulting product was PCR amplified. Clustering and DNA sequencing using the Illumina NextSeq 500 platform was performed according to manufacturer's protocols. A concentration of 1.5 pM of DNA was used. Image analysis, base calling, and quality check was performed with the Illumina data analysis pipeline RTA v1.18.64 and Bcl2fastq v2.17. Per sample, four technical replicates were included in the RNA-Seq experiment. In case of insufficient reads, the sample was re-run on another flow cell to reach satisfactory quantities (≥ 20 M).

Analysis of RNA-Seq data

Analysis of the data was performed using T-REx, a user-friendly webserver which has been optimized for the analysis of prokaryotic RNA-seq-derived expression data (219). The pipeline requires raw RNA expression level data as an input for RNA-Seq data analysis. For data normalisation and determination of the genes, the factorial design statistical method of the RNA-Seq analysis R-package EdgeR is implemented in the T-Rex pipeline. Some samples displayed incomplete rRNA depletion and rRNA mapping reads had to be removed manually prior to analysis.

To analyse the genome-wide pattern in differential gene expression a sliding window analysis was performed essentially as described (188). In short, genome locations (start of the locus tag) were coupled to the locus tags in the T-Rex output. Next, the median log(FC) was calculated for bins of 51 loci with a step size of 1. For each bin of $[X_1, X_2, \dots, X_{51}]$ the median absolute deviation of the median ($MAD = \text{median}(|X_i - \text{median}(X)|)$) was calculated as a robust indication of the distribution around calculated median values. Calculations were performed and three curves (median, median-MAD and median+MAD) were plotted in Microsoft Office Excel 2010 and the graph was prepared for publication using Adobe Photoshop CC and Corel Draw Suite X8.

A GSEA analysis (191) was performed via the Genome2D webserver (219), using our reference genome sequence for *C. difficile* 630Δ*erm*, GenBank identifier LN614756.1 (listed in Genome2D as “*Clostridioides_difficile_630Derm*”)(190). As input a single list of locus tags was used of either up- or down regulated genes. The output was copied to Microsoft Excel 2010. The single_list column was split, and a column was inserted to calculate the significance of the overrepresentation using the formula “(# hits in list/ClassSize)*-log(p-value; 2)” to allow for sorting of the output of the GSEA analysis by significance. Data for the RNA-Seq experiment has been deposited in the Gene Expression Omnibus (GEO) database (220), accession number GSE116503.

General molecular biological techniques

E. coli strain DH5α was used for maintenance of all plasmids. All plasmid transformations into *E. coli* were performed using standard procedures (221). *E. coli* CA434 was used as a donor for conjugation of plasmids into the recipient *C. difficile* (222). Conjugation was performed as previously described (222). Briefly, 1 mL of an overnight culture of donor cells was mixed

with 200 µl of the recipient and spotted onto anaerobic BHI agar plates and incubated for 5-8 hours. After incubation cells were collected and tenfold serial dilutions were plated onto fresh BHI plates containing thiamphenicol and CDSS.

Plasmid DNA was isolated using the NucleoSpin Plasmid (Macherey-Nagel) mini prep kits per manufacturer's instructions. *C. difficile* genomic DNA was isolated using the DNeasy Blood and Tissue kit (Qiagen) with pre-treatment for gram-positives according to instructions of the manufacturer.

Construction of luciferase-reporter fusion plasmids

All PCR reactions for plasmid construction were carried out with Q5 polymerase (New England Biolabs). Putative promoter regions were amplified using *C. difficile* 630Δ*erm* chromosomal DNA (190) as a template.

The P_{CD3412} luciferase reporter plasmid was created by restriction-ligation using the restriction enzymes *KpnI* and *SacI*. P_{CD3412} was amplified using primers oIB-26 and oIB-27 (**Table 5**). The resulting dsDNA fragment was digested and ligated into *KpnI-SacI* digested pAP24 (201), yielding plasmid pIB27. Plasmids pIB68 (P_{CD0350}), pIB69 (P_{CD2963}), pIB74 (P_{CD3614}) and pPH28 (P_{CD0007}) were constructed using a Gibson assembly (223). The plasmid backbone of pAP24 was linearized by PCR using primers oWKS-1580/oWKS-1582, and the predicted promoter areas of CD0350, CD2963 and CD3614 were amplified with primers oIB-80/oIB-94, oIB-82/oIB-95, oIB-92/oIB-100 and oPH-19/oPH-20, respectively. Primers were designed using the NEBuilder assembly tool v.1.12.17 (New England Biolabs) using a 30 bp overlap. For the assembly, 100 ng of vector DNA was assembled to a five-fold molar excess of the PCR fragment of the desired promoter using a homemade Gibson Assembly Master Mix at 50°C for 30 minutes (final concentration: 4 U/µl Taq Ligase (Westburg), 0.004 U/µl T5 exonuclease (New England Biolabs), 0.025 U/µl Phusion polymerase (BioKé), 5% polyethyleneglycol (PEG-8000), 10 mM MgCl₂, 100 mM Tris-Cl pH=7.5, 10 mM dithiothreitol, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dCTP, 0.2 mM dGTP, and 1 mM β-nicotinamide adenine dinucleotide) and transformed into *E. coli* DH5α. Transformants were screened by colony PCR using primers oWKS-1240 and oWKS-1241. Transformants yielding PCR fragments of the correct size were verified by Sanger sequencing.

Construction of *C. difficile* IB56 (Δ sigB)

The up- and downstream regions (both 950 bp each) of the *sigB* coding sequence were amplified with primers oIB-44/oIB-45, and oIB-46/oIB-47, respectively. Vector pMTL-SC7315 (202) was linearized by PCR using primers oWKS-1537/oWKS-1538. Assembly was done according to the method of Gibson (201, 223). The assembled plasmid was transformed into *E. coli* DH5 α and verified using PCR and Sanger sequencing. Generation of the unmarked *sigB* deletion mutant was performed using Allele-Coupled Exchange essentially as described (202, 224). Briefly, pIB54 was introduced into *C. difficile* 630 Δ erm (190) by conjugation. Transconjugants were grown for two days on BHI agar plates supplemented with yeast extract, thiamphenicol and CDSS, struck onto fresh pre-reduced plates and incubated anaerobically at 37°C for two days. Single-crossover integration was confirmed using PCR, and those clones were plated onto non-selective BHI agar plates to allow the second-crossover event to occur. Colonies were then serially diluted and plated onto minimal agar supplemented with 50 μ g/ml 5-fluorocytosine (Sigma) as described (202). DNA was isolated from thiamphenicol-susceptible colonies and the chromosomal deletion was verified by PCR using primers oIB-78/oIB-79 (**Supplemental Figure 3**) as well as Sanger sequencing of the PCR product using primers oIB-53/oIB-76/oIB-78.

Luciferase reporter assay

Strains containing luciferase reporter plasmids were inoculated to an OD₆₀₀=0.05 from an exponentially growing starter culture. Fresh inoculums were grown in BHI broth supplemented with yeast extract, with or without 4 μ g/ml ACX-362E for 5 (for putative *sigB* target genes) or 5.5 (*sigB* promoter) hours. The supernatants from 1mL of culture (harvested by centrifugation for 10 min, 4°C, 8000 rpm) were analyzed in a GloMax[®]-Multi Microplate Multimode Reader (Promega) as described before (201). Statistical significance of the data ($p < 0.05$) was determined by two-way analysis of variance (ANOVA) and a pairwise Tukey-Kramer test using Prism 7 (GraphPad) where appropriate.

Accession numbers

GEO GSE116503; GenBank LN614756.1

Author's contributions

EVE, IMB, EJK and WKS designed experiments. GW and EJK provided reagents or strains. EVE, IMB and IMGJBS performed experiments. EVE, IMB and WKS analyzed data. EVE, IMB and WKS wrote the manuscript. All authors read and approved the manuscript.

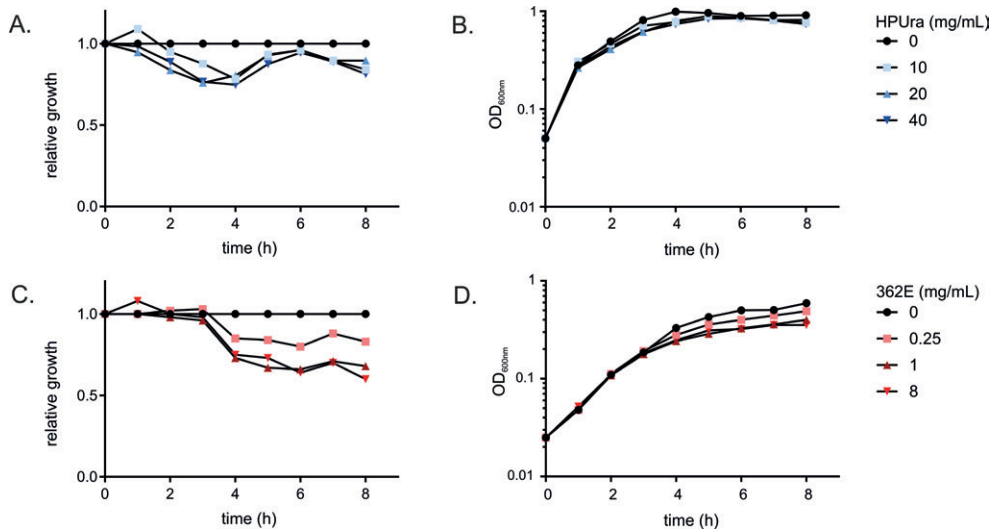
Acknowledgements

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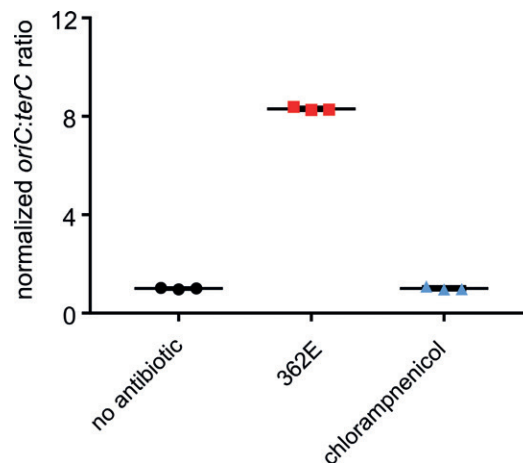
Conflict of interest

EVE and WKS have performed research for Cubist. EJK has performed research for Cubist, Novartis and Qiagen, and has participated in advisory forums of Astellas, Optimer, Actelion, Pfizer, Sanofi Pasteur and Seres Therapeutics and currently holds an unrestricted research grant from Vedanta Biosciences Inc. GW is a consultant for Acurx Pharmaceuticals. The companies had no role in the design of the experiments or the decision to publish. IMB and IMGJSB: none to declare.

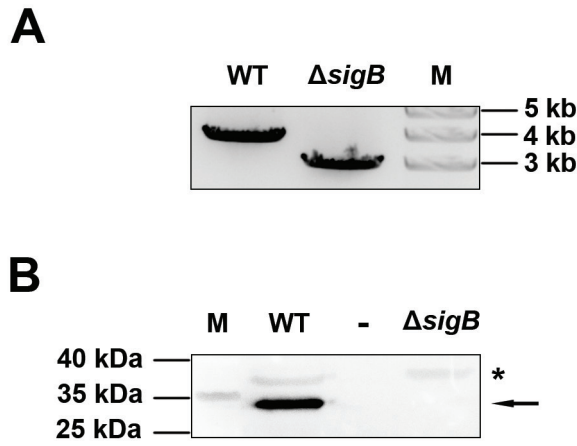
Supplemental information



Supplemental Figure 1. Relative inhibition of growth by varying concentrations of PolC-inhibitors. A) Growth kinetics of a culture grown in the presence of the indicated amount of HPURA, relative to a non-treated culture. **B)** Growth curve of a culture grown in the presence of the indicated amount of HPURA. **C)** Growth kinetics of a culture grown in the presence of the indicated amount of ACX-362E, relative to a non-treated culture. Growth in liquid media was abolished at >2x the MIC (data not shown). **D)** Growth curve of a culture grown in the presence of the indicated amount of ACX-362E.



Supplemental Figure 2. Chloramphenicol does not result in a gene dosage shift. A marker frequency analysis of the effects of sub-inhibitory amounts of ACX-362E (red; 4 μ g/mL) and chloramphenicol (blue; 20 μ g/mL) compared to non-treated cells (black). Datapoints are averages of technical replicates (N=3). Black lines behind the datapoints indicate the average of the biological replicates (N=3) and whiskers indicate the standard deviation of the mean. Data have been normalized compared to the non-treated control. The mean of ACX-362E treated samples is statistically different from the other samples ($p < 0.0001$).



Supplemental Figure 3. Confirmation of the *sigB* mutant strain (IB54). DNA electrophoresis of PCR fragments and immuno blot analysis of whole cell lysates confirm absence of the *sigB* coding sequence and σ^B protein, respectively. **A)** DNA electrophoresis. DNA was isolated from 630 Δerm (WT) and IB54 ($\Delta sigB$) cultured for 15 hours and a PCR using primers oIB-78 and oIB-79 was performed (Table 4). Expected product sizes are 3597 bp (WT) and 2823 bp ($\Delta sigB$). DNA electrophoresis was performed using a 0.5xTAE 1% agarose gel. **B)** Immunoblot analysis of whole cell lysates of 630 Δerm (WT) and IB54 ($\Delta sigB$) cultured for 15 hours. The expected size of the σ^B protein is 34 kDa as indicated by the arrow. A clear signal is detected in the WT sample, but none can be observed in $\Delta sigB$. The asterisk indicates a non-specific cross-reacting protein in the *C. difficile* lysates. Cells from 1 mL of was pelleted (10 min, 4°C, 8000 rpm) and cell pellets were lysed in 250 μ l 1x SDS sample buffer. OD corrected samples were analyzed by SDS-PAGE (12,5%) and transferred onto a 0.45 μ m PVDF membrane. The membrane was probed with a rabbit anti- σ^B antibody in 5% low-fat milk in 0.1% TBST at 4°C overnight. The probed membranes were analyzed with a swine-anti-rabbit-HRP antibody (Agilent) incubated 1:2000 in 5% low-fat milk in 0.1% TBST for 2-3 hours at room temperature. Detection was of chemiluminescent signal was performed using a Uvitec Alliance Q9 Advanced imager (BioSPX), after 5 min incubation with Clarity Western ECL substrate (Bio-Rad). "M" = marker, "-" = no sample loaded.



3

REDEFINING THE *CLOSTRIDIoidES* *DIFFICILE* σ^B REGULON: σ^B ACTIVATES GENES INVOLVED IN DETOXIFYING RADICALS THAT CAN RESULT FROM THE EXPOSURE TO ANTIMICROBIALS AND HYDROGEN PEROXIDE

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Abstract

In many Gram-positive bacteria the general stress response is regulated at the transcriptional level by the alternative sigma factor sigma B (σ^B). In *C. difficile* σ^B has been implicated in protection against stressors such as reactive oxygen species and antimicrobial compounds. Here, we used an anti- σ^B antibody to demonstrate time-limited overproduction of σ^B in *C. difficile* despite its toxicity at higher cellular concentrations. This toxicity eventually led to the loss of the plasmid used for anhydrotetracycline-induced σ^B gene expression. Inducible σ^B overproduction uncouples σ^B expression from its native regulatory network and allowed for the refinement of the previously proposed σ^B regulon. At least 32% the regulon was found to consist of genes involved in the response to reactive radicals. Direct gene activation by *C. difficile* σ^B was demonstrated through *in vitro* run-off transcription of specific target genes (*CD0350*, *CD3614*, *CD3605*, *CD2963*). Finally, we demonstrated that different antimicrobials and hydrogen peroxide induce these genes in a manner dependent on this sigma factor, using a plate-based luciferase reporter assay. Together, our work suggests that lethal exposure to antimicrobials may result in the formation of toxic radicals that lead to σ^B -dependent gene activation.

Importance

Sigma B is the alternative sigma factor governing stress response in many gram-positive bacteria. In *C. difficile*, a *sigB* mutant shows pleiotropic transcriptional effects. Here, we determine genes that are likely direct targets of σ^B by evaluating the transcriptional effects of σ^B overproduction, provide biochemical evidence of direct transcriptional activation by σ^B , and show that σ^B -dependent genes can be activated by antimicrobials. Together our data suggest that σ^B is a key player in dealing with toxic radicals.

Introduction

Disruption of the normal gastrointestinal flora as a result of antimicrobial treatment can lead to a *Clostridioides (Clostridium) difficile* infection (CDI) (225). *Clostridioides difficile* is a gram-positive, spore-forming obligate anaerobe and the primary cause for nosocomial infectious diarrhea (20). Its highly resistant endospores are usually transmitted via the oral-fecal route and germinate into vegetative cells in the colon upon contact with primary bile acids and other inducing factors (58). In the gut vegetative *C. difficile* cells are facing many environmental stressors, including variations in oxygen tension, pH, osmolarity, nutrient availability, and the inflammatory responses of the immune system (150). The bacteria are also faced with antimicrobial compounds produced by the host, the resident microbiota, or given externally during medical therapy (80). The physiological response of *C. difficile* to these insults and the inflammatory responses triggered by CDI can result in the production of Reactive Oxygen Species (ROS), Reactive Nitrogen Species (RNS) and nitric oxide (NO) (20, 172).

Bacteria need to adapt to changing environmental conditions, including stresses, by adapting their physiology in a timely manner. This is achieved by fast transcriptional reprogramming, followed by shortly delayed changes at the translational level (216). The alternative sigma factor sigma B (σ^B , encoded by the *sigB* gene) - which regulates the general stress responses in a variety of Gram-positive organisms - is central to the maintenance of the cellular homeostasis during stress adaptation (148, 226).

Sigma factor B activity in Firmicutes is regulated at the protein level by a partner-switching mechanism in which the anti-sigma factor RsbW binds and inhibits σ^B association with the RNA polymerase under non-stressed conditions. When a σ^B -activating stress is sensed the dephosphorylated anti-anti-sigma factor RsbV sequesters RsbW, allowing for the association of free σ^B with the RNA polymerase core enzyme (148, 227). In *C. difficile*, the phosphatase RsbZ is responsible for RsbV dephosphorylation (169). The tight regulation of σ^B activity by a partner-switching mechanism is necessary as the energy burden associated with σ^B activity was found to be disadvantageous in several different organisms (162, 228).

Despite the burden associated with its expression, σ^B is essential for survival for several pathogenic bacterial species in response to host-dependent stressors or antimicrobials. For

example, in *L. monocytogenes* σ^B is involved in counteracting the effects of the acidic pH encountered in the stomach and upon invasion of intestinal epithelial cells in the lysosome (229, 230). In *Staphylococcus aureus* σ^B overproduction leads to thickening of the cell wall and increased resistance to beta-lactam antimicrobials (170). The *sigB* homologue *sigF* of *Mycobacterium tuberculosis* is induced by small amounts of rifamycin (231). Analogously, *B. subtilis* σ^B is involved in resolving a rifampin-induced growth arrest (171). There is also evidence for the involvement of σ^B in *C. difficile* in the response to antimicrobial substances. Mutants of *sigB* show increased susceptibility to rifampicin and mitomycin C, and are also more sensitive to hydrogen peroxide, nitroprusside and di-ethylamine NONOate (160). However, the underlying molecular mechanisms remain unknown. Finally, indirect activation of σ^B -dependent genes as the result of a gene-dosage shift has been demonstrated for *C. difficile* exposed to DNA-polymerase inhibitors such as the Phase II drug ibezapolstat/ACX-362E (232).

In this study, we demonstrate that σ^B overexpression is detectable and tolerated for short periods of time. This allowed for the experimental identification of a set of genes that is most likely directly regulated by σ^B by performing transcriptome analyses under conditions of acute σ^B overexpression. The obtained results show that genes involved in the oxidative and nitrosative stress response form the core of the regulon. Additionally, we show that various antimicrobials and hydrogen peroxide induce the expression of σ^B -regulated genes in a σ^B -dependent manner, suggesting a link between the lethal exposure to antimicrobials and oxidative and nitrosative stresses in *C. difficile*.

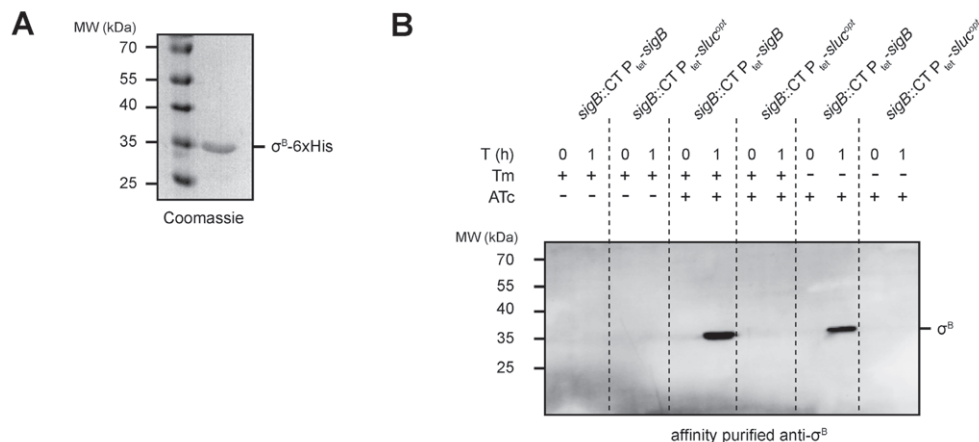


Figure 1. Recombinant σ^B was used to generate a *C. difficile* specific antibody for intracellular detection. **A)** Coomassie Blue-stained 12.5% SDS-PAGE gel of purified recombinant σ^B -6xHis. **B)** Western Blot using affinity purified σ^B antibody (1:500) on strains IB58 (*sigB::CT P_{tet}-sigB*) and IB61 (*sigB::CT P_{tet}-sluc^{opt}*). Cells were grown in lincomycin (20 μ g/mL) and in the presence or absence of thiamphenicol (20 μ g/mL) until OD_{600nm} $\approx$ 0.3, after which the indicated samples were induced with 100 ng/mL ATc. Samples were collected directly after the addition of ATc (or at the time ATc would have been added in the uninduced controls) at T=0h and after 1 hour of induction (T=1).

Results

C. difficile σ^B is measurably overproduced upon induction of the *sigB* gene

Previous investigations of σ^B in *C. difficile* have used a *sigB* mutant and characterized its gene expression in the stationary growth phase in comparison with a wild type strain (160). Though informative, this method is likely to result in indirect effects of σ^B due to stationary phase heterogeneity, prolonged incubation and possible positive or negative feedback in the σ^B regulatory circuit. To circumvent these issues and identify genes likely to be regulated by σ^B directly, we set out to uncouple *sigB* expression from its native regulatory circuit by expressing it from an inducible promoter.

First, in order to confirm overproduction of σ^B , we measured cellular σ^B levels using immunoblotting. For this purpose, we heterologously overproduced and purified σ^B containing a C-terminal His-tag (**Figure 1A**) and used this protein to raise a polyclonal antiserum. Corresponding polyclonal antibodies were affinity-purified to prevent unspecific immune reactions.

Next, we set out to validate the overproduction of σ^B in transconjugant *C. difficile* cells harboring plasmids containing *sigB* under the control of the anhydrotetracyclin (ATc)- dependent promoter P_{tet} (233). For this purpose, σ^B was produced in a *sigB* mutant background (strain IB58; *sigB*::CT P_{tet} -*sigB*). As a control, we introduced a non-related expression construct in the same background (IB61; *sigB*::CT P_{tet} -*sluc*^{opt}) such that this control strain carries a plasmid with the same replicon, resistance marker and inducible promoter.

We expected a signal at approximately 30 kDa in Western blot experiments for cells grown in the presence of inducer ATc for strain IB58, but not for the uninduced cultures of IB58 or the control strain IB61. Additionally, by growing in the presence or absence of thiamphenicol, we investigated whether overproduction of σ^B required selection for the P_{tet} -*sigB* expression plasmid. When strains were grown in BHIY medium supplemented with 20 μ g/mL lincomycin and induced for one hour with or without 100 ng/mL ATc in the presence or absence of 20 μ g/mL thiamphenicol, we did not detect any signal at the molecular weight expected for σ^B in the ATc-induced control samples (*sigB*::CT P_{tet} -*sluc*^{opt}) or in any of the uninduced samples (**Figure 1B**). In contrast, after one hour of induction a clear band of the expected molecular weight of σ^B (~30 kDa) was observed only in the IB58 (*sigB*::CT P_{tet} -*sigB*) samples (**Figure 1B**). Plasmid selection by inclusion of thiamphenicol in the growth medium did not influence σ^B overproduction in this time frame which might have occurred as a trade-off between σ^B overexpression and cellular toxicity (see also further below).

We conclude that the affinity purified rabbit- α - σ^B antibody is specific for σ^B and can be used for its detection in lysates of *C. difficile*. Furthermore, it is possible to uncouple *sigB* expression from its tight regulatory network by ATc-inducible overexpression for one hour *in trans*.

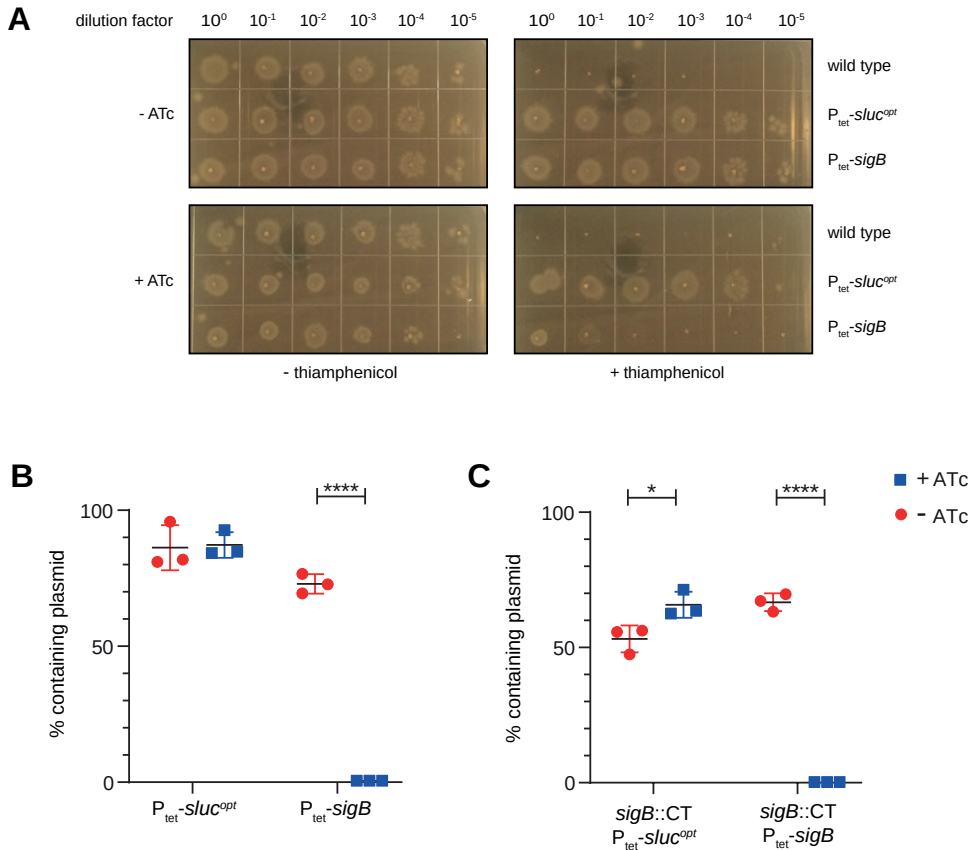


Figure 2. Overexpression of σ^B is toxic for *C. difficile* and leads to plasmid loss. A) Tenfold serial dilutions on BHIY agar plates of 630 Δ erm (wildtype), AP34 (*P_{tet}-sLuc^{opt}*) and JC096 (*P_{tet}-sigB*). Similar results were obtained for strains IB58 and IB61 (data not shown). **B)** Percentage of cells retaining the plasmid in AP34 (*P_{tet}-sLuc^{opt}*) and JC096 (*P_{tet}-sigB*). **C)** Percentage of cells retaining the plasmid in strains IB61 (*sigB::CT P_{tet}-sLuc^{opt}*) and IB58 (*sigB::CT P_{tet}-sigB*). Percentages were calculated based on the ratio of CFU/mL of the paired selective (with thiamphenicol)- and non-selective (without thiamphenicol) plates. * $p < 0.05$ and **** $p \leq 0.0001$ as determined by an unpaired student's t-test, $n = 3$.

Prolonged overexpression of σ^B is lethal and leads to loss of plasmids harboring *P_{tet}-sigB*

Above we showed that it is possible to overproduce σ^B in *C. difficile* and that it is tolerated by the bacterium for one hour. This observation is somewhat at odds with the previously reported toxic nature of overproduced σ^B (148, 169). To reconcile these two observations, the effect of long-term overexpression of σ^B and the stability of the plasmids used for σ^B overproduction under such conditions were investigated. First, overnight cultures of 630 Δ erm

(wildtype), AP34 ($P_{tet}-sluc^{opt}$) and JC096 ($P_{tet}-sigB$) were adjusted for their OD_{600nm} values and ten-fold serially diluted. Subsequently, 2 μ l spots per dilution were made on selective (20 μ g/mL thiamphenicol) and non-selective BHIY agar plates, part of which contained 200 ng/mL ATc to induce P_{tet} -dependent gene expression. All plates were then incubated anaerobically for 24 hours. On plates without thiamphenicol, regardless of the presence of the inducer ATc, comparable growth was observed for all three strains (**Figure 2A**). As expected, when selecting for the plasmid using thiamphenicol no growth was observed for the susceptible 630 Δ *erm* strain (which lacks the *catP* gene contained on the expression vector). In the absence of the inducer, no difference in growth was observed for the vector control strain (AP34; $P_{tet}-sluc^{opt}$) compared to the strain carrying the $P_{tet}-sigB$ plasmid (JC096). However, upon induction of *sigB* expression on selective plates a 3-4 log growth defect was observed for the strain carrying $P_{tet}-sigB$ compared to the vector control strain. We conclude that prolonged induction of *sigB* expression is toxic when cells are cultured in the presence of thiamphenicol. Our results thus corroborate the finding that σ^B overproduction is toxic to *C. difficile* cells in liquid culture (169).

	Control	Target	Conditions	DE	POS	NEG
1	<i>sigB</i> ::CT $P_{tet}-sluc^{opt}$ (IB61)	<i>sigB</i> ::CT $P_{tet}-$ <i>sigB</i> (IB58)	Lincomycin [20 μ g/mL], Thiamphenicol [20 μ g/mL], no ATc	5	4	1
2	<i>sigB</i> ::CT $P_{tet}-sluc^{opt}$ (IB61)	<i>sigB</i> ::CT $P_{tet}-$ <i>sigB</i> (IB58)	Lincomycin [20 μ g/mL], Thiamphenicol [20 μ g/mL], ATc [100 ng/mL]	183 (178)	167 (163)	16 (15)
3	<i>sigB</i> ::CT $P_{tet}-sluc^{opt}$ (IB61)	<i>sigB</i> ::CT $P_{tet}-$ <i>sigB</i> (IB58)	Lincomycin [20 μ g/mL], no Thiamphenicol, no ATc	5	4	1
4	<i>sigB</i> ::CT $P_{tet}-sluc^{opt}$ (IB61)	<i>sigB</i> ::CT $P_{tet}-$ <i>sigB</i> (IB58)	Lincomycin [20 μ g/mL], no Thiamphenicol, ATc [100 ng/mL]	150 (145)	136 (132)	14 (13)

Table 1. Setup of the DNA array and number of differentially expressed (DE) genes, including number of positively (POS) and negatively (NEG) regulated genes. Numbers inbetween brackets correspond to the number of differentially expressed genes after subtracting the differentially expressed genes identified in hybridisations 1 and 3 (that are not dependent on σ^B induction).

The lethality associated with σ^B overproduction was not seen when cells were grown without thiamphenicol in our experiment (**Figure 2A**). We considered two possible explanations for this observation. As thiamphenicol is used for ensuring plasmid maintenance, its absence might result in plasmid loss due to segregation or negative selection pressure when a toxic protein such as σ^B is overproduced. The remaining cells, that no longer express σ^B would consequently be susceptible to thiamphenicol (due to the loss of *catP*) but might outgrow

those carrying the plasmid. Alternatively, the combination of σ^B and thiamphenicol might be toxic to the bacteria. To test whether plasmid loss was the cause of the observed lethality of bacteria overproducing σ^B in the presence of thiamphenicol, cells from the plates without thiamphenicol (with and without ATc) were resuspended in PBS at 1.0 McFarland turbidity, and ten-fold serially diluted in BHI medium. Ten- μ l spots of these dilutions were plated on plasmid-selective (thiamphenicol) and non-selective (no thiamphenicol) plates. Based on the ratio of CFU/mL of the selective and non-selective plates, the percentage of cells which lost their plasmid was calculated. If σ^B overproduction led to the loss of the plasmid under conditions that do not select for its maintenance (no thiamphenicol), we expected significantly reduced growth on plates containing thiamphenicol. Although some plasmid loss was observed under uninduced conditions, as well as for the negative control strain AP34 (P_{tet} -*sluc*^{opt}), all cells originally containing the P_{tet} -*sigB* plasmid (strain JC096) completely lost this plasmid upon induction of σ^B overproduction with ATc (**Figure 2B**). Similar results were obtained for *sigB* mutant strains IB58 (*sigB*::CT P_{tet} -*sigB*) and IB61 (*sigB*::CT P_{tet} -*sluc*^{opt}), indicating the observed effects were solely due to *in trans* σ^B overproduction, and did not result from an interference of the native *sigB* regulatory network (**Figure 2C**). Together, these results are consistent with a model in which the vector with the low-copy number pCD6 replicon are rapidly eradicated upon expression of a gene (here *sigB*) that causes lethal defects (234, 235).

σ^B primarily activates genes relating to oxidative/nitrosative stress responses

Above, we have shown that long-term overproduction σ^B is detrimental and that this leads to loss of the expression plasmid in the absence of thiamphenicol (**Figure 2**), but that σ^B overproduction nevertheless could clearly be demonstrated when induction is limited to 1 hour (**Figure 1C**). Therefore we used the time-limited induction to refine the previously proposed regulon (160) in both the presence and absence of thiamphenicol to strike a balance between potential secondary effects due to toxicity associated with σ^B overproduction (with thiamphenicol), and loss of the expression plasmid from a subpopulation of cells (without thiamphenicol) (**Table 1**). We compared transcriptome data from strain IB58 (*sigB*::CT P_{tet} -*sigB*) to that of strain IB61 (*sigB*::CT P_{tet} -*sluc*^{opt}). IB61 harbors a vector for the inducible expression of a luciferase gene which does not lead to any toxicity and growth phenotype (201).

We expected no or a limited number of genes to be differentially expressed (log2 foldchange (log2FC) of ≤ -1.5 or ≥ 1.5 , and adjusted p-value of <0.05) under non-inducing conditions.

Indeed, we found only five differentially expressed genes in the P_{tet} -*sigB* strain (IB58) compared to the P_{tet} -*sluc*^{opt} control (IB61) strain (hybridizations 1 and 3) (**Dataset S1**). These genes were similarly positively (CD0583, CD0584, both GGDEF domain containing proteins (236); CD2214, CD2215, both potential transcriptional regulators (237)) and negatively (CD1616, an EAL domain protein (236)) regulated in all hybridizations, including those where *sigB* expression was not induced. These results suggest that the basis for the observed differential expression of these genes was vector specific, but not dependent on σ^B induction. These genes were therefore not investigated further and are excluded from the numbers discussed below.

Locus tag	Gene name	log2FC	adjusted p-value	Predicted function
Genes upregulated by aerobic stress and positively regulated by σ^B				
CD630DERM_00530	<i>mrnC</i>	1.9	3.29E-05	Ribonuclease III domain
CD630DERM_01750		8.2	1.11E-06	Oxidoreductase, Fe-S subunit
CD630DERM_01760		8.0	1.52E-06	Oxidoreductase, NAD/FAD binding subunit
CD630DERM_01920	<i>cls</i>	2.6	2.35E-04	Cardiolipin synthetase 1
CD630DERM_03500		1.9	4.63E-05	Putative hydrolase, HAD superfamily
CD630DERM_03510		1.8	5.66E-05	Conserved hypothetical protein
CD630DERM_05600	<i>nfo</i>	4.0	3.5E-05	Endonuclease IV
CD630DERM_05610		2.0	2.39E-05	Putative aldo/keto reductase; putative ferredoxin
CD630DERM_05650	<i>nth</i>	3.7	5.66E-06	Endonuclease III
CD630DERM_05660		2.5	6.62E-06	Putative tRNA/rRNA methyltransferase
CD630DERM_05800	<i>gapN</i>	6.2	1.38E-06	Glyceraldehyde-3-phosphate dehydrogenase (NADP(+)) (GADPH)
CD630DERM_11250		5.5	1.15E-05	Nitroreductase-family protein
CD630DERM_11570	<i>norV</i>	4.0	5.16E-06	Anaerobic nitric oxide reductase flavorubredoxin (FIRd) (FlavoRb)
CD630DERM_13410	<i>exoA</i>	1.8	6.76E-05	Exodeoxyribonuclease
CD630DERM_14630		1.8	1.77E-04	Conserved hypothetical protein
CD630DERM_15240		4.8	1.88E-05	Putative rubrerythrin
CD630DERM_15260	<i>pyrC</i>	1.6	1.98E-04	dihydroorotase
CD630DERM_15760		1.6	2.71E-02	putative arylesterase
CD630DERM_16220		2.7	1.03E-04	Putative lipoprotein
CD630DERM_16230		6.5	4.21E-06	NADH-oxygen oxidoreductase
CD630DERM_16240	<i>vanR</i>	1.6	8.84E-05	two-component response regulator
CD630DERM_16900	<i>trxA</i>	5.3	1.31E-06	Thioredoxin reductase
CD630DERM_16910	<i>trxB</i>	5.3	1.62E-06	Putative thioredoxin-disulfide reductase
CD630DERM_17780		2.6	1.19E-05	Conserved hypothetical protein
CD630DERM_17790		2.4	1.46E-05	Conserved hypothetical protein
CD630DERM_18180	<i>ispH</i>	1.8	5.35E-05	4-hydroxy-3-methylbut-2-enyl diphosphate reductase
CD630DERM_18220	<i>bcp</i>	6.1	2.70E-05	Putative thiol peroxidase
CD630DERM_18970		1.8	3.20E-05	Conserved hypothetical protein

Locus tag	Gene name	log2FC	adjusted p-value	Predicted function
Genes upregulated by aerobic stress and positively regulated by σ^B				
CD630DERM_19430		3.3	3.62E-06	Conserved hypothetical protein
CD630DERM_20460		5.5	5.60E-06	Conserved hypothetical protein
CD630DERM_21170	<i>trxB2</i>	5.0	1.86E-05	Thioredoxin reductase
CD630DERM_21650		2.2	1.04E-04	Transcriptional regulator, HTH-type
CD630DERM_24760		4.6	5.84E-06	Conserved hypothetical protein
CD630DERM_27960	<i>cwp10</i>	5.1	2.47E-05	Cell surface protein
CD630DERM_27970		5.1	1.75E-05	Putative calcium-binding adhesion protein
CD630DERM_29930		3.8	3.07E-05	Conserved hypothetical protein
CD630DERM_30380		3.9	2.21E-05	Conserved hypothetical protein
CD630DERM_30390		4.1	1.07E-05	Putative ATPase
CD630DERM_30400		2.1	8.46E-05	Conserved hypothetical protein
CD630DERM_30420		2.6	2.47E-05	Putative membrane protein
CD630DERM_33070		3.8	1.51E-06	Putative phosphoesterase
CD630DERM_33100		3.1	6.31E-06	Putative D-isomer specific 2-hydroxyacid dehydrogenase
CD630DERM_33110		2.9	1.19E-05	Conserved hypothetical protein
CD630DERM_34080		2.3	3.38E-05	Putative DNA mismatch repair ATPase MutS
CD630DERM_34090	<i>scoC/hprK</i>	2.4	1.74E-05	PTS system, HPr kinase/phosphorylase
CD630DERM_34100	<i>uvrC</i>	2.7	1.19E-05	Excinuclease subunit C
CD630DERM_34730	<i>atpC2</i>	1.6	2.90E-04	ATP synthase C chain
CD630DERM_34760	<i>atpZ</i>	1.6	2.42E-04	putative ATP synthase protein
CD630DERM_36100		4.6	2.38E-05	Conserved hypothetical protein
CD630DERM_36140		2.5	1.07E-05	Conserved hypothetical protein, DUF1130 family
Other genes involved in oxidative/nitrosative stress positively regulated by σ^B				
CD630DERM_01740	<i>cooS</i>	8.3	1.20E-06	Carbon monoxide dehydrogenase
CD630DERM_12790	<i>iscS2</i>	2.8	2.00E-03	Cysteine desulfurase
CD630DERM_12800		3.0	1.56E-03	Putative NifU-like protein
CD630DERM_14740		5.0	1.28E-05	Putative rubrerythrin (Rr)
CD630DERM_15940	<i>cysK</i>	3.4	4.44E-03	O-acetyl-serine thiol-lyase A (O-acetyl-sulphydrylase)(OAS-TL)
CD630DERM_18230		6.8	5.93E-06	Conserved hypothetical protein, UPF0246 family
CD630DERM_21660	<i>msrAB</i>	4.7	1.24E-05	Peptide methionine sulfoxide reductase MsrA/MsrB

Table 2. Selected genes differentially expressed upon overexpression of σ^B . Genes positively regulated by σ^B and aerobic stress and other genes involved in oxidative/nitrosative stress (160, 216) are highlighted here. CD numbers corresponding to the published annotation of strain CD630 (199) can be derived by removing “630DERM_” and removing the last digit (in case of a 0) or replacing it with an A (in the case of a 1).

Upon induction of *sigB* expression 145 genes were differentially expressed when strains were cultured without thiamphenicol (hybridization 4), and 178 genes were differentially expressed when thiamphenicol was present during cultivation (hybridization 2) (**Figure 3**,

Table 1 and Dataset S1). The majority showed an increase in expression upon induction of *sigB* expression (132 in the samples without thiamphenicol, and 163 in the samples with thiamphenicol), consistent with its function as a sigma factor (238), while a minority revealed a decreased expression (13 in the samples without thiamphenicol, and 16 in the samples with thiamphenicol). Of note, we observed only a minor difference in the number of differentially expressed genes between the cells grown in the absence and presence of thiamphenicol (33 genes). Together, these results demonstrate a high level of consistency in the σ^B regulon, despite potential plasmid-loss (when grown in the absence of thiamphenicol) or toxic effects (when grown in the presence thiamphenicol). Our results also show that σ^B primarily activates gene expression.

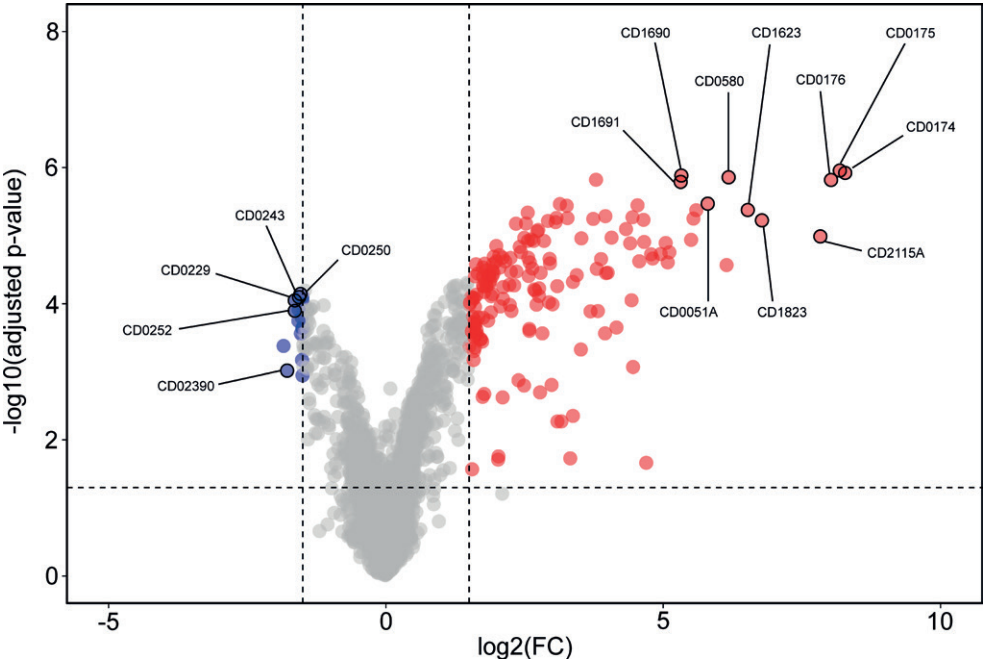


Figure 3. Volcano plot of the transcriptome analysis of the σ^B regulon. Graphical representation of differential gene regulation upon overproduction of σ^B . Dashed lines indicated the significance threshold: $|\log_2FC| > 1.5$ and adjusted p-value < 0.05 . Genes significantly up-regulated by σ^B are indicated in red, and genes down-regulated are indicated in blue. The top-10 of up-regulated genes and 5 selected down-regulated genes are annotated in the figure. An interactive version of the graph is available for exploration via the URL provided in the **Text S1**.

We focused our further analyses on the data obtained from hybridization 2 (with ATc and thiamphenicol), as this condition provided the broadest dataset (178 differentially expressed genes) for the re-definition of the σ^B regulon under our experimental conditions (**Dataset S1**).

Of the 163 genes up-regulated by σ^B the vast majority appeared to be associated with an response to oxidative stress, since they encode various oxidoreductases, peroxidases, and thioredoxin reductases (**Table 2**). Notably, approximately 51% of the 98 genes previously found to be up-regulated under aerobic stress (216) were also positively regulated by σ^B (**Table 2**). Five additional genes associated with aerobic/nitrosative stress (CD0174/*cooS*, CD1279/*iscS2*, CD1280, CD1594/*cysK*, CD1823 and CD2166/*msrAB*) were also found to be induced by σ^B , in agreement with previous findings (160).

Our findings are recapitulated in a volcano plot (239), which clearly shows that genes with lower expression upon *sigB* induction (in blue) cluster close to the significance threshold whereas those with increased expression (in red) show a larger fold change (**Figure 3**). We calculated the Manhattan distance for each data point (**Dataset S2**), and discuss the proteins encoded by the top-10 of the differentially expressed genes below.

CD0051A is a small hypothetical protein of unknown function. It does not contain any recognizable domains and a secondary structure prediction using Phyre2 does not give any clues as to its potential function (240). CD0580 (GapN) is annotated as an glyceraldehyde-3-phosphate dehydrogenase (GADPH), a key glycolytic enzyme, and contains an aldehyde dehydrogenase domain. Interestingly, its activity has been shown to be redox-controlled in other bacteria and has been implicated in the response to reactive oxygen and nitrogen species (241-243). CD1623 is a putative oxidoreductase with similarity to FAD flavoproteins and rubredoxins. CD1690 (TrxA) and CD1691 (TrxB) are likely encoded in the same operon (244), and form a thioredoxin/thioredoxin-disulfide reductase couple. CD0174 (*CooS*; IPR010047), CD0175 and CD0176 are likely also encoded in a single operon (244), and function as carbon monoxide dehydrogenase, and two putative oxidoreductases. As mentioned above, CD0174 has been implicated in aerobic/nitrosative stress and it is likely that CD1623, CD1690 and CD1691 also function in this pathway. Finally, CD2115A encodes another small hypothetical protein and as for CD0051A, no function could be assigned on the basis of secondary structure prediction.

As the σ^B regulon that we define here is substantially smaller than that previously reported, the major conclusion is that at least 32% of the σ^B regulon is involved in positively regulating oxidative/nitrosative stress responses. In the previous investigation of the σ^B regulon they were approximately 3.2% (~32/1000) (160). Overall, we conclude that the core functions of the σ^B regulon lie in the regulation of the detoxification response to oxygen- and nitro-radicals.

In vitro run-off transcriptions demonstrate direction activation of P_{CD0350} , P_{CD2963} , P_{CD3412} and P_{CD3605} by σ^B

Gene expression can directly or indirectly be influenced by σ^B , and to date no attempts have been made to discriminate these possibilities biochemically (160, 169). Despite the short time of induction and the uncoupling of σ^B from its normal regulatory network, our analyses could possibly also have picked up indirect effects. To determine if the transcription of selected genes is directly activated by σ^B , *in vitro* transcription run-off reactions were performed using purified σ^B_{6xHis} and RNA polymerase core enzyme (RNAP^{core}) on the upstream regions of a selection of genes. The genes CD0350 (encoding a putative hydrolase involved in oxidative stress; **Table 2**), CD2963 (encoding an L,D-transpeptidase), CD3412 (encoding UvrB, involved in nucleotide excision repair), CD3605 (encoding a ferredoxin) and CD3614 (encoding a hypothetical protein involved in oxidative stress; **Table 2**) were selected on the basis of differential expression in our transcriptome analyses (**Dataset S1**) and those of others (160), availability of reporter constructs that could be used to generate a template for the *in vitro* transcription reactions (232) and/or the presence of a putative σ^B consensus upstream in the upstream region (169). The gene CD0872 (maltose O-acetyltransferase) was not differentially expressed in our transcriptome data and was thus included as a negative control. The promoter of the toxin A gene (*tcdA*) in combination with purified TcdR was used as a positive control for the assay, as previously described (245). As expected, no *in vitro* transcript was observed for a linear DNA fragment containing P_{CD0872} incubated with purified σ^B_{6xHis} and RNAP^{core} under our experimental conditions, whereas a specific product was obtained for the positive control P_{tcdA} in the presence of TcdR and RNAP^{core} (**Figure 4**). An RNAP^{core}- and σ^B_{6xHis} -specific signal was observed for fragments containing the putative promoter regions of the genes CD0350, CD2963, CD3412 and CD3605, demonstrating that expression of these genes was directed by σ^B . For the fragments containing the putative promoter of CD3614 we did not get a consistent product in the *in vitro* transcription experiments, though some smearing is visible in the lane with RNAP^{core} and σ^B_{6xHis} . As CD3614 demonstrates clear differential expression in the DNA array experiments and its upstream region harbors the σ^B consensus sequence $WGWT-N_{13-17}-(G/T)GGTWA$ (160), we consider it likely that this gene is directly regulated by σ^B and our failure to obtain a discrete signal is due to our experimental conditions or the lack of an auxiliary factor in our *in vitro* assays.

Overall, we provide the first biochemical evidence for direct σ^B -dependent activation of several genes identified via transcriptome analyses as part of the σ^B regulon in *C. difficile*.

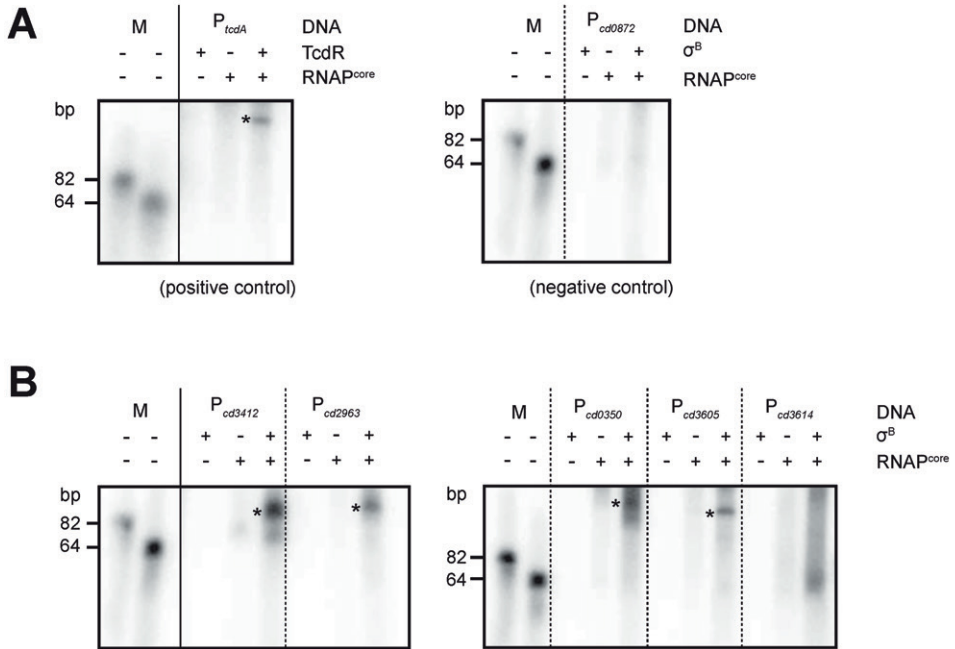


Figure 4. *In vitro* run-off transcription of selected promoter regions. Samples were run on an 8% urea gel. The two bands corresponding to 82 bp and 64 bp are end-labelled oligonucleotides. Reactions without sigma factor (σ^B or TcdR, respectively) or RNAP^{core} were analyzed as controls. Asterisks indicate the presence of distinct transcripts. RNAP^{core} = *E. coli* RNA polymerase core enzyme (NEB). **A)** Controls for the *in vitro* run-off transcriptions. Purified TcdR, a sigma factor demonstrated to activate *tcdA* transcription *in vitro* (245), was used with P_{tcdA} (pCD22) as a positive control for the assay. P_{cd0872} (derived from pIB21) shows no altered transcription in the DNA array analysis and was taken along as negative control. **B)** *In vitro* run-off transcriptions for selected genes induced by σ^B overproduction.

Antimicrobials and hydrogen peroxide activate σ^B -directed gene transcription

The redefined σ^B regulon pointed towards a substantial role for σ^B in coordinating the oxidative- and nitrosative stress response, which could result from antimicrobial treatment. In order to test for the activation of σ^B -dependent promoters by antimicrobials, we set up a plate-based luciferase reporter assay. In this assay cells harboring σ^B -dependent luciferase reporter constructs were plated on BHIY agar to give confluent growth and exposed to antimicrobials either through an epsilometer test (E-test) or through a filter disc. Subsequently, luciferase activity was imaged (for details, see Materials and Methods). A strain harboring P_{tet}-*sluc*^{opt} (AP34) served as negative control, as this promoter is not expected to respond in a σ^B -dependent manner (**Figure 5A**). First, the σ^B -dependent response to metronidazole was investigated. Metronidazole, formerly used as a first line treatment for CDI, is believed to cause DNA damage

through the formation of nitro-radicals although its exact mode of action remains unclear (172, 246). To survey a full spectrum of metronidazole concentrations, we evaluated luminescence after 24h of incubation of an metronidazole E-test. If metronidazole treatment results in σ^B -dependent activation of gene transcription, we expect to see a luciferase signal in the wild type, but not in a *sigB* knockout background. In agreement with this, activation of P_{CD0350} was observed at the edge of the halo resulting from the metronidazole E-test in the wild type background, but not in the *sigB* knockout strain (**Figure 5B**). No signal was observed for the negative control $P_{tet}-sluc^{opt}$ (**Figure 5A**). The observed activation σ^B -dependent activation of gene expression at the edge of the halo, but not further into the plate suggests that the metronidazole induced, σ^B -dependent activation of P_{CD0350} occurred close to the Minimal Inhibitory Concentration (MIC). Expression of the luciferase from P_{CD2963} was found to be strictly dependent on σ^B as no luciferase activity was observed in the *sigB* knockout strain. However, there was limited to no increase in reporter gene expression in the presence of metronidazole. Metronidazole strongly activated transcription from P_{CD3412} at MIC levels of metronidazole, but this appeared to be independent of σ^B in this assay since in the *sigB* mutant a similar induction was observed. Finally, in a manner comparable to P_{CD0350} , the activation of P_{CD3614} was strongly induced by metronidazole at values close to MIC in a σ^B -dependent manner, but residual activity was observed in the *sigB* knockout strain independent of metronidazole levels. We noted that metronidazole-induced promoter activation appeared to occur on the inside of the E-test halo, which might be attributed to the secretion of the luciferase reporter.

The observed diverse regulatory responses at different tested promoters during the treatment of *C. difficile* with metronidazole (with respect to basal level, σ^B dependence and induction) pointed towards a more complex regulatory network with the participation of σ^B , but also influenced by other factors. Antimicrobial-driven (and σ^B -dependent) activation of σ^B target genes could be specific to metronidazole or represent a more general response to cellular (toxic) stresses. Therefore, we evaluated the effects of different antimicrobial compounds and the radical producer H_2O_2 as a positive control (160), using the P_{CD0350} reporter construct, as this promoter demonstrated the clearest σ^B dependent activation in the presence of metronidazole (**Figure 5B**). We tested the cell wall biosynthesis inhibitor vancomycin, the protein synthesis inhibitor lincomycin and the DNA polymerase inhibitor ibezapolstat (formerly known as ACX-362E) (247). We observed clear activation of P_{CD0350} in the presence of all added stressors, but not for a negative control containing water (**Figure 5C**).

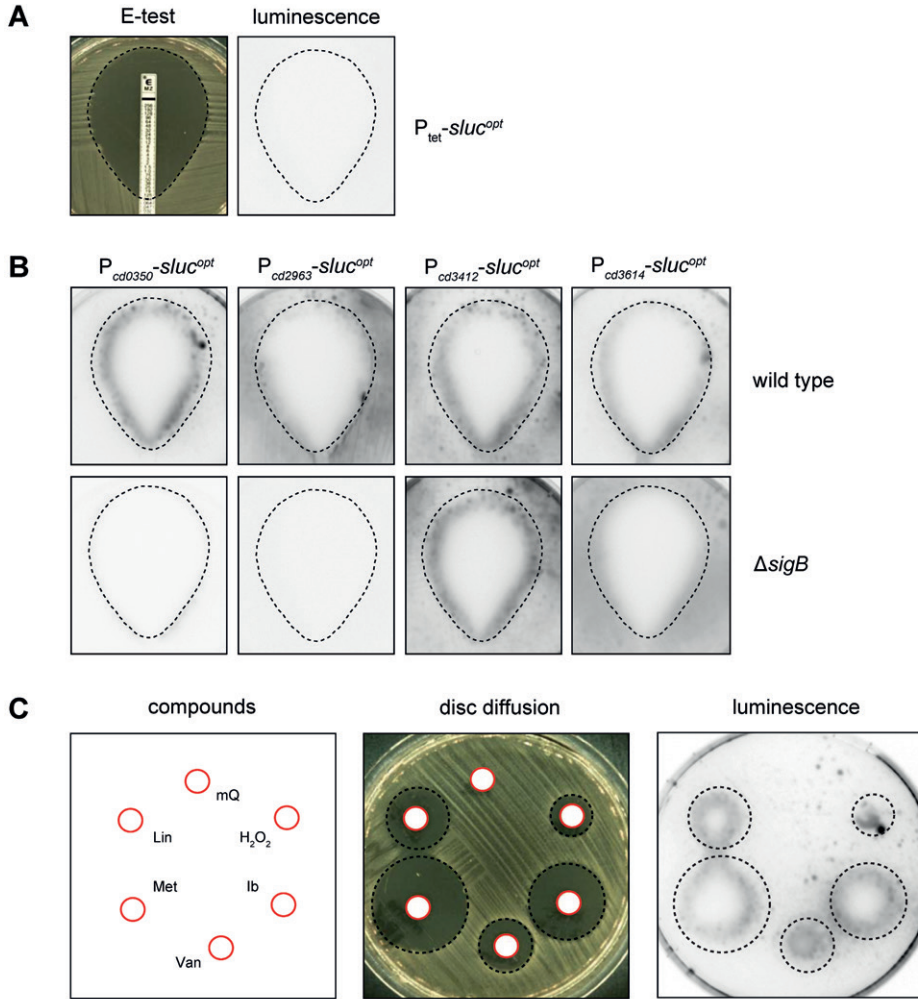


Figure 5. Plate-based luciferase assay shows σ^B -dependant promotor activity from antimicrobial and hydrogen peroxide exposure. **A)** Setup of the assay. E-test halos (left panel) were sprayed with luciferase substrate and imaged (right panel). The dotted line indicates the location of the halo based on the left panel. Strain AP34 ($P_{tet}-sluc^{opt}$) shows no signal due to the absence of inducer. **B)** Luciferase reporters of different σ^B regulated promoters were tested for luciferase signal after a metronidazole E-test. Halos are indicated by the dashed lines as in panel A. **C)** Luciferase activity of the P_{cd0350} luciferase reporter was imaged in the presence of discs containing 10 μ L of various compounds: sterile H_2O (mQ), Lincomycin (3000 μ g/ml; Lin), metronidazole (200 μ g/ml; Met), vancomycin (200 μ g/ml; Van), Ibezapolstat (400 μ g/ml; Ib) and hydrogen peroxide (1M; H_2O_2). Halos and the location of the different stressors are indicated by red circles (disc) and black dashed circles (halo). Signals outside the halo in these images are representative for the signals across the plate. Experiments were performed at least in triplicate with qualitatively similar results.

We conclude that, at least for the σ^B dependent promoter of CD0350, activation does not only occur upon exposure to lethal levels of metronidazole, but also with unrelated antimicrobials and toxic stressors such as hydrogen peroxide.

Discussion

In this work we have demonstrated by Western blotting using an affinity purified anti- σ^B antibody that σ^B can be overproduced for a limited period of time, sufficient for transcriptome analyses. The induced production of σ^B in a *sigB* mutant background yielded highly consistent results, despite potential toxicity and plasmid-loss (**Figure 2**), and the results were used to redefine the surprisingly large σ^B regulon previously proposed (160). As our approach more accurately measures changes in transcription directly related to σ^B production, the refined regulon described here is much smaller (**Dataset S1**). Its size is fully in line with that of the σ^B regulon of other gram-positive bacteria such as *L. monocytogenes* (~130 genes), *B. subtilis* (~150 genes) and *S. aureus* (~200 genes) (148). The redefined regulon underscores the importance of σ^B in responding to oxidative/nitrosative stresses as genes implicated in such processes are significantly enriched in the smaller regulon.

The majority of the genes in our regulon were found to be induced, rather than repressed, by σ^B . This is in line with sigma factors acting as specificity determinants for transcription initiation (238). Similar observations have been made for the σ^B regulon of *L. monocytogenes* (248, 249). For the first time, direct evidence of *C. difficile* σ^B -dependent gene activation is provided by the results of the *in vitro* run-off transcriptions (**Figure 4**), which demonstrate that RNAP^{core} and σ^B are sufficient to generate transcripts from P_{CD0350}, P_{CD2963}, P_{CD3412} and P_{CD3605}. Notably, these experiments pave the way for a further *in vitro* characterization of this sigma factor in *C. difficile* including validation of the σ^B binding sequence and the interplay with other regulators.

Although the promoters of CD3412 (*uvrB*) and CD3614 were reported to have a σ^B consensus sequence and are differentially expressed upon σ^B overexpression (160, 247), our results clearly demonstrate that they can also be expressed in a σ^B -independent manner (**Figure 5B**). This is most notable for P_{CD3412}, which is still activated by metronidazole in the absence of σ^B , in line with results obtained with ibezapolstat in a different study (232). Both metronidazole

and ibezapolstat treatment can cause DNA damage, and DNA-damage dependent induction of CD3412 therefore likely depends on a σ^B -independent pathway.

The observed σ^B -dependent gene repression is expected to be indirect (σ^B induces the transcription of a repressor gene), or the result of competition (σ^B competes with other sigma factors for RNAP) as sigma factors by their very nature induce gene expression (238). We consider the second scenario more likely for the following reasons. First, little overproduction of σ^B was detected after 30 minutes of induction. This leaves only a limited time for indirect effects to occur in our setup. Second, the majority of genes down-regulated upon overexpression of σ^B fall into a single functional group (flagellar motility). These genes are known to be regulated by the dedicated sigma factor, σ^D (250), supporting the model of sigma factor competition. Strikingly, in *L. monocytogenes* σ^B activity (indirectly) also results in down-regulation of flagellar gene expression, but this is mediated by the repressor MogR (251). Protein BLAST analyses revealed that *C. difficile* does not possess a MogR homologue. Nevertheless, the conserved inverse correlation between the σ^B -dependent general stress response and bacterial motility could represent a cost-saving strategy for bacterial cells (252). The indirect mechanism underlying the observed σ^B -dependent down-regulation in *C. difficile* remains to be determined.

There appears to be an intriguing link between σ^B and the response to toxic compounds, as a *sigB* mutant was more susceptible to rifampicin and mitomycin C (160) and exposure to antimicrobials (metronidazole, vancomycin, lincomycin and ibezapolstat) or hydrogen peroxide leads to σ^B -dependent promoter activation (**Figure 5C**). The mechanism behind the latter is unclear. It has been suggested that antimicrobials at toxic concentrations can influence metabolism and respiration (253, 254), potentially resulting in the formation of bactericidal concentrations of radical species (124, 255, 256). A strong connection between σ^B and oxidative (and/or nitrosative) stress in *C. difficile* (**Table 2**) and other bacteria (160, 171, 216), as well as a recently described radical scavenging strategy that increases tolerance to antimicrobials (257) are consistent with such a model. However, additional research is necessary to determine exactly how these processes occur and are influenced by antimicrobials in anaerobic organisms under anoxic conditions.

In conclusion, we have demonstrated that σ^B is directly involved in metabolic and oxidative stress responses and that lethal stresses may influence these processes, resulting in activation of σ^B targeted genes.

Materials and Methods

Construction of σ^B expression- and luciferase-reporter vectors

All oligonucleotides used in this study can be found in **Table 3**. Plasmids and strains are listed in **Table 4**. All PCR products used for sequencing or plasmid synthesis were generated with Q5 high-fidelity polymerase (NEB). The P_{T7} -*sigB*_{6xHis} expression vector pIB14 was created by restriction-ligation using restriction enzymes *NdeI* and *XhoI*. Using primers oIB-1 and oIB-2 on *C. difficile* 630 Δ *erm* chromosomal DNA, the *sigB* CDS was amplified by PCR. The resulting DNA fragment was digested and ligated into *NdeI*-*XhoI* digested pET21b(-) vector, generating expression vector pIB14. Plasmids pIB27, pIB68, pIB69 and pIB74 have been described previously (232). The CD0872 promoter area was amplified using primers oIB-14 and oIB-15 and the P_{CD0872} luciferase-reporter plasmid was created by restriction-ligation using restriction enzymes *KpnI* and *SacI* in digested pAP24 backbone, generating plasmid pIB21. The P_{CD3605} luciferase-reporter plasmid was generated by Gibson assembly as described (232) using primers oIB-90 and oIB-99, yielding plasmid pIB73. A plasmid containing P_{tet} -*sigB* was generated by cloning the 630 Δ *erm* *sigB* CDS amplified with oWKS-1498 and oWKS-1499 in pMiniT (NEB E1202) per manufacturer's instructions. Using restriction enzymes *SacI* and *BamHI* this PCR fragment was cloned into pRPF185, yielding pWKS1760. All plasmids were verified by Sanger sequencing.

Bacterial strains and growth conditions

Strains of *E. coli* were grown aerobically at 37°C in Luria-Bertani broth (Affymetrix) supplemented with ampicillin (50 μ g/mL), kanamycin (50 μ g/mL) and/or chloramphenicol (20 μ g/mL) when required. Plasmids were maintained in *E. coli* strains DH5 α or MDS42 (Scarab Genomics) under appropriate antimicrobial selection and cells were transformed using standard procedures (221). For plasmid conjugation into recipient wild type *C. difficile* 630 Δ *erm* and the isogenic *sigB* mutant, *E. coli* strain CA434 was used as a donor strain as previously described (222). *C. difficile* strains were cultured anaerobically at 37°C in either a Don Whitley VA-1000 or A55 workstation.

Cells were cultured in Brain Heart Infusion (BHI, Oxoid) broth supplemented with 0.5% (w/v) yeast-extract (BHIY) and 20 μ g/ml thiamphenicol when appropriate. Unless additional antimicrobials/stressors were added (metronidazole E-test and sterile pads supplemented with different stressors), medium was supplemented with *C. difficile* Selective Supplements (CDSS, Oxoid).

Name	Sequence (5' - 3')	Description	Source
Cdi-sigB-F	GTAGCTAAATGCTACACATTAC	Verification of <i>sigB</i> Clostron mutant	This study
Cdi-sigB-R	CAGTCATCTGTGATATCCCTAG	Verification of <i>sigB</i> Clostron mutant	This study
EBSuniversal	CGAAATTAGAAACTTGGTTCAGTAA	Verification of <i>sigB</i> Clostron mutant	(258)
ErmRAM-F	ACGGTTATATTGATAAAATAATAGTGGG	Verification of <i>sigB</i> Clostron mutant	(258)
ErmRAM-R	ACCGGTGCGACTCATAGAATTATTTCTCCCG	Verification of <i>sigB</i> Clostron mutant	(258)
oIB-1	TAGCCATATGAATAATGCTAGCTAATGCTACAC	Forward primer for <i>sigB</i> cloning in pET21b, containing a NdeI restriction site	This study
oIB-2	ACTGCTCGAGTAAATTTTTTTCATATCTTTTTTCAG	Reverse primer for <i>sigB</i> cloning in pET21b, containing an XhoI restriction site	This study
oIB-14	GTACGGTACCTTTACATATACATATATGTAGAAAAAC	Forward primer for P _{CD0872} <i>kpnI</i> restriction site	This study
oIB-15	GGTAGAGCTCATAGTTACTCCTTTTGTGTTAATTTG	Reverse primer for P _{CD0872} <i>SacI</i> restriction site	This study
oIB-26	GGAAGGTACCGTTGAATAAAGTATTTATTTTCCATG	Forward primer for P _{CD3412} containing a <i>kpnI</i> restriction site	(232)
oIB-27	GGTAGAGCTCAGTATCACTCCTTTTTCGAAAC	Reverse primer for P _{CD3412} containing a <i>SacI</i> restriction site	(232)
oIB-80	ctagcataaaataagaagcctgcatgtgcAAATTACGAAAAAGCTTGC	Forward primer for P _{CD0350}	(232)
oIB-82	ctagcataaaataagaagcctgcatgtgcTTGTGTTAAGGGATTTTGAAG	Forward primer for P _{CD2963}	(232)
oIB-90	ctagcataaaataagaagcctgcatgtgcATGTAAAGAAAGCCGAAAGAA	Forward primer for P _{CD3605}	This study
oIB-92	ctagcataaaataagaagcctgcatgtgcGAATAAAAAAGGGTGTGTC	Forward primer for P _{CD3614}	(232)
oIB-94	agctataataatttttacttggtgtcatTTTACCTCCATGTAACATTATTG	Reverse primer for P _{CD0350}	(232)
oIB-95	agctataataatttttacttggtgtcatTAATTAATCCTTCCTACATTGTAATTAC	Reverse primer for P _{CD2963}	(232)
oIB-99	agctataataatttttacttggtgtcatATTTACGCCCTCCATATTG	Reverse primer for P _{CD3605}	This study
oIB-100	agctataataatttttacttggtgtcatATAAACACCCCTCTATTCTTTG	Reverse primer for P _{CD3614}	(232)
oWKS-1498	GAGCTCTGCAGTAAAGAGAAAAATTTTATGAAAAATGAGCTAATGCTACAC	Forward primer for <i>sigB</i> CDS	This study
oWKS-1499	GGATCTTATAAATTTTTTTCATATCTTTTTTTCAG	Reverse primer for <i>sigB</i> CDS	This study
oWKS-1513	GAGCTCAAAATTTGAATTTTTTATAGGGGGAATAACCATGCATCATCACCATCAC	Oligonucleotide used for end labelling	This study
oWKS-1516	CACGGTTCGAAATCGGTACTGGCTTTCC		
oWKS-1506	GAGCTCAAAATTTGAATTTTTTATAGGGGGAATAACCATGCATCATCACCATCAT	Oligonucleotide used for end labelling	This study

Table 3. Oligonucleotides used in this study. Restriction sites are underlined, 30 bp overlapping regions as used in Gibson Assembly are indicated in lowercase letters.

The *sigB* Clostron mutant described in this manuscript was generated as described (258), using pMTL007C-E2_ sigB171s::intron_ermB, synthesized by DNA2.0 (now ATUM). Design of the retargeted intron was performed with the Perutka algorithm, via the Clostron website (<http://clostron.com/>). The mutant was verified using primers Cdi-sigB-F, Cdi-sigB-R, EBSuniversal, ErmRAM-F and ErmRAM-R.

Overproduction, purification and affinity purification of σ^B_{6xHis} for synthesis of a polyclonal anti- σ^B antibody

Overproduction and purification of σ^B_{6xHis}

Overexpression of σ^B_{6xHis} was performed by using *Escherichia coli* (*E. coli*) Rosetta (DE3) pLysS cells (Novagen) harboring the *E. coli* expression plasmid pIB14. These cells were cultured in Luria-Bertani (LB) broth and induced with 0.5 mM IPTG for one hour starting at an optical density $\approx$ 0.6. Cells were collected by centrifugation at 4°C and the resulting cell pellets were resuspended in lysis buffer (pH = 8.0, 50 mM NaH_2PO_4 , 300 mM NaCl, 5mM β -mercaptoethanol, 0.1% NP-40 and Complete protease inhibitor cocktail (CPIC, Roche Applied Science). Through the addition of 1 mg/mL lysozyme and sonication (6 times 20 seconds) cells were lysed. The lysate was drawn through a blunt 1.2 mm needle and was clarified by centrifugation at 13000 $\times g$ at 4°C for 25 minutes. Recombinant σ^B_{6xHis} was purified from the supernatant on TALON Superflow resin (GE healthcare) per manufacturer's instructions. Proteins were dialyzed and stored in buffer (pH=8.0) containing 50 mM NaH_2PO_4 , 300 mM NaCl and 12% glycerol. Protein concentrations were determined using a Bradford assay (Biorad). Two mL of σ^B_{6xHis} protein solution containing 2 mg/mL protein was sent to BioGenes GmbH (Berlin) for generation of a polyclonal rabbit-anti- σ^B antibody.

Affinity purification of the polyclonal anti- σ^B antibody

Affinity purification of the antibody was performed to increase specificity of σ^B detection. Approximately 350 μg of purified σ^B_{6xHis} protein was loaded onto an SDS-PAGE gel. After electrophoresis and transfer of proteins to a PVDF membrane using standard blotting procedures, purified σ^B_{6xHis} protein was visualized by Ponceau S staining and the membrane containing the protein was cut as small as possible whilst retaining the region with the protein. The membrane was destained and washed with TBST buffer (500 mM NaCl, 20 mM Tris-base, 0.05% v/v Tween-20, pH = 7.4) twice for 5 minutes at room temperature. The membrane

was then pre-eluted by soaking in acidic glycine solution (100 mM, pH = 2.5) for 5 minutes prior to washing with TBST twice for five minutes at room temperature. Subsequently the membrane was blocked in 5% non-fat milk powder solution (Campina Elk, dissolved in TBST buffer) for one hour at room temperature after again washing twice with TBST for 5 minutes. Serum containing anti- σ^B antibody was incubated on the membrane overnight at 4°C. After three 5-minute washes with TBST, the membrane was washed twice for 5 minutes in PBS. Affinity purified antibody was eluted from the membrane by adding acidic glycine solution and incubating for 10 minutes at room temperature. The pH of the eluate was adjusted to 7.0 through the addition of 1 M Tris-HCl (pH = 8.0). This step was repeated twice more and the eluates were pooled and centrifuged (1 min maximum speed) to remove precipitated protein and membrane particles. Bovine serum albumin (BSA) and sodium azide were added to the affinity purified anti- σ^B antibody to end concentrations of 1 mg/mL and 5 mM respectively, and the affinity purified antibody was stored at -80°C.

Characterization of the σ^B regulon

σ^B overproduction in *C. difficile*

Exponentially growing starter cultures of *C. difficile* strain IB58 and IB61 were diluted to an $OD_{600}=0.05$ in BHIY medium supplemented with 20 μ g/mL lincomycin and thiamphenicol (20 μ g/mL) where appropriate. Cells were grown until $OD_{600} \approx 0.3$ after which a 1-mL sample was taken for control by Western blotting and gene expression was induced with 100 ng/mL ATc for one hour. Subsequently, 1 mL of sample was taken for control by Western Blotting and 50 mL was collected by centrifugation and stored at -20°C until RNA extraction. Non-induced samples were treated and collected identically, except that no ATc was added at $OD_{600} \approx 0.3$. All samples were corrected for OD_{600} prior to analysis by Western Blot.

	Name	Relevant features	Source
Plasmids	pAP24	P _{tet} - <i>sluc</i> ^{opt} ; <i>catP</i>	(201)
	pCD22	P _{tcdA} ; <i>catP</i>	(259)
	pIB14	P _{T7} - <i>sigB</i> _{6xHis} ; <i>amp</i>	This study
	pIB21	P _{CD0872} - <i>sluc</i> ^{opt} ; <i>catP</i>	This study
	pIB27	P _{CD3412} - <i>sluc</i> ^{opt} ; <i>catP</i>	(232)
	pIB68	P _{CD0350} - <i>sluc</i> ^{opt} ; <i>catP</i>	(232)
	pIB69	P _{CD2963} - <i>sluc</i> ^{opt} ; <i>catP</i>	(232)
	pIB73	P _{CD3605} - <i>sluc</i> ^{opt} ; <i>catP</i>	This study
	pIB74	P _{CD3614} - <i>sluc</i> ^{opt} ; <i>catP</i>	(232)
	pMTL007C-E2_ sigB171s::intron_ermB	<i>sigB</i> -retargeted intron (<i>ermB</i>)	This study
	pWKS1750	P _{tet} - <i>sigB</i> ; <i>catP</i>	This study
Strains	630Δ <i>erm</i>	MLS-susceptible derivative of <i>Clostridioides difficile</i> strain 630	(190) or (260)
	AP34	630Δ <i>erm</i> pAP24; <i>thia</i> ^R	(201)
	DH5α	<i>Escherichia coli</i> F <i>endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20</i> φ80 <i>dlacZ</i> Δ <i>M15</i> Δ(<i>lacZYA-argF</i>) <i>U169 hsdR17</i> (r _K ⁻ m _K ⁺) λ ⁻	Laboratory stock
	IB14	Rosetta (DE3) pLysS pIB14; <i>amp</i> ^R , <i>chlor</i> ^R	This study
	IB18	630Δ <i>erm</i> <i>sigB</i> :: <i>CT</i> ; <i>erm</i> ^R / <i>linco</i> ^R	This study
	IB37	630Δ <i>erm</i> pIB27; <i>thia</i> ^R	(232)
	IB56	630Δ <i>erm</i> Δ <i>sigB</i>	(232)
	IB58	IB18 pWKS1750; <i>thia</i> ^R , <i>erm</i> ^R / <i>linco</i> ^R	This study
	IB61	IB18 pAP24; <i>thia</i> ^R , <i>erm</i> ^R / <i>linco</i> ^R	This study
	IB95	630Δ <i>erm</i> pIB68; <i>thia</i> ^R	(232)
	IB96	630Δ <i>erm</i> pIB69; <i>thia</i> ^R	(232)
	IB98	IB56 pIB27; <i>thia</i> ^R	(232)
	IB99	IB56 pIB68; <i>thia</i> ^R	(232)
	IB100	IB56 pIB69; <i>thia</i> ^R	(232)
	IB108	630Δ <i>erm</i> pIB74; <i>thia</i> ^R	(232)
	IB111	IB56 pIB74; <i>thia</i> ^R	(232)
	JC096	630Δ <i>erm</i> pWKS1750; <i>thia</i> ^R	This study

Table 4. Plasmids and strains used in this study.

RNA extraction

Bacterial RNA was extracted and analyzed as previously described (261). Briefly, cell pellets were lysed for 30 mins at room temperature in enzymatic lysis buffer consisting of 15 mg/mL lysozyme and TE buffer. Further disruption of cells was performed by vigorous mechanical lysis for 3 minutes in RLT buffer to which one spatula of glass beads was added. After samples were centrifuged (3 minutes at 10.000 rpm at 4°C) and 100% ethanol was added to the supernatant, RNA was purified using the Qiagen RNeasy Kit protocol according to manufacturer's instructions.

DNA contamination was removed by using RNase-free DNase I (Qiagen) twice prior to elution of the RNA samples in H₂O. RNA quality and integrity numbers (RINs) were assessed with a Bioanalyzer 2100 (Agilent) and RNA 6000 Nano Reagents (Agilent). Only samples with an RIN of ≥ 7 were used for further analysis.

DNA microarray and data analysis

A customized whole-genome DNA microarray of 630 Δ erm was used (8x15K format, Agilent) (261). Quadruplicate samples were analyzed for the DNA microarray. Using the ULS fluorescent labelling kit for Agilent arrays (Kreatech), 1 μ g of total RNA was used for labelling with either Cy3 (P_{tet}-sigB) or Cy5 (P_{tet}-sluc^{opt}). After pooling and fragmentation, 300 ng of labelled RNA per sample was hybridized according to the two-color microarray protocol from Agilent. DNA microarrays were scanned with an Agilent C scanner and analyzed as described (261). A gene was considered differentially expressed if the log₂ foldchange (log₂ FC) was ≤ -1.5 or ≥ 1.5 and the p-value was < 0.05 . Results were visualized in VolcanoseR (239), and are available as an interactive graph via the URL contained in **Text S1**. The data used in the visualization has been deposited at Zenodo for this purpose (doi: 10.5281/zenodo.3945936). Full transcriptome data has been deposited in the GEO database, and can be accessed through the identifier GSE152515.

In vitro transcription

DNA oligonucleotides oWKS-1506 and oWKS-15136 (64- and 82 bp, respectively) were end-labelled with γ -³²P-ATP using T4 Polynucleotide Kinase (PNK, Invitrogen) and used as a size indicator for the *in vitro* transcription reactions. For the end labelling reaction, 1 μ l γ -³²P-ATP was incubated together with 200 pmol oligonucleotide and 1 μ l (10 U) PNK in Forward Reaction buffer (70 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 100 mM KCl, 1 mM 2-mercaptoethanol) at 37°C for 30 minutes. For the *in vitro* run-off transcriptions sigma factors and RNA polymerase core enzyme were preincubated with PCR amplified promoter areas (for P_{CD0350'}, P_{CD0872'}, P_{CD2963'}, P_{CD3412}, P_{CD3605} and P_{CD3614}) or *Xba*I linearized pCD22 (P_{tcdA}) for 30 minutes at 37°C prior to start of the reaction. PCR products of the promoter areas as used for the *in vitro* transcription reactions were loaded on and excised from agarose gels and purified using a NucleoSpin™ Gel and PCR Clean-up kit (Macherey-Nagel). *In vitro* transcription reactions mixtures contained 1 μ l (1 U) *E. coli* RNAP^{core} (NEB M0550S), 16 pmol sigma factor, 0.5 pmol DNA, 10 mM NTP mix and 0.3 μ l α -³²P-ATP in reaction buffer (40mM Tris-HCl, 150 mM KCl, 10 mM MgCl₂, 1 mM DTT, 0.01% triton X-100, pH=7.5) and were incubated for 15 minutes at 37°C. Transcripts and

labelled oligonucleotides to be used as a size indication were purified using P-30 Bio-Gel spin columns (Biorad). All reactions were stopped in gel loading buffer II (Invitrogen) containing 95% Formamide, 18 mM EDTA and 0.025% each of SDS, xylene cyanol, and bromophenol blue at 95°C for 5 minutes and loaded on 8% monomeric UreaGel (SequaGel, National Diagnostics). Gels were dried and exposed to PhosphorImager screens overnight (approximately 17 hours) and imaged with a Typhoon 9410 scanner (GE Healthcare).

Spot assay for viability upon σ^B overproduction in *C. difficile* and vector stability assay

C. difficile overnight pre-cultures were corrected for OD₆₀₀ and subsequently tenfold serially diluted in BHI medium. Two- μ l spots of each dilution were plated on selective (20 μ g/mL thiamphenicol) and unselective square (90x15 mm, VWR international) BHI plates with or without 200 ng/mL anhydrotetracycline (ATc). Growth was evaluated after 24 hours and subsequently swabs were taken from all strains grown on unselective BHI agar plates with- and without 200 ng/mL ATc for the vector stability assay. These swabs used for the vector stability assay were resuspended in PBS to a turbidity of 1.0 McFarland, adjusted for their OD₆₀₀-values and tenfold serially diluted in non-selective BHI medium. Of these serially diluted suspensions, 10- μ l spots of each dilution were then plated on selective (20 μ g/mL thiamphenicol + CDSS) and non-selective (BHI + CDSS) plates and CFU/mL was counted after 24- 48 hours of growth. The percentage of cells retaining the plasmid was calculated as $[\text{CFU/mL}]_{\text{selective}} / [\text{CFU/mL}]_{\text{non-selective}} \times 100\%$. If no growth was detected on selective plates containing thiamphenicol the percentage of plasmid maintained was set as 0%. To calculate statistical significance between % plasmid maintained in strains induced or not induced by ATc, an unpaired student's t-test was used.

Plate based luciferase assay with metronidazole E-test and disk diffusion

Strains harboring luciferase-reporter plasmids were grown on prereduced, selective BHI plates for 24 hours. Subsequently bacterial suspensions corresponding to 1.0 McFarland turbidity were applied on BHI agar supplemented with 0.5% yeast-extract after which a metronidazole E-test or plain disks were applied. Disks were spotted with 10 μ l each of sterile H₂O, 1 M H₂O₂, 3000 μ g/mL lincomycin, 200 μ g/mL metronidazole, 400 μ g/mL ibezapolstat and 200 μ g/mL vancomycin. After 24 hours of growth luciferase activity was visualized by spraying 1:100 reconstituted NanoGlo Luciferase substrate (Promega N1110) on the agar plate using a disposable spray flask. One spray corresponded to approx. 250 μ l reconstituted NanoGlo Luciferase substrate. Luminescence was recorded using a Uvitec Alliance Q9 Advanced imager

(BioSPX) after 10 sec exposure time per plate. Luciferase were conjugated into a *sigB* knockout made by Allelic Coupled Exchange (202) whereas a ClosTron mutant background was used for the DNA arrays. However, no differences between these backgrounds have ever been observed in our assays.

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4

PLASMID-MEDIATED METRONIDAZOLE RESISTANCE IN *CLOSTRIDIoidES* *DIFFICILE*

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Abstract

Metronidazole was until recently used as a first line treatment for potentially life-threatening *Clostridioides difficile* (CD) infection. Although cases of metronidazole resistance have been documented, no clear mechanism for metronidazole resistance or a role for plasmids in antimicrobial resistance has been described for CD. Here, we report genome sequences of seven susceptible and sixteen resistant CD isolates from human and animal sources, including isolates from a patient with recurrent CD infection by a PCR ribotype (RT) 020 strain, which developed resistance to metronidazole over the course of treatment (Minimal Inhibitory Concentration [MIC] = 8 mg/L). Metronidazole resistance correlates with the presence of a 7-kb plasmid, pCD-METRO. pCD-METRO is present in toxigenic and non-toxigenic resistant (n=23), but not susceptible (n=563) isolates from multiple countries. Introduction of a pCD-METRO-derived vector into a susceptible strain increases the MIC 25-fold. Our finding of plasmid-mediated resistance can impact diagnostics and treatment of CD infections.

Introduction

Clostridioides difficile (*Clostridium difficile*) is a Gram-positive obligate anaerobe capable of causing *Clostridioides difficile* Infection (CDI) upon disruption of the normal intestinal microbiota (225). Although it is one of the major causes of nosocomial infectious diarrhea, community-acquired CDI is becoming more frequent (62, 262). CDI infection poses a significant economic burden with an estimated cost at €3 billion per year in the European Union and impairs the quality of life in infected individuals (45, 53). The incidence of CDI has increased over the last two decades with outbreaks caused by epidemic types such as PCR ribotype (RT) 027 (NAP1/BI)(44). CDI is not restricted to this type, however, as infections caused by RT001, RT002, RT014/020 and RT078 are frequently reported in both Europe and the United States (20, 52). Metronidazole is used for the treatment of mild-to-moderate infections and vancomycin for severe infections, though vancomycin is increasingly indicated as a general first-line treatment (80, 263-269). Fidaxomicin has recently also been approved for CDI treatment, but its use is limited by high costs (80). Fecal Microbiota Transplantation (FMT) is effective at treating recurrent CDI (rCDI) that is refractory to antimicrobial therapy (15). Reduced susceptibility and resistance to clinically used antimicrobials, including metronidazole, has been reported and this, combined with the intrinsic multiple drug-resistant nature of *C. difficile*, stresses the importance for the development of better diagnostics and new effective treatment modalities (20).

Routine antimicrobial susceptibility testing is generally not performed for *C. difficile* and consequently, reports of resistance to metronidazole are rare (51, 270, 271). Longitudinal surveillance in Europe found that 0.2% of clinical isolates investigated were resistant to metronidazole (271), but reported rates from other studies vary from 0-18.3% (90, 272-274). These differences may reflect geographic distributions in resistant strains, or differences in testing methodology and breakpoints used (178, 275). Moreover, metronidazole resistance can be unstable, inducible and heterogeneous (124). Finally, metronidazole resistance appears to be more frequent in non-toxigenic strains such as those belonging to RT010, which have a 7-9 fold increase in Minimal Inhibitory Concentration (MIC) values compared to RT001, RT027 and RT078 (90, 275).

Metronidazole is a 5-nitroimidazole prodrug that upon intracellular reductive activation induces cellular damage through nitro-radicals (124). It is not only used in the treatment of CDI, but also an important drug for treating parasitic infections and as prophylactic antimicrobial in for instance abdominal surgery (120, 124). Mechanisms associated with metronidazole resistance described in other organisms include the presence of 5-nitroimidazole reductases (*nim* genes), altered pyruvate-ferredoxin oxidoreductase (PFOR) activity and adaptations to (oxidative) stress (124). The knowledge on resistance mechanisms in *C. difficile* is very limited, but may involve modulation of core metabolic and stress pathways as well (140, 141). Of note, levels of metronidazole achieved in the colon are generally low and this could be relevant for the selection of resistant strains (276).

Here, we present a case of a patient with rCDI due to an initially metronidazole susceptible (MTZ^S) RT020 strain which developed resistance to metronidazole over time. We analyze the genome sequences of these toxigenic MTZ^S and metronidazole resistant (MTZ^R) strains, together with 5 MTZ^S and 11 MTZ^R non-toxigenic RT010 strains. We identify pCD-METRO, a 7-kb plasmid conferring metronidazole resistance. This plasmid is internationally disseminated and also occurs in epidemic types. We thus report a clinically relevant phenotype associated with plasmid-carriage in *C. difficile*.

Results

In-patient development of a metronidazole resistant strain

A 54 year old kidney-pancreas transplant patient with a medical history of Type I diabetes mellitus, vascular disease and a double lower leg amputation was on hemodialysis when developing diarrhea. The patient was subsequently diagnosed with CDI and a toxigenic metronidazole sensitive (MIC=0.25 mg/L) RT020 strain was isolated from the fecal material of the patient. Treatment with metronidazole was started, leading to initial resolution of the symptoms (**Figure 1**). Two more episodes of CDI occurred during which the patient was treated primarily with vancomycin (but also metronidazole) prior to an FMT provided by the Netherlands Donor Feces Bank. At the start of the second episode a MTZ^S RT020 strain was once more isolated.

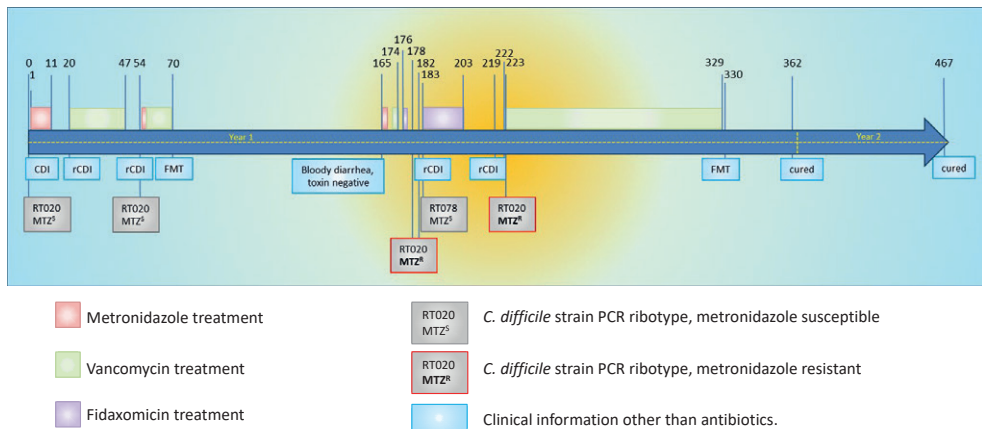


Figure 1. Timeline of the course of the antibiotic treatment and rCDI in the patient. Dates and timepoints on which treatment was initiated or stopped and *C. difficile* isolates were recovered are indicated above the timeline. (r)CDI was diagnosed when both a toxin enzyme-immune assay and nucleic acid amplification test were positive for *C. difficile* in combination with a physician's assessment of symptoms consistent with CDI. Yellow highlighting indicates the time where pCD-METRO positive MTZ^R *C. difficile* was isolated. Source data are provided as a Source Data file.

Three months after the first FMT, the patient once again developed bloody diarrhea and two more episodes of rCDI were diagnosed which were treated with a vancomycin and a fidaxomicin regime. At two instances, RT020 strains were again isolated from the fecal material of the patient. Strikingly, these two clinical isolates were now phenotypically resistant to metronidazole

(MIC=8 mg/L as determined by agar dilution). Ultimately the patient was cured by a second FMT. We hypothesized that the rCDI episodes were due to clonal RT020 strains that persisted despite antimicrobial therapy and an FMT. Clonal MTZ^S and MTZ^R strains would allow us to determine the underlying genetic changes that resulted in metronidazole resistance. To determine the relatedness between these RT020 isolates whole genome sequencing (WGS) was performed (**Table 1**). We also included 2 more MTZ^R RT020 strains and a non-related RT078 strain isolated from the same patient and 4 MTZ^S and 8 MTZ^R RT010 strains from our laboratory collection (**Supplemental Dataset 1**) to perform single nucleotide polymorphism (SNP) analyses. Strains were considered resistant to metronidazole with MIC values >2 mg/L according to the EUCAST epidemiological cut-off value (87). All strains resistant to metronidazole (n=12) showed cross-resistance to the nitroimidazole drug tinidazole.

Assembly of the MTZ^R RT020 strain IB136 (**Supplemental Dataset 2**) resulted in a genome of 4166362 bp with 57 contigs, and an average G+C-content of 28.5% (N50= 263391 bp, mapping rate 98.97%). A BLAST comparison between this genome and the NCBI nt database showed that the genome is closest to the genome of strain LEM1 (277). As expected, 5/6 strains isolated from the patient (all RT020) showed 100% identity over the majority of all contigs, suggesting they are highly similar. All RT020 strains were found to be of multi-locus sequence type (ST) 2, consistent with data from others (278). The sixth strain (IB137), was a clear outlier and was identified as being closest to the RT078 reference strain M120 (279). This is consistent with another ribotype (RT078) and sequence type (ST11) assignment. All RT010 strains belonged to ST15.

Resistance does not correlate with a SNP

Previous studies analyzing the mechanism behind metronidazole resistance in *C. difficile* only studied a single isolate each (138, 141). We performed a core genome SNP analysis on selected strains (n=18; **Table 1**), comparing MTZ^S (n=6) and MTZ^R (n=12) strains within and between the different PCR ribotypes (RT010, RT020 and RT078).

The evolutionary rate of *C. difficile* has been estimated at 0-2 SNPs per genome per year but might vary based on intrinsic (strain type) and extrinsic (selective pressure) factors (280). Our analysis identified a single SNP in the MTZ^R RT020 (IB136), compared to the MTZ^S RT020 strains derived from the same patient, conclusively demonstrating that these strains are clonal.

Considering the time of isolation of the susceptible and resistant isolates, this implies the MTZ^S RT020 strain most likely acquired metronidazole resistance. In contrast, between the MTZ^S and MTZ^R RT010 isolates (which come from diverse human and animal sources) 457 SNPs were detected. Moreover, RT010 and RT020 were separated by >25000 SNPs.

The SNP identified in the RT020 strains discriminating the MTZ^S from the MTZ^R isolates is located in a conserved putative cobalt transporter (CbiN, IPR003705). However, the SNP is not observed in the MTZ^R RT010 strains. Thus, metronidazole resistance is either multifactorial or not contained within the core genome. We did not investigate the contribution of this SNP to metronidazole resistance further.

MTZ^R *C. difficile* strains contain a 7-kb plasmid

Next, we investigated extrachromosomal elements (ECEs), which can include plasmids. Although plasmids containing antimicrobial resistance determinants have been described in Gram-positive bacteria, they appear to be more common in Gram-negatives (281). Plasmids in *C. difficile* are known to exist, but no phenotypic consequences of plasmid carriage have been described to date (282). The investigation of the pan-genome of all sequenced strains, including a prediction of ECEs predicted by an in-house pipeline similar to PLACNET (283, 284), showed a single contig that was present in all MTZ^R strains (4.6%- 19.27% of reads mapped, with a minimum of 479497), but absent from MTZ^S strains, of both RT010 and RT020 (0% of reads mapped with a maximum 327 reads). Circularization based on terminal repeats yielded a putative plasmid of 7056bp with a G+C-content of 41.6% (**Figure 2A**). Correct assembly was confirmed by PCR (**Figure 2B**) and Sanger sequencing.

To confirm the circular nature of the contig, total DNA isolated from the MTZ^R RT010 strain IB138 before and after PlasmidSafe DNase (PSD, Epicentre) (282) treatment was analyzed by PCR using primers specific for chromosomal DNA (*gluD*) and the putative plasmid (**Figure 2C**). A positive signal for *gluD* was only observed in samples that had not been treated with PSD, demonstrating that PSD treatment degrades chromosomal DNA to below the detection limit of the PCR. By contrast, a signal specific for the putative plasmid was visible both before and after PSD treatment. Consequently, we conclude that our whole genome sequence identified a legitimate 7-kb plasmid.

A total of 8 open reading frames (ORFs) were annotated on the plasmid (**Figure 2A**). ORF1-5 encode a hypothetical protein (ORF1), a MobC-like relaxase/Arc-type ribbon-helix-helix (ORF2; PF05713), a MobA/VirD2 family endonuclease relaxase protein (ORF3; PF03432), a hypothetical protein with a MutS2 signature (ORF4), and a predicted replication protein (ORF5), respectively. ORF6 is a small ORF that is likely a pseudogene, and the remaining ORFs encode a metallohydrolase/oxidoreductase protein (ORF7; IPR001279) and a Tn5-like transposase gene (ORF8; PF13701). Intriguingly, ORF6 showed homology on the protein level to the 5-nitroimidazole reductase (*nim*) gene *nimB* (33% identity, 54% positives over 61 amino acids) described in *Bacteroides fragilis* (CAA50578.1) and found in both metronidazole resistant- and susceptible isolates of anaerobic Gram-positive cocci (136, 285). The ORF lacks the region encoding the N-terminal part of the Nim protein, and the Phyre2-predicted protein structure shows it lacks the catalytic site residues. Of note, the plasmid sequences from all strains are highly similar. Compared to the plasmid of strain IB136, only strains IB143, IB144 and IB145 contained a single SNP resulting in a Y286S mutation within the Tn5-like transposase ORF (**Supplemental Figure 1**).

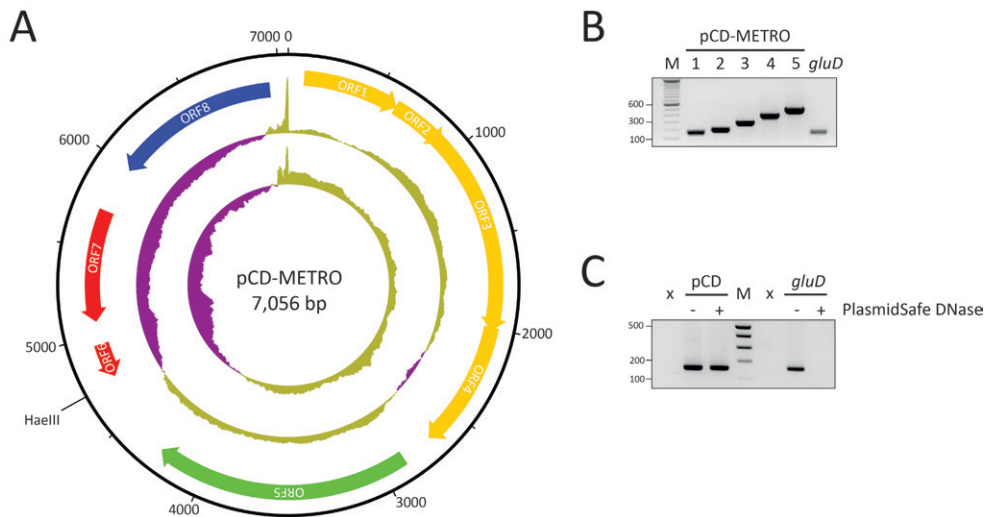


Figure 2. pCD-METRO is a 7-kb plasmid. **A)** Structure of plasmid pCD-METRO and its ORFs. The two innermost circles represent GC content (outer circle) and GC skew (innermost circle) (both step size 5nt and window size 500nt; , above average in yellow, below average in purple). The unique *Hae*III site used to construct pCD-METRO^{shuttle} (see methods) is indicated. **B)** Gene specific PCR products amplifying regions of ORFs 6 (lane 1+2), ORF5 (lane 3), ORF7 (lane 4) and ORF3 (lane 5), and a chromosomal locus (*gluD*) (lane 6). **C)** The product of plasmid specific amplification (targeting ORF6) or chromosomal specific amplification (*gluD*) before and after PlasmidSafe DNase treatment. Source data are provided as a Source Data file.

Together, these results show that all of the MTZ^R strains, but none of the MTZ^S strains, sequenced in this study contain a plasmid, hereafter referred to as pCD-METRO (for **p**lasmid from **C.** *difficile* associated with **metronidazole** resistance).

pCD-METRO is found in strains from different countries

Two clinical isolates with stable metronidazole resistance have been described and we evaluated the presence of pCD-METRO in the assembled genome sequences from these strains using BLAST (138, 141). We failed to identify pCD-METRO in the draft genome of a toxigenic NAP1 isolate that acquired stable metronidazole resistance through serial passaging under selection (138). We did identify pCD-METRO (fragmented over multiple contigs) in the draft genome a non-toxigenic Spanish RT010 strain with stable metronidazole resistance (strain 7032989), whereas neither the reduced-susceptible strain nor the susceptible strain from the same study contained the plasmid (141). We confirmed these results using PCR, as described for strain IB138 (**Figure 3; lanes SP**), demonstrating pCD-METRO is indeed present in strain 7032989. These data show that the presence of pCD-METRO may explain at least part of the cases of metronidazole resistance described in literature. We did not detect pCD-METRO in the sequence read archive in entries labelled as *C. difficile*, or otherwise.

Our observations above raise the question how prevalent pCD-METRO is in MTZ^R *C. difficile* isolates and if there is a bias towards specific types or geographic origins. As metronidazole resistance in *C. difficile* is rare, we expanded our collection of clinical isolates through our network (including the ECDC) (n=76) and with selected strains from the Tolevamer (n=42) and MODIFY (n=46) clinical trials (111, 174, 286). To correct for interlaboratory differences in typing and antimicrobial susceptibility testing, all strains were retyped by ribotyping and tested for metronidazole resistance using agar dilution according to Clinical & Laboratory Standards Institute (CLSI) guidelines in our laboratory with inclusion of appropriate control strains (13, 86). Although these strains, with the exception of the Tolevamer strains, were characterized as having altered metronidazole susceptibility by the senders (n=122), agar dilution performed in our own laboratory classified nearly all of these strains as metronidazole susceptible (MIC <2 mg/L). We expected pCD-METRO to be present in MTZ^R strains, but not in MTZ^S strains.

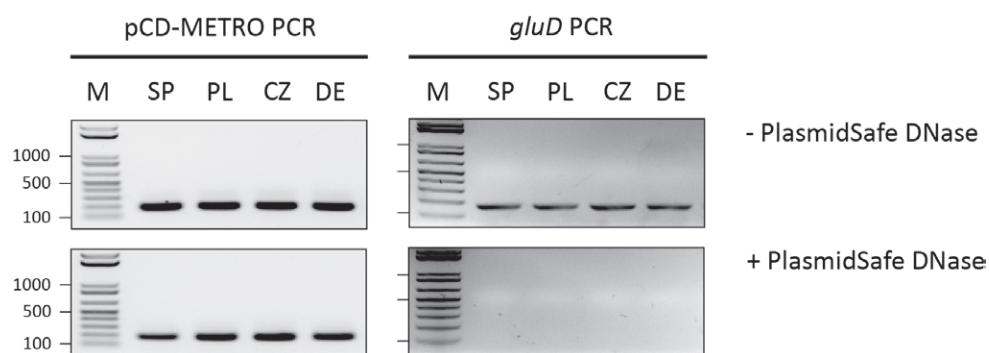


Figure 3. pCD-METRO is internationally disseminated. PCR analysis of strains 7032989 (RT010, Spain) (SP), LUMCMM190960 (RT027, Poland) (PL), LUMCMM19 0880 (RT010, Czech Republic) (CZ) and P016134 (RT010, Germany) (DE). The product of plasmid specific amplification (targeting ORF8) or chromosomal specific amplification (*gluD*) before and after PlasmidSafe DNase treatment are shown. Source data are provided as a Source Data file.

We identified three additional metronidazole resistant strains: a RT027 isolate from Poland (LUMCMM19 0960; MIC>8 mg/L), a RT010 isolate from the Czech Republic (LUMCMM19 0880; MIC>8 mg/L) and a RT010 isolate from Germany (P016134; MIC>8 mg/L) (**Table 1**). A PCR on PSD-treated chromosomal DNA isolated from these strains yielded a positive signal using primers targeting the plasmid, but not the chromosome (**Figure 3, lanes PL/CZ/DE**), demonstrating all three strains contain pCD-METRO. WGS showed that pCD-METRO in strain LUMCMM19 0960 was identical to that of strain IB136, whereas LUMCMM19 0880 contained a single SNP resulting in a D131N substitution in ORF1. We also screened our laboratory collection of RT010 strains from human and animal sources and identified 7 more MTZ^R strains (as determined by both agar dilution and epsilometer tests [E-test]), 6 of which were positive for pCD-METRO (86%; **Supplemental Dataset 1**). A single RT010 strain (LUMCMM19 0830) tested MTZ^R resistant in agar dilution according to CLSI guidelines (MIC=4 mg/L) (86), but this strain was negative for pCD-METRO in both PCR and WGS. Using E-tests, we found this strain to be susceptible to metronidazole (MIC=0.19 mg/L) when grown on standard laboratory Brain-Heart Infusion (BHI) agar but resistant (MIC=16 mg/L) on Brucella Blood Agar (BBA), suggesting a contribution of medium components (possibly haem) to the resistance phenotype (**Figure 4**). On both media RT010 control strains IB138 and IB140 are resistant and susceptible, respectively (**Figure 4**), with 2-4-fold differences in MIC between the medium conditions. Thus, all pCD-METRO containing strains in this study show medium-independent metronidazole resistance with a MIC₂ ≥ 8 mg/L in agar dilution (22/22). By contrast, all susceptible isolates (n=563) lacked pCD-METRO.

Taken together, our results show that pCD-METRO is internationally disseminated and can explain metronidazole resistance in both non-toxigenic- and toxigenic isolates of *C. difficile*, including those belonging to epidemic ribotypes such as RT027.

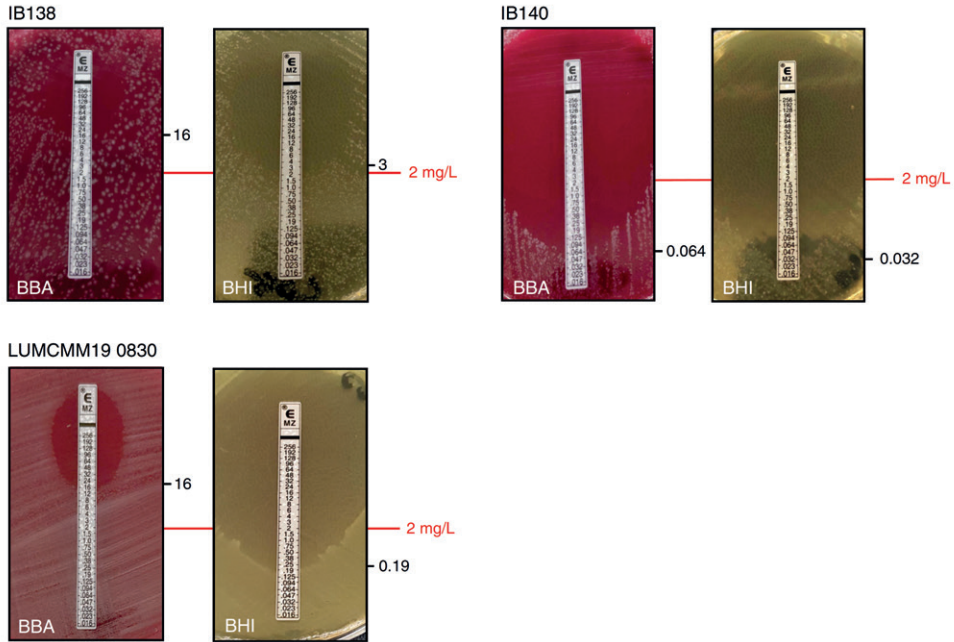


Figure 4. Medium-dependent metronidazole resistance. Strains were grown as described under Antimicrobial Susceptibility Testing in the Methods section and spread onto either Brucella Blood Agar (BBA) plates, or onto BHI/CDSS agar plates (BHI). E-tests were placed, and plates were incubated for 48h before imaging. 2 mg/L is the EUCAST epidemiological cut-off for metronidazole that was used to define resistance in this study (87). E-test values for the indicated strains are shown next to their respective panels. The images represent 3 independent repeats with a single replicate per condition. Source data are provided as a Source Data file.

pCD-METRO is likely acquired via horizontal gene transfer

Our whole genome sequence analysis suggested the acquisition of pCD-METRO by a toxigenic RT020 strain during treatment of rCDI. We made use of longitudinal fecal samples that were stored during treatment to investigate the presence of pCD-METRO in total fecal DNA at various timepoints. Total DNA derived from the fecal sample harboring the MTZ^s RT020 was positive for the presence of pCD-METRO (**Figure 5**). This indicates that pCD-METRO was present in the gut reservoir of the patient. Post-FMT, pCD-METRO was no longer detected in total fecal DNA, suggesting that the fecal transplant reduced levels of pCD-METRO containing *C. difficile* and/or the donor organism to below the limit of detection of the assay. Fecal samples were

stored in the absence of cryoprotectant and as a result we were unable to reculture the possible donor organism.

Though we cannot exclude the possibility that the MTZ^R RT020 strain was already present at the moment the MTZ^S RT020 strain was isolated, our results indicate that pCD-METRO was most likely acquired through horizontal gene transfer between the MTZ^S *C. difficile* strain and an as-of-yet uncharacterized donor organism in the gut of the patient.

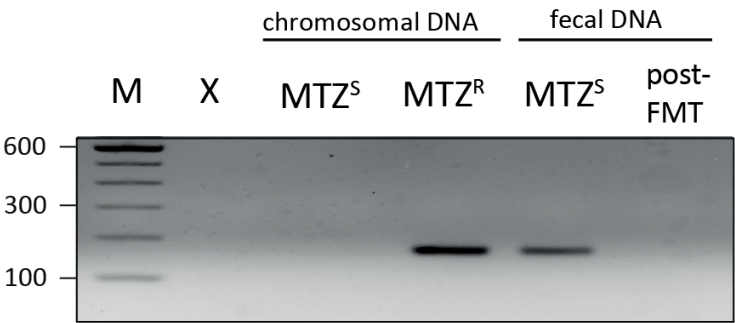


Figure 5. pCD-METRO is detectable in fecal total DNA. pCD-METRO is detectable in fecal total DNA from the same sample from which a MTZ^S RT020 *C. difficile* was isolated. Shown are the results from a PCR targeting ORF6, but similar results were obtained for other plasmid-specific primer sets. Source data are provided as a Source Data file.

PCR-based identification of metronidazole resistant strains

We implemented a PCR targeting pCD-METRO in our routine surveillance and *ad hoc* typing, as part of the Dutch National Reference Laboratory (NRL) for *C. difficile*. In the period February–August 2019, we characterized 721 strains by ribotyping, and identified a single pCD-METRO positive strain (LUMCMM19 0348) by PCR. These preliminary data suggest a prevalence of <0.14% in an endemic setting in the Netherlands. The identified strain belonged to RT020 and was confirmed to be MTZ^R in an E-test on BBA (**Supplemental Figure 2**). As described for the patient case above, we were able to identify an earlier RT020 isolate from the same patient (LUMCMM18 0002) that was pCD-METRO negative and MTZ^S (**Supplemental Figure 2**). WGS revealed that the susceptible and resistant strains were identical (0 SNPs difference), but differed as expected in carriage of pCD-METRO. pCD-METRO in the MTZ^R isolate contained two SNPs compared to the plasmid of strain IB136; a G>A conversion located intergenically between ORF6 and ORF7, and a mutation resulting in a V13A mutation in ORF8 (**Supplemental Figure 1**). Our data suggests that selection by metronidazole is crucial in the acquisition of, or selection for, pCD-METRO containing *C. difficile*.

pCD-METRO confers metronidazole resistance in C. difficile

Above, we have clearly established a correlation between the presence of pCD-METRO and metronidazole resistance. Next, we sought to unambiguously demonstrate that acquisition of pCD-METRO, and not any secondary events, lead to metronidazole resistance. To generate isogenic strains with or without pCD-METRO, we introduced a shuttle module in the unique HaeIII restriction site of the plasmid and introduced the resulting vector, pCD-METRO^{shuttle} (pIB86; **Supplemental Figure 3**), into the RT012 laboratory strain 630 Δ erm using standard methods(190). Metronidazole E-tests showed a reproducible 15-to-20-fold increase in the MIC from 0.064/0.19 mg/L for the strain without pCD-METRO^{shuttle} to 2-4 mg/L for the strain with pCD-METRO^{shuttle} (**Figure 6**). These results were confirmed using agar dilution, that showed a >24-fold increase (>5 doubling dilutions) from 0.125-0.25 mg/L to 8 mg/L or higher upon introduction of pCD-METRO^{shuttle} (**Table 1**).

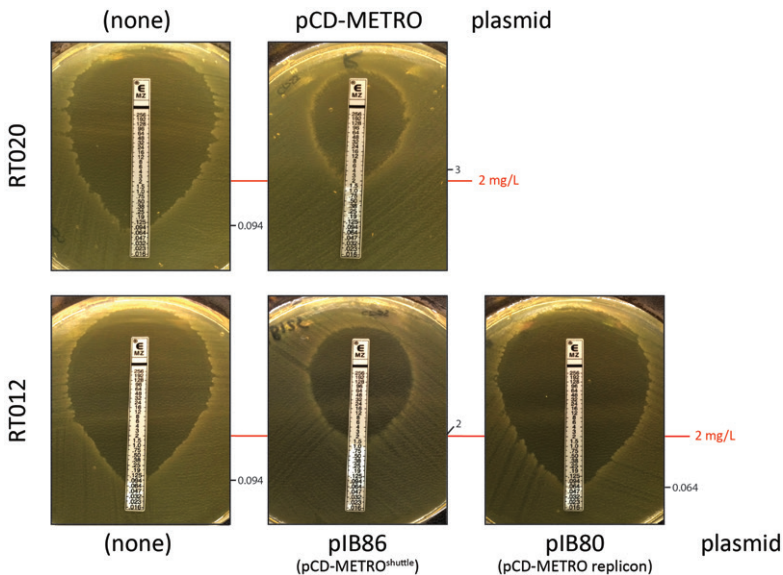


Figure 6. pCD-METRO confers metronidazole resistance. RT020 without plasmid (MTZ^S, strain IB132), RT020 with pCD-METRO (MTZ^R, strain IB133), RT012 without plasmid (MTZ^S, strain 630 Δ erm), RT012 with pIB86 (pCD-METRO^{shuttle}, MTZ^R, strain IB125), RT012 with pIB80 (MTZ^S, IB90; pIB80 contains the pCD-METRO replicon but lacks the other ORFs of pCD-METRO). IB90 and IB125 are 630 Δ erm-derivatives (190). E-tests were performed on BHI agar plates with CDSS. Identical results were obtained on plates without CDSS. The images represent 3 independent repeats with a single replicate per condition. 2 mg/L indicates the EUCAST epidemiological cut-off for metronidazole that was used to define resistance in this study (87). E-test values for the indicated strains are shown next to their respective panels. Source data are provided as a Source Data file.

Name	Characteristics	PCR ribotype*	Toxin profile**	MTZ resistance***	Source	Reference
630Δerm	wild type	012	A+ B+ CDT-	0.125 (S)	Laboratory	(190)
IB125	630Δerm pIB86 (pCD-METRO ^{shuttle}); Thia ^R	012	A+ B+ CDT-	≥8 (R)	Laboratory	This study
IB132	pCD-METRO-	020	A+ B+ CDT-	0.25 (S)	Human	This study
IB133	pCD-METRO +	020	A+ B+ CDT-	8 (R)	Human	This study
IB134	pCD-METRO +	020	A+ B+ CDT-	8 (R)	Human	This study
IB135	pCD-METRO +	020	A+ B+ CDT-	8 (R)	Human	This study
IB136	pCD-METRO +	020	A+ B+ CDT-	8 (R)	Human	This study
IB137	pCD-METRO-	078	A+ B+ CDT+	0.125 (S)	Human	This study
IB138	pCD-METRO +	010	A- B- CDT-	>8 (R)	Human	This study
IB139	pCD-METRO-	010	A- B- CDT-	1 (S)	Human	This study
IB140	pCD-METRO-	010	A- B- CDT-	0.25 (S)	Human	This study
IB141	pCD-METRO-	010	A- B- CDT-	0.125 (S)	Human	This study
IB142	pCD-METRO-	010	A- B- CDT-	0.125 (S)	Human	This study
IB143	pCD-METRO +	010	A- B- CDT-	>8 (R)	Animal	This study
IB144	pCD-METRO +	010	A- B- CDT-	>8 (R)	Animal	This study
IB145	pCD-METRO +	010	A- B- CDT-	>8 (R)	Animal	This study
IB146	pCD-METRO +	010	A- B- CDT-	>8 (R)	Animal	This study
IB147	pCD-METRO +	010	A- B- CDT-	>8 (R)	Animal	This study
IB148	pCD-METRO +	010	A- B- CDT-	>8 (R)	Animal	This study
IB149	pCD-METRO +	010	A- B- CDT-	>8 (R)	Animal	This study
IB151 (P016134)	pCD-METRO +	010	A- B- CDT-	>8 (R)	Unknown	(287)
IB30	630Δerm pIB20 (pCD6 replicon, P _{CD0716} - <i>sluc^{opt}</i>); Thia ^R	012	A+ B+ CDT-	0.25 (S)	Laboratory	This study
IB90	630Δerm pIB80 (pCD-METRO replicon, P _{tet} - <i>gusA</i>); Thia ^R	012	A+ B+ CDT-	0.125 (S)	Laboratory	This study
LUMCMM18 0002	pCD-METRO-	020	A+ B+ CDT-	ND (S)	Human	This study
LUMCMM19 0348	pCD-METRO +	020	A+ B+ CDT-	ND (R)	Human	This study
LUMCMM19 0830	pCD-METRO-	010	A- B- CDT-	4 (R)	Unknown	This study
LUMCMM19 0880	pCD-METRO +	010	A- B- CDT-	>8 (R)	Unknown	This study
LUMCMM19 0970 (7032989)	pCD-METRO +	010	A- B- CDT-	>8 (R)	Unknown	(141)
LUMCMM190960	pCD-METRO +	027	A+ B+ CDT+	>8 (R)	Human	(111)

Table 1. Strains described in this study. Listed are strains mentioned in the main body of the manuscript. For a complete overview of all strains used, see Supplemental Dataset 1. * PCR ribotype determined at the LUMC standard PCR ribotyping ** toxin profile determined by multiplex PCR. *** Metronidazole MIC values in mg/L as determined by agar dilution conform CLSI guidelines. S = susceptible (MIC<2 mg/L), R = resistant (MIC>2 mg/L) based on the EUCAST epidemiological cut-off for metronidazole (87). ND= not determined by agar dilution, but only by E-test (Supplemental Figure 2).

As controls, we included the MTZ^S (IB132) and a MTZ^R (IB133) RT020 strain isolated from the patient. In agreement with the MIC values determined by agar dilution (MIC=0.25 mg/L and MIC=8 mg/L), these isolates showed a MIC corresponding to those observed for the MTZ^S and MTZ^R RT012 isolates, respectively (**Figure 6**).

Overall, our results show that acquisition of pCD-METRO is sufficient to raise the MIC of *C. difficile* to values greater than the epidemiological cut-off value defined by EUCAST(87).

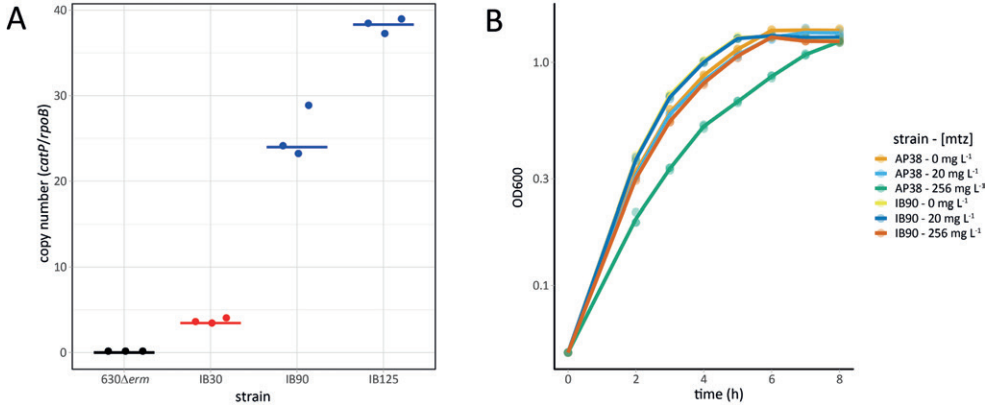


Figure 7. The pCD-METRO replicon sustains a high plasmid copy number. **A)** 630Δerm is the wild type RT012 laboratory strain. IB30: 630Δerm + pIB20 (contains pCD6 replicon); IB90: 630Δerm + pIB80 (contains pCD-METRO replicon); IB125: 630Δerm + pCD-METRO^{shuttle} (pIB86, contains pCD-METRO replicon). Copy number is determined as the ratio of a plasmid locus (*catP*) relative to a chromosomal locus (*rpoB*) as determined by qPCR on total DNA. Data from strains containing a plasmid with the pCD6-replicon are indicated in blue, data from strains containing a plasmid with the pCD-METRO replicon are indicated in red. Individual data points are plotted using symbols. Horizontal lines indicate median values. 630Δerm vs IB30: $P=0.1018$; IB90 vs IB125: $P=0.0002$; other comparisons: $P<0.0001$ as determined by two-sided ANOVA and Tukey's test. Experiments were performed in triplicate on three different technical replicates. **B)** A strain derived from the laboratory RT012 strain 630Δerm harboring a plasmid containing the pCD-METRO replicon (IB90) has a growth advantage over a strain containing a plasmid with the pCD6 replicon (AP38) when cultured at high levels of thiamphenicol (Tm). AP38: 630Δerm + pAP24 (pCD6 replicon); IB90: 630Δerm + pIB80 (pCD-METRO replicon). Growth is measured as an increase in optical density at 600nm (OD600). Solid line indicates the mean of the individual measurements that are shown using dots ($n=3$ biologically independent samples). Source data are provided as a Source Data file.

pCD-METRO contains a high copy number replicon

Read depth of pCD-METRO in our WGS data indicates an estimated copy number of 100-200, in stark contrast with the pCD6 replicon commonly used in shuttle vectors for *C. difficile* (copy number 4-10) (234). We wanted to establish the functionality of the predicted replicon and determine the copy number sustained by this replicon in RT012 strains.

A pRPF185-based vector (233) (pIB80) was constructed in which the conventional pCD6 replicon was replaced by a 2-kb DNA fragment of pCD-METRO that includes ORF5, encoding the putative replication protein (**Supplemental Figure 4**). Transconjugants containing this

vector were readily obtained in the RT012 laboratory strain 630 Δ *erm*, demonstrating this region contains a functional replicon.

Next, we compared the relative copy number of the plasmids in overnight cultures by qPCR (234). Based on the ratio of plasmid-locus *catP* to the chromosomal locus *rpoB*, the copy number of pCD6-replicon vector was approximately 4, concordant with results of others (234). By contrast, the copy number of vectors with the pCD-METRO replicon ranges from approximately 25 (for pIB80, in IB90) to 38 (pCD-METRO^{shuttle}, in IB125) (**Figure 7A**). We hypothesized that a higher plasmid copy number would also lead to more copies of the resistance marker on the plasmid and thus to a possible increase of resistance to the corresponding antibiotic. Indeed, a strain harboring a *catP*-containing plasmid with the pCD-METRO replicon demonstrates a growth advantage over a strain harboring a similar plasmid with the pCD6 replicon when exposed to high levels (256 mg/L) of thiamphenicol. No significant difference in growth was observed at low concentrations (20 mg/L) of thiamphenicol (**Figure 7B**). As pIB90 containing strains are not MTZ^R (**Figure 6**), resistance to metronidazole is not mediated by a higher copy number plasmid per se, but is dependent on a determinant specific to pCD-METRO.

A difference between the read-depth estimate and the qPCR can be explained by technical bias or differences in strain background. Nevertheless, our experiments clearly demonstrate that the pCD-METRO replicon sustains plasmid levels that are approximately 10-fold greater than that of currently used replicons.

We investigated whether the relatively high copy number of pCD-METRO imposes a metabolic cost, by evaluating the growth of strains with and without plasmid in the absence or presence of varying concentrations of metronidazole (**Figure 8**). In the absence of metronidazole, the growth of susceptible and resistant strains of both RT012 and RT020 is indistinguishable (**Figure 8A**). This was not due to loss of pCD-METRO from the resistant strain, as all colonies tested after the growth experiment had retained the plasmid. With increasing amounts of metronidazole, susceptible strains show a clear growth defect already at the lowest concentration of metronidazole tested (0.125 mg/L), whereas resistant strains do not markedly differ in growth from the control culture at concentrations below the epidemiological cut-off (2mg/L) (**Figure 8B and 8C**). These values are in agreement with the E-tests performed on the same media (**Figure 6**). We conclude that carriage of the plasmid does not affect growth rate

in the absence of metronidazole, despite the high copy number, and confers a clear growth advantage in the presence of metronidazole.

We attempted to cure metronidazole resistant strains of pCD-METRO using serial passaging on non-selective liquid or solid medium. Despite our efforts (Supplemental Methods), we failed to obtain colonies that lacked pCD-METRO, even after non-selective culturing for >50 generations. We hypothesize that the high copy number of pCD-METRO contributes to its stability.

Together, these results demonstrate that pCD-METRO encodes a functional replicon that is responsible for a high copy number in *C. difficile* and is efficiently maintained in the absence of selection.

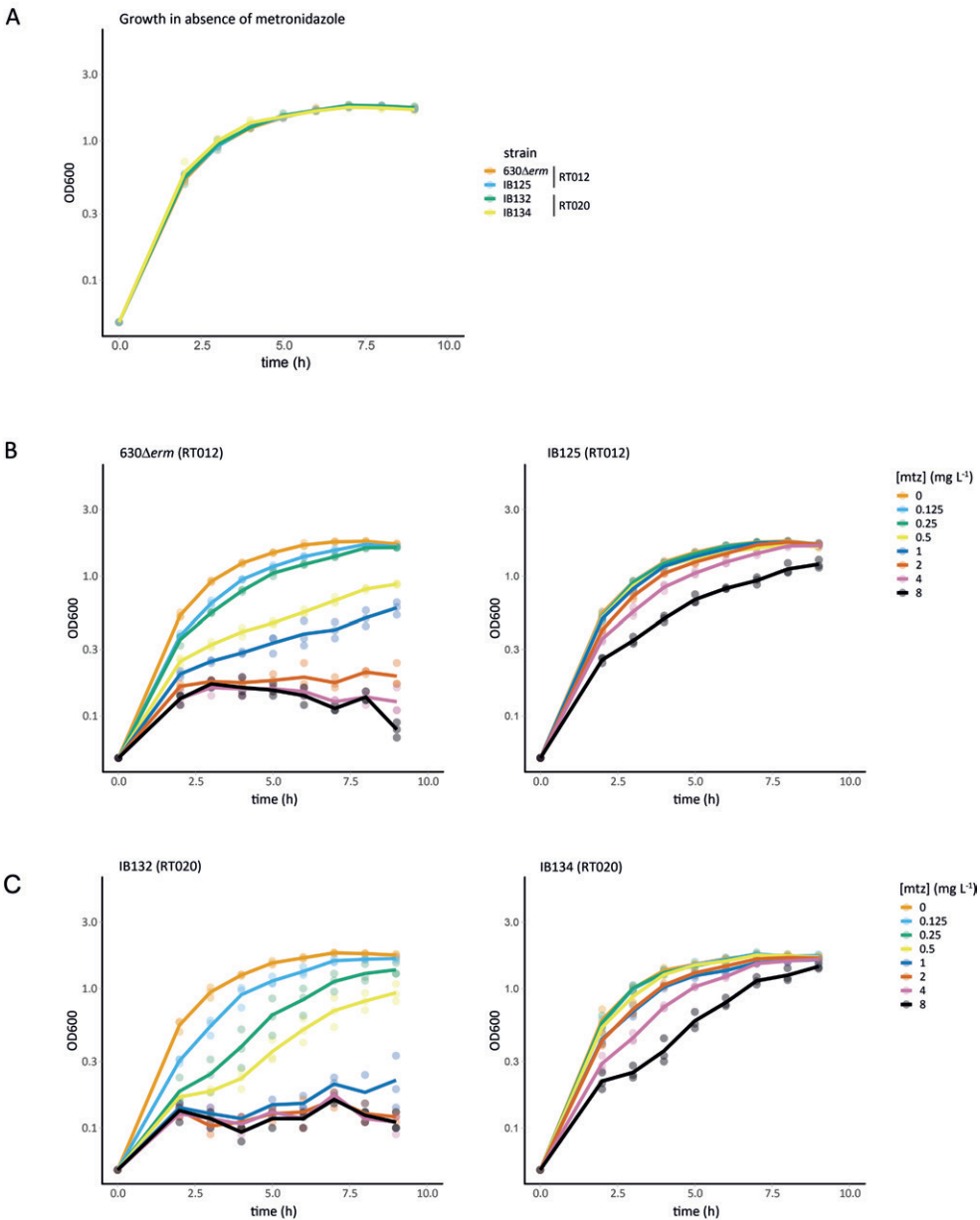


Figure 8. The effects of carriage of pCD-METRO on growth. A) Growth as monitored by an increase in optical density at 600nm (OD600) of various strains in the absence of metronidazole. **B)** Growth of RT012 strains in the presence of various concentrations of metronidazole [mtz]. **C)** Growth of RT020 strains in the presence of various concentrations of metronidazole [mtz]. Individual measurements are shown as semi-transparent dots. The median of the replicates is shown as a solid line. Source data are provided as a Source Data file.

Discussion

In this study we describe the a plasmid linked to resistance against a clinically relevant antimicrobial in *C. difficile*. We show that the high-copy number plasmid pCD-METRO is internationally disseminated, present in diverse PCR ribotypes- including those known to cause outbreaks – and we provide evidence for the possible horizontal transmission of the plasmid. Our data suggests a possible prevalence of <0.14 % (1/721; endemic) to 3.9% (22/563; collection enriched for metronidazole resistant strains) of the plasmid, in line with previous observations (288).

Though the presence of plasmids in *C. difficile* has been known for many years, no phenotypes associated with plasmid carriage have been described (282, 284, 289). We show that introduction of pCD-METRO in susceptible strains leads to stable and medium-independent metronidazole resistance. Plasmids may play a broader role in antimicrobial resistance of *C. difficile*. A putative plasmid containing the aminoglycoside/linezolid resistance gene *cfrC* was recently identified *in silico*, but in contrast to our work no experiments were presented to verify the contig was in fact a plasmid conferring resistance (73). The presence of an antimicrobial resistance gene does not always result in resistance, and DNA-based identification of putative resistance genes without phenotypic confirmation may lead to an overestimation of the resistance frequencies (271, 290, 291).

At present, it is unknown which gene(s) on pCD-METRO are responsible for metronidazole resistance. Nitroimidazole reductase (*nim*) genes have been implicated in resistance to nitroimidazole type antibiotics (124). Though the presence of a truncated *nim* gene on pCD-METRO is intriguing, we do not believe this gene to be responsible for the phenotype for several reasons. Structural modelling of the predicted protein shows that it lacks the catalytic domain, and introduction of the ORF under the control of an inducible promoter (**Supplemental Dataset 1** and **Supplemental Table 2**) did not confer resistance in our laboratory strain. Moreover, the RT027 strain R20291 encodes a putative 5-nitroimidazole reductase (R20291_1308) and is not resistant to metronidazole, implying the presence of a *nim* gene is not causally related to metronidazole resistance in *C. difficile* as also noted by others (124). Further research is necessary to determine the mechanism for metronidazole resistance in *C. difficile* conferred by pCD-METRO, and to investigate the contribution of the high copy number (**Figure 6**) to the resistance phenotype.

Our work, combined with that of others, suggests that metronidazole resistance is multifactorial and other factors than pCD-METRO can cause or contribute to metronidazole resistance in *C. difficile*. For instance, pCD-METRO may not explain low level resistance, heterogeneous resistance, or stable resistance resulting from serial passaging of isolated strains under metronidazole selection (124, 138, 141, 292). We also observed that absolute MIC values in agar dilution experiments differed between MTZ^R isolates of different PCR ribotypes despite carriage of pCD-METRO, suggesting a contribution of chromosomal or other extrachromosomal loci to absolute resistance levels. Though the SNP we identified in the RT020 strain IB136 was not found in other MTZ^R strains of RT010/RT020/RT027, we cannot exclude that it contributes to the resistance in this particular strain. We also observed strong medium dependent effects: the MICs obtained on BBA are generally higher than those on BHI (**Figure 4**), underscoring the importance of using standard conditions for susceptibility testing. Notably, for at least one RT010 strain (LUMCMM19 0830) this lead to conversion of the resistance phenotype. Clearly, medium components (possibly iron or haem) contribute to metronidazole resistance. This is in line with suggested metabolic changes in MTZ^R strains that do not harbour pCD-METRO (138, 140, 141).

The pCD-METRO plasmid is internationally disseminated (**Table 1, Figure 3**), although further research is necessary to determine how prevalent the plasmid is in metronidazole resistant *C. difficile* isolates. This study attempted to enrich for metronidazole resistant strains as this resistance is scarce in *C. difficile*. We received strains which were reported to be metronidazole resistant by the senders. However, when performing antimicrobial susceptibility testing for these strains with agar dilution in our own laboratory, virtually all strains had MIC values below the epidemiological cut-off value from EUCAST for metronidazole and were considered susceptible. For this reason, we ended up having very few metronidazole resistant isolates of other PCR ribotypes than RT010 (RT020 and RT027). It is not entirely clear how these differences came into existence. Depending on handling of the sample material and freeze-thawing cycles, it is possible that inducible metronidazole resistance, unrelated to pCD-METRO, was initially measured and that this was lost after storage and lack of selection (138). Considering the apparent stability of pCD-METRO, we do not think that the discrepancies are due to loss of the plasmid during passaging on non-selective media.

Based on the apparent stability of the pCD-METRO plasmid, we consider acquisition rather than loss of pCD-METRO a more likely explanation for the clonality of the RT020 patient isolates. As *C. difficile* has not been demonstrated to be naturally competent for transformation, this suggests that pCD-METRO may be transmissible. Horizontal gene transfer is consistent with the observed level of sequence conservation between the RT010, RT020 and RT027 pCD-METRO plasmids sequenced in this study (**Supplemental Figure 1**). Nevertheless, we failed to demonstrate intraspecies transfer with different donor and recipient strains of *C. difficile* under laboratory conditions (Supplemental Methods), suggesting that the strains tested (or possibly the species) lack a determinant required for transfer. Together with its size and the presence of mobilization genes (**Figure 2A**), we therefore hypothesize that pCD-METRO is mobilizable from an uncharacterized donor organism (293). We screened the complete sequence read archive of the NCBI (paired-end Illumina data) for potential sources of the plasmid, but failed to identify any entries with reliable mapping (>1% of data) to pCD-METRO.

As more reports are published associating metronidazole with higher treatment failure (276), a shift in consensus for using metronidazole as first line treatment for mild to moderate CDI is occurring (294). The reason for treatment failure is currently unknown, but no correlation between MTZ^R *C. difficile* isolates and treatment failure seems to exist (290). We also observed that clinical isolates from subjects in which metronidazole treatment failed, were metronidazole susceptible and pCD-METRO negative (**Supplemental Dataset 1**) (286). These observations, however, do not rule out a role for (other) metronidazole resistant organisms, potentially harboring pCD-METRO, in treatment failure. Indeed, levels of metronidazole at the end of the colon and in fecal material are low (most likely due to absorption of the drug in the small intestine in the absence of diarrhea) (276), and members of the microbiota involved in inactivation or sequestering of metronidazole may allow for growth of MTZ^S species (295–298).

Our observation of a putatively transmissible plasmid associated with metronidazole resistance in *C. difficile* and the gut microbiome has implications for clinical practice. First, it warrants a further investigation into the role of the plasmid in metronidazole treatment failure in CDI, and – more broadly – in metronidazole resistance of organisms other than *C. difficile*. Second, though this work can be seen as one more argument against the use of metronidazole as a first line treatment of CDI, detection of the plasmid in fecal material might also guide treatment decisions (i.e. pCD-METRO harboring patients are excluded from metronidazole treatment).

And finally, screening by PCR of fecal donor material intended for FMT might be desirable to reduce the possibility of transferring pCD-METRO from hitherto uncharacterized donor organisms to *C. difficile* in patients.

Materials and Methods

Strains

The 22 strains sequenced as part of this study were isolated from a single patient at the Leiden University Medical Center (LUMC) or derived from the collection of the Dutch NRL for *C. difficile*, which is hosted at the LUMC and also includes animal isolates. Informed consent (approved by the Medical Ethical Committee of the LUMC) was given for the use of the patient samples for research purposes. Other clinical isolates (n=567) were obtained through the NRL and partners in the *C. difficile* typing network of the European Centre for Disease Prevention and Control (ECDC), or were previously collected as part of the ECDIS study and the Tolevamer and MODIFY I+II clinical trials(111, 174, 286). Strain IB136, for which the genome sequence is available from the European Nucleotide Archive (accession CAADHH010000000) has been deposited in the National Collection of Type Cultures (NCTC14385) of Public Health England.

Whole genome sequencing and analysis

DNA was extracted from 9mL of stationary growth phase cultures grown in BHI (Oxoid) broth using a QIAasympyphony (Qiagen, The Netherlands) with the QIAasympyphony DSP Virus/Pathogen Midi Kit according to the manufacturer's instructions. All samples were sequenced on an Illumina HiSeq4000 (all samples except those mentioned hereafter) or NovaSeq (LUMCMM18 0002, LUMCMM19 0348, LUMCMM19 0880 and LUMCMM19 0960) platform with read length 150bp in paired-end mode. All *C. difficile* samples isolated from the patient were assembled using an inhouse pipeline, which includes various QC and comparative measures, assembly with six different assemblers as well as scaffolding, but which are not used for all steps and assemblers. Ultimately, Edena v3.131028 was used on the non-trimmed reads with an overlap range between 76 and 146 (299). Reads were mapped back to all assemblies for quality control purposes with Bowtie2 v2.3.1, and SAM files were converted to sorted and indexed BAM files with Samtools v1.5 to obtain mapping rates to the assembly (300, 301). After this step, all contigs from assemblies with a length smaller than 304 bp were discarded, as well as contigs

corresponding to the phiX phage spike in (GenBank accession number J02482.1). To remove contaminating contigs, contigs from all assemblies were compared with Blastn v2.6 against the NCBI database (download July 10, 2018, standard parameters, except evalule of 0.0001) (302, 303). Taxonomy was estimated with the LCA algorithm as implemented in MEGAN, except that only Blast matches with a minimum length of 100bp, and as well only matches not deviating more than 10% in length from the longest match were considered (304). Filtering was performed on phylum level and the dominant phylum was determined by the amount of base pairs in the assigned blast matches. All contigs assigned to another phylum were discarded. For quality control, the expected genome size was estimated with kmerspectrumalyzer download August 2013 and Jellyfish v1.1.11 (305, 306). Using bedtools genomecov v2.2.16 the read coverage of the assemblies were calculated (307). All sequence ranges larger than 20 bp with less than 50% coverage and with a larger distance than 200 bp from the contig end were manually inspected, unless only Ns were contained in the sequence. Final evaluation was performed with the values for N50, assembly size (in relation to predicted genome size), mapping rate and manual inspection of low coverage sites. The assembly being evaluated as being best was performed with the Edena assembler and an overlap of 126 (IB136) or 136 (LUMCMM19 0348), with contamination and length filtering, without additional scaffolding and gapfilling.

Annotation was performed with an in-house pipeline as described before (284). This annotation was furthermore manually reviewed and the annotations from the assembly of *C. difficile* 630 (based on Blastn comparison on gene level) were transferred where applicable.

Extrachromosomal elements were predicted as described before (283, 284). To identify plasmids similar to the pCD-METRO, a homology search was performed with Blastn (with an e-value of 0.0001). A further search for non-assembled plasmid sequences was performed. All samples sequenced in paired-end mode on Illumina machines were downloaded from the NCBI with eutils prefetch, and mapped to the plasmid sequence with bowtie2 v2.3.1 to the plasmid sequence. The option -no-mixed was used to suppress incorrectly mapping pairs (303).

SNP typing was performed after selecting the best reference assembly. The SNP typing was performed with the in-house pipeline Basti based on the biopet framework (308). This pipeline performs mapping to the reference assembly with bowtie2 v2.3.1 and SNP typing

with BCFtools v1.1-134 (309). Groups were investigated for homozygous SNPs differentiating them. Heterozygous SNPs were discarded. Genomic comparisons between assemblies were performed with Blastn (standard parameters, except an e-value of 0.0001). All programs were executed with standard parameters unless otherwise specified. Multi-locus sequence type was determined using stringMLST v0.6.2(310) with default settings.

Antimicrobial susceptibility testing and ribotyping

All strains were characterized by standardized PCR ribotyping and tested multiple times for metronidazole resistance by agar dilution according to CLSI guidelines, with the inclusion of appropriate microbiological controls (13, 86). No formal breakpoints have been defined for metronidazole resistance in *C. difficile*; here we use the EUCAST epidemiological cut-off of 2 mg/L to define resistance (87). Details of all strains and their characteristics are available in **Table 1** and **Supplemental Dataset 1**. For E-tests (BioMerieux), bacterial suspensions corresponding to 1.0 McFarland turbidity were applied on BHI agar supplemented with 0.5% yeast extract (Sigma-Aldrich) and *Clostridium difficile* Selective Supplement (CDSS, Oxoid) or on Brucella Blood Agar plates without antimicrobials. MIC values were read after 48 hours of incubation as recommended by CLSI (86).

Molecular biology techniques

Escherichia coli was cultured aerobically at 37°C in Luria-Bertani (LB) broth, supplemented with 20 mg/L chloramphenicol and 50 mg/L kanamycin when appropriate. *C. difficile* was cultured in BHI supplemented with 0.5% yeast extract, CDSS and 20 mg/L thiamphenicol when appropriate, in a Don Whitley VA-1000 workstation (10% CO₂, 10% H₂ and 80% N₂ atmosphere).

Plasmids and oligonucleotides are listed in **Supplemental Tables 1 and 2**, respectively. pIB86 (pCD-METRO^{shuttle}, **Supplemental Figure 3** and **Supplemental File 1**) was constructed using Gibson assembly using HaeIII-linearized pCD-METRO and a fragment from pRPF185 (Addgene 106367) (233). This fragment was obtained by PCR, and contained the requirements for maintenance in, and transfer from, *E. coli*. Cesium chloride purified pCD-METRO (see below) was linearized using restriction endonuclease HaeIII. Primers oWKS-1663 and oWKS-1664 annealed on pRPF185 generating a PCR shuttle-fragment containing pBR322ori-catP-oriT-traJ. To assemble pIB86, 100 ng of insert was assembled against a four-fold molar excess of linearized pCD-METRO backbone using a homemade Gibson Assembly Master Mix (4 U/μl

Taq Ligase (Westburg), 0.004 U/μl T5 exonuclease (New England Biolabs), 0.025 U/μl Phusion polymerase (BioLabs), 5% polyethyleneglycol (PEG-8000), 10 mM MgCl₂, 100 mM Tris-Cl pH=7.5, 10 mM dithiothreitol, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dCTP, 0.2 mM dGTP, and 1 mM β-nicotinamide adenine dinucleotide) for 30 minutes at 50°C and transformed into MDS42 cells. Transformants were screened by colony PCR using primers oBH-5 and oWKS-1387. The entire sequence of pIB86 was verified by Sanger sequencing using primers oBH-1, oBH-5, oBH-6, oBH-8, oBH-9, oBH-10, oBH-11, oBH-12, oIB-120-, oIB-121, and oIB-122, oWKS-1241-, oWKS-1383, oWKS-1388, oWKS-1537, oWKS-1539, oWKS-1540, oWKS-1574, oWKS-1656, oWKS-1658, oWKS-1659, oWKS-1661, oWKS-1663, oWKS-1664 and oWKS-1678. Plasmid pIB80 (**Supplemental Figure 4** and **Supplemental File 2**) was constructed by ATUM (Newark, CA) and contains a pCD-METRO derived fragment inserted in between the *KpnI* and *NcoI* sites of pRPF185 (233).

Transfer of plasmids from *E. coli* CA434 to *C. difficile* 630Δ*erm* (190) was done using standard methods (222). Routine DNA extractions were performed using the Nucleospin Plasmid EasyPure (Macherey-Nagel) and DNeasy Blood and Tissue (Qiagen) kits after incubating the cells in an enzymatic lysis buffer according to instructions of the manufacturers.

Isolation of cloning-grade pCD-METRO

Plasmid pCD-METRO was extracted from 400 mL of culture containing the MTZ^R strain IB138 using the Macherey-Nagel Nucleobond Xtra Midi kit. Using the CsCl₂ plasmid purification method this plasmid prep was further cleaned as summarized hereafter. pCD-METRO plasmid prep was added to TE buffer (10 mM Tris pH=8.0, 1 mM EDTA) and CsCl₂ was added to a density of 1g/g. Approximately 220 μg/ml ethidium bromide was added to this solution after which samples were spun down in a Beckman Coulter Optima XE-90 ultracentrifuge for 17 hours at 65,000 rpm, 20°C. Bands were visualized with UV light and plasmid DNA was collected by withdrawing the lowest of the two resulting bands with an 18 gauge needle. To remove ethidium bromide 1x vol/vol 5M NaCl saturated N-butanol was used to remove the upper (purple) phase after centrifugation. Samples were ethanol precipitated twice prior to resuspending purified plasmid DNA in TE buffer.

Plasmid copy number determination

Real time quantitative PCR (qPCR) experiments were performed essentially as described (234). In short, total DNA was isolated after 17h of growth using a phenol-chloroform extraction protocol and diluted to a concentration of 10 ng/ μ l. 4 μ l of the diluted DNA sample was added to 6 μ l of a mixture containing SYBR Green Supermix (Bio-Rad) and gene-specific primers (0.4 μ M total) for a total volume of 10 μ l per well. Gene specific primers used were targeting *rpoB* (chromosome) and *catR* (plasmid) and copy number was calculated using the ΔC_T method. Experiments were performed in triplicates on three different technical replicates. Statistical significance was calculated using two-way analysis of variance (ANOVA) and Tukey's test for multiple comparisons (Prism 8, GraphPad) (**Supplemental Table 3**).

Preparation of Figures

Agarose gel and E-test images were acquired using a BioRad GelDoc XR, processed in Adobe Photoshop CC 2018 (Adobe). Plasmid maps were generated using Artemis and DNAPlotter (311, 312). Graphs were generated using PlotTwist (<https://huygens.science.uva.nl/PlotTwist/>) and PlotsOfData (<https://huygens.science.uva.nl/PlotsOfData/>). All figures were prepared for publication in Adobe Illustrator CC 2018 (Adobe).

Data availability

Sequence data that support the findings of this study are available in the European Nucleotide Archive under BioProject number PRJEB24167 (<https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJEB24167>) with accession numbers ERR2232520- ERR2232537, ERR3611150-ERR3611153 and ERR3772426. The annotated genome assembly for IB136, including pCD-METRO, can be found under accession number CAADHH010000000 (<https://www.ebi.ac.uk/ena/browser/view/CAADHH010000000>) and as **Supplemental Dataset 2**. The source data underlying Figs 1, 2b, 2c, 3, 4, 5, 6, 7 and 8 and **Supplemental Figure 2** are provided as a Source Data file.

Code availability

Computer code related to the analysis from this paper is based on published tools, as described in the Methods, and details are available from the authors on request.

Acknowledgements

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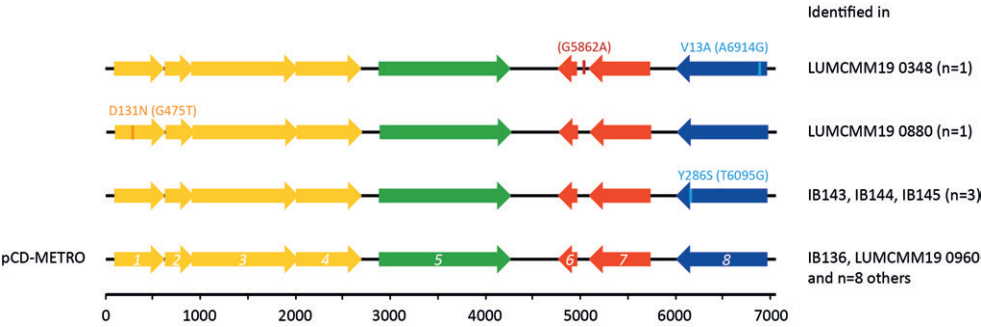
Author statements

Performed experiments: IMB, ES, CH, IMJGBS, JC, WKS. Analyzed data: BVHH, IMB, WKS, JC. Contributed patient samples and metadata: EMT, EJK. Contributed reagents: RB, BVHH. Drafted manuscript: IMB, BVHH, JC, EJK, WKS. All authors edited and approved the final version of the manuscript. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

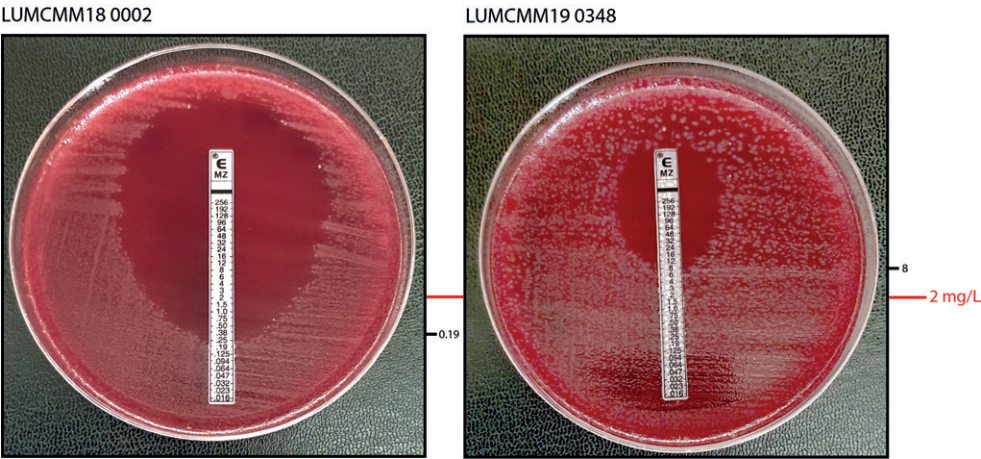
Competing interests

WKS has performed research for Cubist and has received speaker fees from Promega. EJK has performed research for Cubist, Novartis and Qiagen, and has participated in advisory forums of Astellas, Optimer, Actelion, Pfizer, Sanofi Pasteur and Seres Therapeutics. EJK, BVHH and EMT currently hold an unrestricted research grant from Vedanta Biosciences. These companies had no role in the design and execution of the experiments for this study or the decision to publish. IMB, ES, RB, CH, JC and IMGJSB: none to declare. Part of this data has been presented at the International Clostridium difficile Symposium 2018, the Scientific Spring Meeting of the KNVM/NVMM 2019, ECCMID 2019, CLOSPATH11 (2019) and Bacterial Morphogenesis, Survival and Virulence: Regulation in 4D (2019).

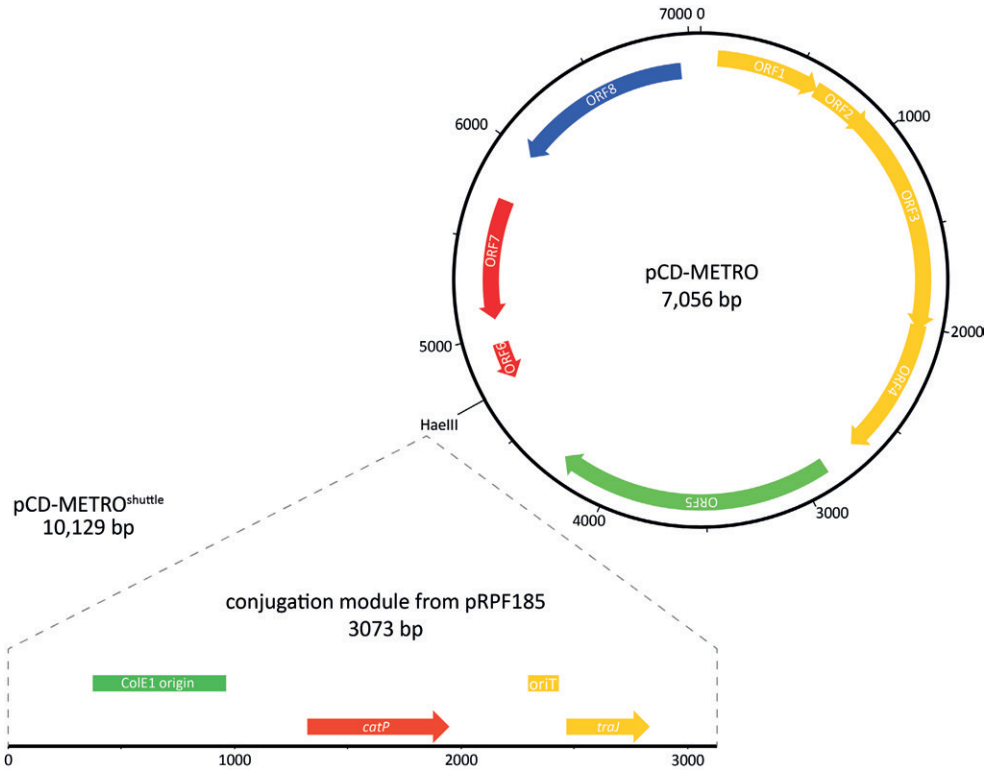
Supplemental information



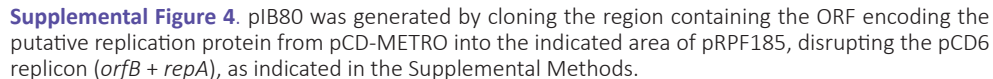
Supplemental Figure 1. Variants of pCD-METRO as identified in this study. Assembly and SNP calling was performed as described in Supplemental Methods. Strain IB136 is the reference for pCD-METRO, and most strains are similar to this plasmid. Variants are indicated with vertical bars. Amino acid changes are indicated in single letter genetic code for non-synonymous substitutions (in ORFs). Nucleotide changes are given in between brackets with the relevant nucleotide position in relation to the scale bar below (forward strand).



Supplemental Figure 2. Metronidazole resistance of RT020 strains obtained from routine surveillance. Strains LUMC19 0348 (pCD-METRO negative) and LUMC19 0830 (pCD-METRO positive) from the patient were tested for metronidazole resistance using E-test. 2 mg/L is the EUCAST epidemiological cut-off for metronidazole that was used to define resistance in this study. Source data are provided as a Source Data file.



Supplemental Figure 3. Schematic representation of the construction of pCD-METROshuttle (pIB86): the PCR-amplified conjugation module from pRPF185 was inserted in the unique HaeIII restriction site through Gibson assembly as described in Supplemental Methods.



Name	Relevant features	Source/reference
pRPF185	tetR P _{tet} - <i>sLuc</i> ^{opt} ; catP	A Oliveira Paiva et al., 2016
pCD-METRO	(see: accession number ERZ807316)	This study
pIB20	pRPF185 P _{CD0716} - <i>sLuc</i> ^{opt} ; catP	This study
pIB76	tetR P _{tet} -nimB; catP	This study
pIB80	tetR P _{tet} - <i>gusA-pCD-METROreplicon</i>	This study
pIB86	pCD-METRO containing shuttle fragment; pCD-METRO-oriT- <i>pBR322-traI catP</i> ; catP	This study

Supplemental Table 1. plasmids used in this study

Name	Sequence (5'→3')	Description	Source
oBH-1	CCTCGTAGAATCCGGTGCAA	Forward primer annealing to <i>orf6</i> of pCD-METRO	This study
oBH-2	TATTTCTTGCCGCTGAGGT	Reverse primer annealing to <i>orf6</i> of pCD-METRO	This study
oBH-3	GCAGACGGTTGGTGATTCG	Forward primer annealing to <i>orf6</i> of pCD-METRO	This study
oBH-4	GTATTTCTTGCCGCTGAGG	Reverse primer annealing to <i>orf6</i> of pCD-METRO	This study
oBH-5	AGTAGGGGTCGGTACTTCT	Forward primer annealing to <i>orf5</i> of pCD-METRO	This study
oBH-6	CTTCGGTTCGCTGTTTGG	Reverse primer annealing to <i>orf5</i> of pCD-METRO	This study
oBH-7	GGCTCAGACACTTCTACCGC	Forward primer annealing to <i>orf7</i> of pCD-METRO	This study
oBH-8	CCCCTTCAGGGGTGTTTTCT	Reverse primer annealing to <i>orf7</i> of pCD-METRO	This study
oBH-9	GGGCTATAAGACCGACTGGC	Forward primer annealing to <i>orf3</i> of pCD-METRO	This study
oBH-10	AACGGTCTCTACCTCCGTCA	Reverse primer annealing to <i>orf3</i> of pCD-METRO	This study
oBH-11	ACTTACACTGCAACCGGTGC	Forward primer annealing to <i>orf8</i> of pCD-METRO	This study
oBH-12	TCGTGCTTGTGAGGTGAGT	Reverse primer annealing to <i>orf8</i> of pCD-METRO	This study
oIB-103	CATATAAGATAAAAAATATTTGAAAAATAC	Forward primer used for sequencing <i>rpoB</i>	This study
oIB-104	GATATGACCTAGCAAAAGTTGGTAG	Forward primer used for sequencing <i>rpoB</i>	This study
oIB-105	AGAAATCACCATATAGAAAATTTGATAAAG	Forward primer used for sequencing <i>rpoB</i>	This study
oIB-106	GGAGAAACTGAACCTTACTGCTGAG	Forward primer used for sequencing <i>rpoB</i>	This study
oIB-107	GACAGATGAAGACCAAGAAATAGAAAG	Forward primer used for sequencing <i>rpoB</i>	This study
oIB-108	ATTGTCTTATTTTCTTGAGAAGCC	Forward primer used for sequencing <i>rpoB</i>	This study
oIB-120	GCCGAAAAAGAAAACTGCCGGG	Sequencing primer for pCD-METRO ^{shuttle}	This study
oIB-121	CTTACTCAACGGAAGTGATGAGAG	Sequencing primer for pCD-METRO ^{shuttle}	This study
oIB-122	CGGTTATAGTACACTGGCTTG	Sequencing primer for pCD-METRO ^{shuttle}	This study
RP314	GAAGGTTGACCCGGAATCAT	Forward qPCR primer for <i>catP</i>	EM Ransom et al., 2015
RP315	CGCAACGGTATGGAACAATC	Reverse qPCR primer for <i>catP</i>	EM Ransom et al., 2015
TEQ009	AAGAGCTGGATTCGAAGTGCCTGA	Forward qPCR primer for <i>rpoB</i>	EM Ransom et al., 2015
TEQ010	ACCGATATTTGGTCCCTCTGGAGT	Reverse qPCR primer for <i>rpoB</i>	EM Ransom et al., 2015
oWKS-1070	GTCTTGGATGGTTGATGAGTAC	Forward primer in <i>gluD</i>	WK Smits et al., 2018
oWKS-1071	TTCTTAATTTAGCAGCAGCTTC	Reverse primer in <i>gluD</i>	WK Smits et al., 2018
oWKS-1241	CACCGACAGCAAGGCAAGACCG	Sequencing primer for pCD-METRO ^{shuttle}	E Van Eijk et al., 2019
oWKS-1387	CAGATGAGGGCAAGCGGATG	Sequencing primer for pCD-METRO ^{shuttle}	This study
oWKS-1388	CGTCGGTGAGCCAGAGTTTC	Sequencing primer for pCD-METRO ^{shuttle}	This study
oWKS-1537	TAGGGTAACAAAAACACCG	Sequencing primer for pCD-METRO ^{shuttle}	PT van Leeuwen et al., 2016

Name	Sequence (5'→3')	Description	Source
oWKS-1539	GGATTTCACATTTGCCGTTTGTAAAC	Sequencing primer for pCD-METRO ^{shuttle}	PT van Leeuwen et al., 2016
oWKS-1540	GATCTTTTCTACGGGTCTGAC	Sequencing primer for pCD-METRO ^{shuttle}	PT van Leeuwen et al., 2016
oWKS-1574	AAACAAACACCGCTGGTAG	Sequencing primer for pCD-METRO ^{shuttle}	This study
oWKS-1656	TAGCGGATCCAAGCGTTCTGAACGCACTG	Sequencing primer for pCD-METRO ^{shuttle}	This study
oWKS-1658	TAGCGGATCCGGGCTTACTTCTGGGTATCC	Sequencing primer for pCD-METRO ^{shuttle}	This study
oWKS-1659	TAGCGGTACCTGTTGCCTGCTTCCTGTATG	Sequencing primer for pCD-METRO ^{shuttle}	This study
oWKS-1661	TAGCGGTACCATCCACGCACCAACAC	Sequencing primer for pCD-METRO ^{shuttle}	This study
oWKS-1663	CAAGACGGTCGGCGTTGCGCTCGAAGATGG	Forward primer for PCR of shuttle fragment <i>pBR322ori-catP</i>	This study
	ATAAAATAAAATAGAGGCTATAAATAGC	<i>oriT-tral</i>	
oWKS-1664	CGATAAAATCTTGATTGATGAAGTACAAGGTTAG	Reverse primer for PCR of shuttle fragment <i>pBR322ori-catP</i>	This study
	TAGGTGCTTTTAAAAAC	<i>oriT-tral</i>	
oWKS-1678	CCATCTTCGAGGCAACG	Sequencing primer for pCD-METRO ^{shuttle}	This study

Supplemental Table 2. oligonucleotides used in this study.

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Row Factor	6,189	2	3,094	F (2, 6) = 1,344	P=0,3294
Column Factor	2945	3	981,8	F (3, 6) = 426,4	P<0,0001
Residual	13,82	6	2,303		
Tukey's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Significant?	Summary	Adjusted P Value
630Δerm vs. IB30	-3,543	-7,832 to 0,7457	No	Ns	0,1018
630Δerm vs. IB90	-25,25	-29,54 to -20,96	Yes	****	<0,0001
630Δerm vs. IB125	-38,07	-42,36 to -33,78	Yes	****	<0,0001
IB30 vs. IB90	-21,71	-26,00 to -17,42	Yes	****	<0,0001
IB30 vs. IB125	-34,53	-38,82 to -30,24	Yes	****	<0,0001
IB90 vs. IB125	-12,82	-17,11 to -8,531	Yes	***	0,0002

Supplemental Table 3. Statistics associated with the qPCR analysis. These data relate to **Figure 7A** of the main text of the manuscript.



5

HEME IS CRUCIAL FOR MEDIUM-DEPENDENT METRONIDAZOLE RESISTANCE IN CLINICAL ISOLATES OF *CLOSTRIDIoidES DIFFICILE*

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Abstract

Until recently, metronidazole was the first-line treatment for *Clostridioides difficile* infection and it is still commonly used. Though resistance has been reported due to the plasmid pCD-METRO, this does not explain all cases. Here, we investigate resistance to metronidazole in a collection of clinical isolates of *C. difficile*. We find that nearly all isolates demonstrate a heme-dependent increase in the minimal inhibitory concentration (MIC) for metronidazole, which in some cases leads to isolates being qualified as resistant (MIC > 2 mg/L). Moreover, whole genome sequence analysis reveals a single nucleotide polymorphism in the heme responsive gene *hsmA*, which defines a metronidazole resistant lineage of PCR ribotype 010 / multilocus sequence type 15 isolates that also includes pCD-METRO containing strains. Together our data demonstrate that heme is crucial for medium-dependent metronidazole resistance in *C. difficile*.

Introduction

Clostridioides difficile is a gram-positive, anaerobic enteropathogen capable of causing a *C. difficile* infection (CDI) upon disruption of the normal intestinal microbiota by for instance antimicrobial therapy (20, 225). It is the primary cause of nosocomial diarrhea, but is also found in cases of community-acquired disease (20, 313). Although the use of antibiotics is a risk factor for CDI, antimicrobials are also used to treat the infection. Until recently, metronidazole was considered the drug-of-choice for treatment of mild CDI. Though vancomycin and fidaxomicin are currently indicated as first-line therapeutics for the treatment of CDI, (80, 264) metronidazole is still commonly used (83, 84).

Our understanding of the mechanisms of resistance to metronidazole in *C. difficile* is still limited. For Clostridia, studies are complicated by reports of unstable, inducible metronidazole resistance which is lost upon removal of antibiotic pressure or after the strain undergoes freeze-thawing cycles (124, 134). Recently, however, we have demonstrated that metronidazole resistance in diverse strains of *C. difficile* can be mediated by the plasmid pCD-METRO through a mechanism that is not yet understood (235). Notably, the presence of pCD-METRO explains at least part of independently reported cases of metronidazole resistance (141, 314, 315). However, strains that lack pCD-METRO can still be resistant to metronidazole. For instance, we have previously identified a pCD-METRO negative strain that demonstrated medium-dependent metronidazole resistance (235). This suggests that other, potentially chromosomal, determinants contribute to resistance.

Information on pathways that could contribute to resistance comes from laboratory strains with evolved resistance to metronidazole. Using a laboratory-evolved PCR ribotype (RT) 027 strain exhibiting stable metronidazole resistance, mutations were identified in genes affecting electron transport and iron utilization, but the individual contribution of these mutations to the resistance phenotype was not further investigated (138). More recently, a mutator strain defective in DNA mismatch repair was evolved in the presence of metronidazole (139). The study discovered mutations in a gene encoding an iron transporter (*feoB1*) in metronidazole resistant strains and showed that sequential mutations in *nifJ* (encoding the pyruvate-ferredoxin oxidoreductase, PFOR), *xdh* (encoding xanthine dehydrogenase) or *iscR* (encoding an iron-sulfur cluster regulator) could further increase metronidazole resistance (139). Though studies

with laboratory evolved strains are informative, it is unclear how these findings translate to metronidazole resistant strains isolated from subjects outside the laboratory.

Here, we leverage the potential of strains collected within a project to develop a detailed understanding of the epidemiology and clinical impact of CDI across Europe (COMBACTE-CDI) that were investigated for metronidazole resistance. Four strains demonstrated stable metronidazole resistance, but lacked pCD-METRO. We show that there is a heme-dependent increase in the minimal inhibitory concentration (MIC) for metronidazole across PCR ribotypes and that a higher MIC, at least in a subset of strains, correlates with a specific mutational signature in the gene *hsmA*.

Results

Metronidazole resistance is observed in the COMBACTE-CDI strain collection

The Combatting Bacterial Resistance in Europe – *Clostridioides difficile* infections (COMBACTE-CDI) is a multi-centre European-wide project with an aim to provide detailed understanding of the epidemiology and clinical impact of CDI across the whole healthcare economy in Europe. Sites testing both in-patient and community samples were recruited from 12 countries across Europe. All diarrheal fecal samples (regardless of tests requested by physician) were submitted to a central laboratory (Leeds, UK) on two selected days between July and November 2018. From these samples *C. difficile* was isolated and tested by PCR ribotyping. The metronidazole Minimum Inhibitory Concentration (MIC) for 213 clinical isolates (Belgium n=3, France n=4, Greece n=4, Ireland n=1, Italy n=23, Netherlands n=8, Poland n=29, Romania n=37, Slovakia n=1, Spain n=43, Sweden n=12, UK n=48) were determined by Wilkins-Chalgren agar dilution. (271) Of these, 22 isolates (10%) were found to be resistant to metronidazole using the EUCAST criteria as a cut-off (MIC $\geq$ 2 mg/L) and were sent to the *C. difficile* reference laboratory of the Leiden University Medical Center for further study. (87) When possible, an RT-matched isolate with an MIC<2 mg/L from the COMBACTE-CDI collection was also provided. PCR ribotypes submitted were RT002 (n=3); RT010 (n=7); RT016 (n=1); RT018 (n=3); RT027 (n=12); RT176 (n=1); RT181 (n=4) and RT198 (n=1) (**Table 1**).

As metronidazole is generally quite rare, our findings underscore the importance for investigating large collections of clinical isolates to enrich for strains that are resistant, in order to investigate possible underlying causes of resistance.

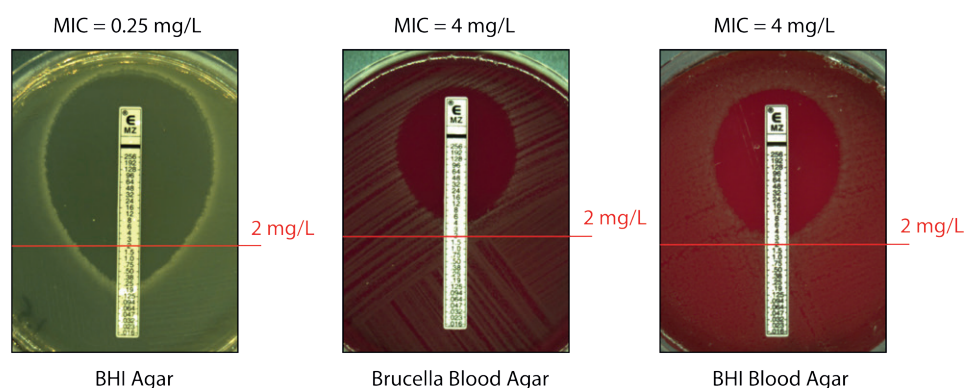


Figure 1. Medium-dependent resistance to metronidazole is independent of base broth. Strain GSK241 was grown and resuspended to 2.0 McFarland turbidity and spread on BHI agar, Brucella Blood Agar (supplemented with 1 µg/ml vitamin K and 5 µg/ml hemin) and BHI Blood Agar (BHI broth supplemented with 5% sheep blood, 1 µg/ml vitamin K and 5 µg/ml hemin). An E-test was placed and plates were incubated for 48h prior to imaging. Red lines indicate the epidemiological cut-off value for metronidazole as determined by EUCAST and have been used to determine resistance in this study (87). Numbers between brackets at the top of the image correspond to the reported MIC value on BHI agar, Brucella blood agar and BHI blood agar, respectively.

Low level resistance to metronidazole is not due to carriage of the pCD-METRO plasmid

We further investigated the clinical isolates from the COMBACTE-CDI strain collection (n=32; **Table 1**). To correct for interlaboratory differences and to make the results directly comparable to our previous study, (235) we performed PCR ribotyping and antimicrobial susceptibility testing by agar dilution according to CLSI standards on Brucella Blood agar (BBA) in a second laboratory (235, 316). A single strain showed discrepant results in PCR ribotyping and was therefore excluded from further analysis. We found 4/31 strains resistant to metronidazole (13% of the preselected isolates, **Table 1**). Three of these 4 strains belonged to RT010 (MIC=4 mg/L) and the fourth isolate belonged to RT016 (MIC=2 mg/L). All of these strains were also identified as resistant in the initial susceptibility testing in Leeds. The interlaboratory difference in antimicrobial susceptibility may be explained by differences in testing methodology, but were not further investigated here.

To determine whether the observed resistance was due to the presence of the pCD-METRO plasmid, we performed a reference assembly of the sequence reads obtained from whole genome sequencing of these isolates against the pCD-METRO reference sequence (obtained from the European Nucleotide Archive BioProject number PRJEB24167). No reliable mapping

of reads to the reference sequence was found and – in line with this finding – the strains were negative in a PCR assay directed against pCD-METRO (data not shown) (235). We therefore conclude that these strains do not carry pCD-METRO and that a different mechanism confers metronidazole resistance in these isolates.

Resistance to metronidazole is medium dependent

We have previously described a strain that demonstrated medium-dependent metronidazole resistance (MIC=4 mg/L) independent of pCD-METRO (235). In order to test if the metronidazole resistant phenotype of the four strains from the COMBACTE-CDI study could similarly be medium-dependent, strain GSK241 (MIC=4 mg/L) was plated on BHI agar that is routinely used in our laboratory, BBA (containing 5% defibrinated sheep blood, 1 µg/ml vitamin K and 5 µg/ml hemin), and BHI blood agar (BHI agar supplemented with 5% defibrinated sheep blood, 1 µg/ml vitamin K and 5 µg/ml hemin) after which a metronidazole E-test was applied. We found that strain GSK241 was susceptible to metronidazole (MIC=0.25 mg/L) on BHI medium but resistant (MIC≥2 mg/L) on both BBA agar and BHI blood agar (**Figure 1**). These results indicate that components present in blood agar are responsible for this medium-dependent resistant phenotype.

We wondered whether the medium-dependent change in MIC values was a general characteristic of *C. difficile* irrespective of the resistance phenotype, or specific to the resistant strains. For this reason, we tested selected COMBACTE-CDI strains by E-test on both BHI agar and BBA (**Table 2**). All strains were clearly susceptible on BHI agar (MIC < 0.5 mg/L) but, with the exception of GSK234, showed a 4-to-32-fold increase in MIC when tested on BBA compared to BHI agar. This medium-dependent increase in MIC was not restricted to a specific RT, as the phenotype was seen for strains belonging to diverse types (RT010, RT016, RT018, RT027, RT176, RT181 and RT198).

Taken together, our data suggest that components present in BBA/BHI blood agar result in reduced susceptibility of strains to metronidazole through a general mechanism. In the case of strains GSK184, GSK241, GSK242NEW and GSK313 this leads to these strains being qualified as metronidazole resistant.

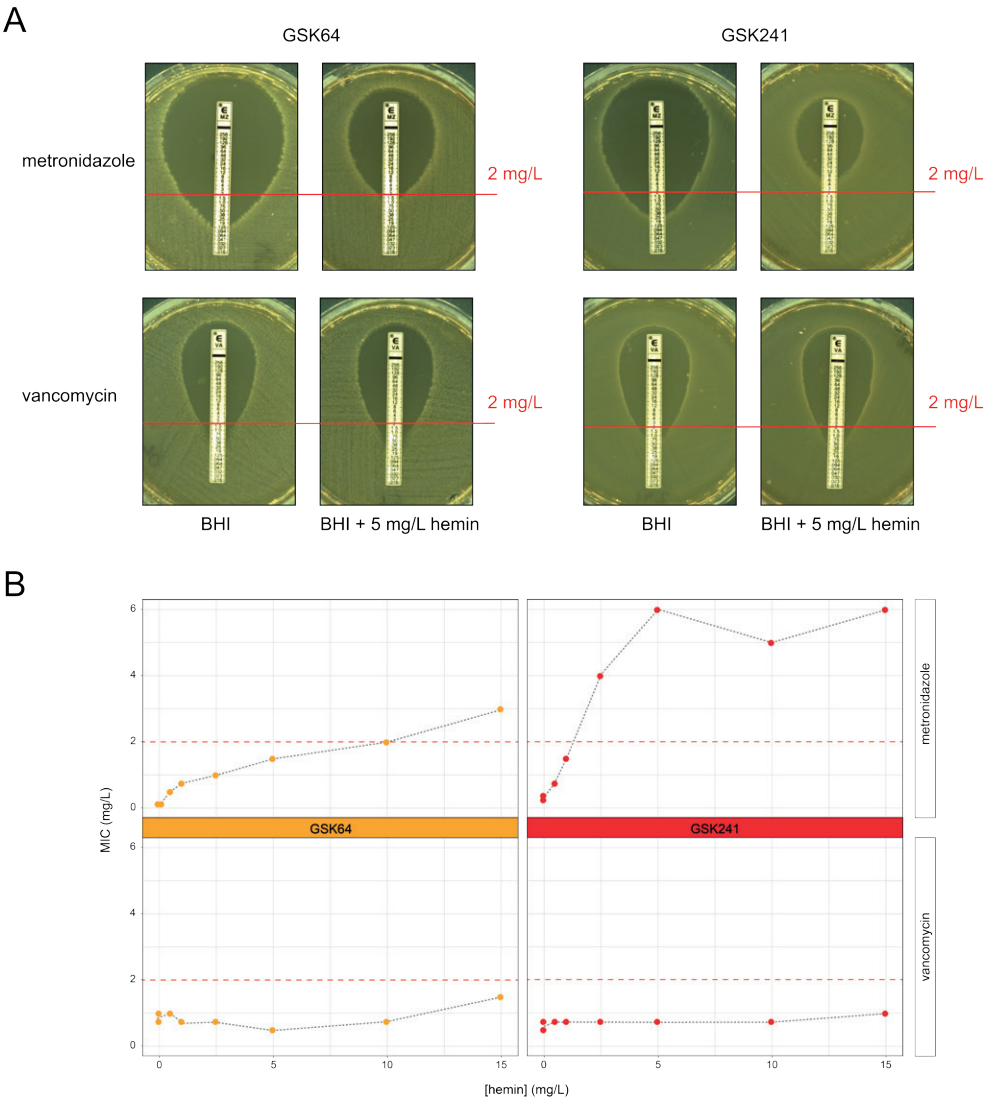


Figure 2. Heme supplementation increases the MICs to metronidazole, but not to vancomycin. A) Strains GSK64 and GSK241 were resuspended in PBS to 2.0 McFarland turbidity and plated on BHI agar and on BHI agar supplemented with 5 μ g/ml hemin. Subsequently E-tests for metronidazole and vancomycin were applied and plates were incubated for 48h prior to imaging. Red lines indicate the epidemiological cut-off value for metronidazole as determined by EUCAST and have been used to determine resistance in this study (87). For a complete overview of the MICs to metronidazole of these strains on BHI agar and BHI agar supplemented with hemin, see Table 2. B) Typical metronidazole (top) and vancomycin (bottom) E-test results performed on strains GSK64 (orange) and GSK241 (red) and when grown on BHI supplemented with various concentration of hemin (0; 0.5; 1; 2.5; 5; 10; 15 mg/L). The dashed line indicates the epidemiological cut-off value for metronidazole and vancomycin as determined by EUCAST (2 mg/L).

Isolate	Location of participant at time of sample	Country of origin	PCR ribotype	MIC (mg/L)
GSK234	Inpatient	Sweden	002	0.25
GSK6	Community doctor	UK	002	0.25
GSK7	Inpatient	UK	002	0.125
GSK22bis	Inpatient	UK	010	0.125
GSK211	Inpatient	Romania	010	0.25
GSK241	Inpatient	France	010	4
GSK242new	Inpatient	France	010	4
GSK246	Inpatient	France	010	0.5
GSK313	Inpatient	Spain	010	4
GSK184	Inpatient	Poland	016	2
GSK39	Community doctor	Italy	018	0.5
GSK302	Inpatient	Italy	018	0.5
GSK303	Inpatient	Italy	018	0.25
GSK54	Inpatient	UK	027	0.25
GSK55	Inpatient	Romania	027	0.25
GSK60	Inpatient	Poland	027	0.5
GSK61	Inpatient	Poland	027	0.5
GSK62	Inpatient	Poland	027	0.5
GSK63	Inpatient	Poland	027	0.5
GSK64	Inpatient	Poland	027	0.5
GSK65	Inpatient	Poland	027	0.5
GSK179	Inpatient	Poland	027	0.5
GSK318	Inpatient	Poland	027	0.5
GSK325	Inpatient	Poland	027	0.5
GSK327	Inpatient	Poland	027	0.5
GSK258	Inpatient	Slovakia	176	0.5
GSK110	Inpatient	Romania	181	0.5
GSK113	Inpatient	Romania	181	0.25
GSK114	Inpatient	Poland	198	0.5
GSK180	Community doctor	Romania	181	0.5
GSK190	Inpatient	Romania	181	0.25

Table 1. Characteristics of the strains from this study. MIC refers to the minimal inhibitory concentration for metronidazole as determined by agar dilution at the Leiden University Medical Center (see Materials and Methods).

Heme is required for medium-dependent differences in metronidazole susceptibility

The fact that the medium-dependent increase in MIC was observed for both BBA and BHI blood agar (**Figure 1**) suggests that the phenotype is independent of the base broth and is likely to be mediated by the supplementation with vitamin K, hemin, and/or blood.

For practical purposes, we evaluated the effect of hemin on the metronidazole resistance phenotype of strains GSK64 (metronidazole susceptible) and GSK241 (metronidazole resistant). We found that supplementation of BHI with 5 µg/ml hemin raised the metronidazole MIC to levels similar to those observed for the BBA and BHI blood agar plates (**Table 2; Figure 2A**).

Strain name	PCR Ribotype	E-test MIC BHI	E-test MIC BBA	E-test MIC BHI + hemin
GSK234	002	0.064	0.064	0.064
GSK241	010	0.25	4	6
GSK242new	010	0.25	6	6
GSK313	010	0.38	4	8
GSK246	010	0.125	1	1
GSK184	016	0.25	2	ND
GSK39	018	0.125	1.5	1
GSK318	027	0.125	1	1
GSK327	027	0.125	2	2
GSK325	027	0.125	1.5	ND
GSK60	027	0.125	4	ND
GSK61	027	0.125	2	1.5
GSK62	027	0.094	1.5	ND
GSK63	027	0.125	1.5	ND
GSK64	027	0.125	1.5	1.5
GSK65	027	0.094	1	ND
GSK179	027	0.125	1.5	ND
GSK258	176	0.16	1	1.5
GSK110	181	0.125	1	1.5
GSK180	181	0.094	1	1
GSK114	198	0.125	2	2

Table 2. Metronidazole minimal inhibitory concentrations on different agar media as determined by E-test. Strains were tested on BHI agar, BHI agar supplemented with 5 µg/ml hemin or BBA as determined by E-test. ND = not determined. MIC = minimal inhibitory concentration of metronidazole under the conditions tested

We extended this finding to a selection of COMBACTE-CDI strains and found that, with the exception of strain GSK234, all tested strains showed an 8-to-24-fold increase in metronidazole MIC on BHI supplemented with hemin compared to BHI alone (**Table 2**). These results mirror those obtained for the E-test on BBA, indicating that hemin is the main determinant of medium-dependent differences in metronidazole MIC for these strains. Under our experimental conditions, the hemin-dependent increase in MIC appears to be specific to metronidazole, as no increase in MIC was observed for vancomycin (**Figure 2A**).

We next assessed the MIC of both metronidazole and vancomycin of strains GSK64 and GSK241 with a range of 0-15 µg/ml hemin. Both strains show a gradual increase in MIC for metronidazole that in the case of GSK241 saturates at > 5µg/ml hemin; the MIC for GSK64 appears to increase further at higher concentration of hemin. In contrast, no increase in vancomycin MIC was seen even in the presence of the highest concentrations of hemin tested (**Figure 2B**).

Altogether these results demonstrate that the presence of heme is crucial for a medium-dependent resistance phenotype in *C. difficile* and that this appears to be specific for metronidazole.

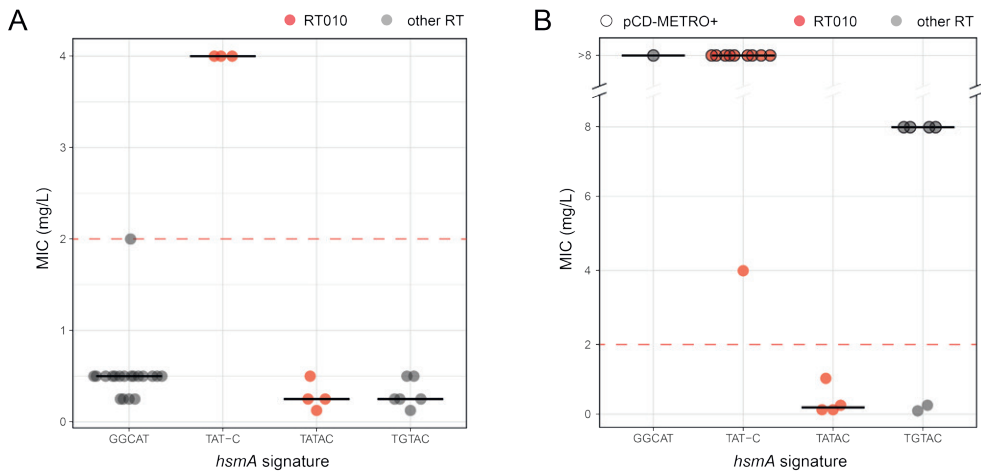


Figure 3. TAT-C signature in *hsmA* correlates to metronidazole resistance in RT010 isolates. The horizontal black bars represent the median MIC. **A)** *hsmA* signature sequences based on SNP analysis in the COMBACTE-CDI clinical isolates. Strains containing the GGCAT signatures belong to RT016, RT027, RT176, RT181 and RT198. The TAT-C and TATAC signatures correspond to RT010 in this study, whereas RT002 and RT018 containing sequence TGTAC. **B)** *hsmA* signature based on SNP analysis in the clinical isolates sequenced in the pCD-METRO study (235). The signature sequence GGCAT is found in RT027, TAT-C and TATAC in RT010 and TGTAC in RT012 and RT020.

An *hsmA* genetic signature is associated with increase metronidazole MICs in PCR ribotype 010 strains

Recent work has shown that four genes (*hsmA*, *hsmR*, *hatT*, *hatR*) are differentially regulated in response to heme, and that the products of the *hsmAR* operon improve growth of a *C. difficile* PCR ribotype 027 strain in the presence of metronidazole (257, 317). For this reason we performed a single nucleotide polymorphism (SNP) analysis on the genes *hatR*, *hatT*, *hsmR*

and *hsmA* using the sequences from the RT027 strain R20291 (GenBank accession FN545816) as a reference.

Using variant positions, we identified signatures for these genes for each strain from the COMBACTE-CDI collection. In the case of *hatR*, *hatT* and *hsmR* these signatures were conserved within a PCR ribotype, and even across closely related PCR ribotypes (e.g RT027, RT198, RT181 and RT176 share the same signatures) (**Supplemental Table 1**). However, for *hsmA*, we observed two distinct but related signatures within a single PCR ribotype. SNPs were identified in the 399-bp *hsmA* gene at positions 129, 249, 366, 372 and 392, resulting in a 5-base pair signature sequence. This signature is GGCAT for the RT027 and RT027-like isolates, TGTAC in RT002 and RT018 isolates and TATAC in some RT010 isolates (**Figure 3A**). Interestingly, the signature sequence TAT-C was found in all 3 metronidazole resistant RT010 isolates. The deletion at position 372 results in a frameshift and alters the primary amino acid sequence of the C-terminus of the HsmA protein.

In order to validate the significance of the signature, we performed the same *hsmA* SNP analysis on whole genome sequences obtained from another collection of isolates enriched for metronidazole resistance (235). This collection contains RT010, RT020 and RT027 isolates which have been characterized with respect to metronidazole MIC and pCD-METRO carriage. We expected to find a similar clustering of *hsmA* signature sequence and PCR ribotype and predicted that the previously described RT010 isolate showing medium-dependent resistance (MIC=4 mg/L) would carry the 1-bp deletion in *hsmA*. Indeed, we found this was the case (**Figure 3B**). Strikingly, all highly resistant RT010 strains that carried pCD-METRO also contained the TAT-C *hsmA* signature sequence (**Figure 3B**).

We wondered how frequently this deletion could be found in RT010 isolates, as metronidazole resistance is most commonly observed in this RT. For this reason, we performed whole genome SNP (wgSNP) analysis through the Enterobase platform on sequences available in the Sequence Read Archive (SRA) of multilocus sequence type 15 (ST15; which includes RT010) (n=57) and ST15-like strains (n=4) as well as the sequences of the RT010 isolates described earlier in this study (n=21) (235, 284, 318, 319). We found that the TAT-C signature in *hsmA* was detected in a specific lineage of ST15- and ST15-like strains originating from different countries (**Figure 4**). One out of 61 (1.6%) of the ST15- and ST15-like isolates was found to contain the 1-bp *hsmA*

deletion (accession number ERR125985), but no metadata was available for this strain in the SRA to confirm metronidazole resistance. Interestingly, pCD-METRO carriage is distributed throughout the lineage with the TAT-C *hsmA* signature, suggesting that pCD-METRO may be preferentially acquired in strains with pre-existing low-level metronidazole resistance.

Altogether our results demonstrate that a specific signature of *hsmA*, resulting in an altered C-terminal protein sequence, is associated with heme-dependent metronidazole resistance as well as pCD-METRO carriage in RT010 strains of *C. difficile*.

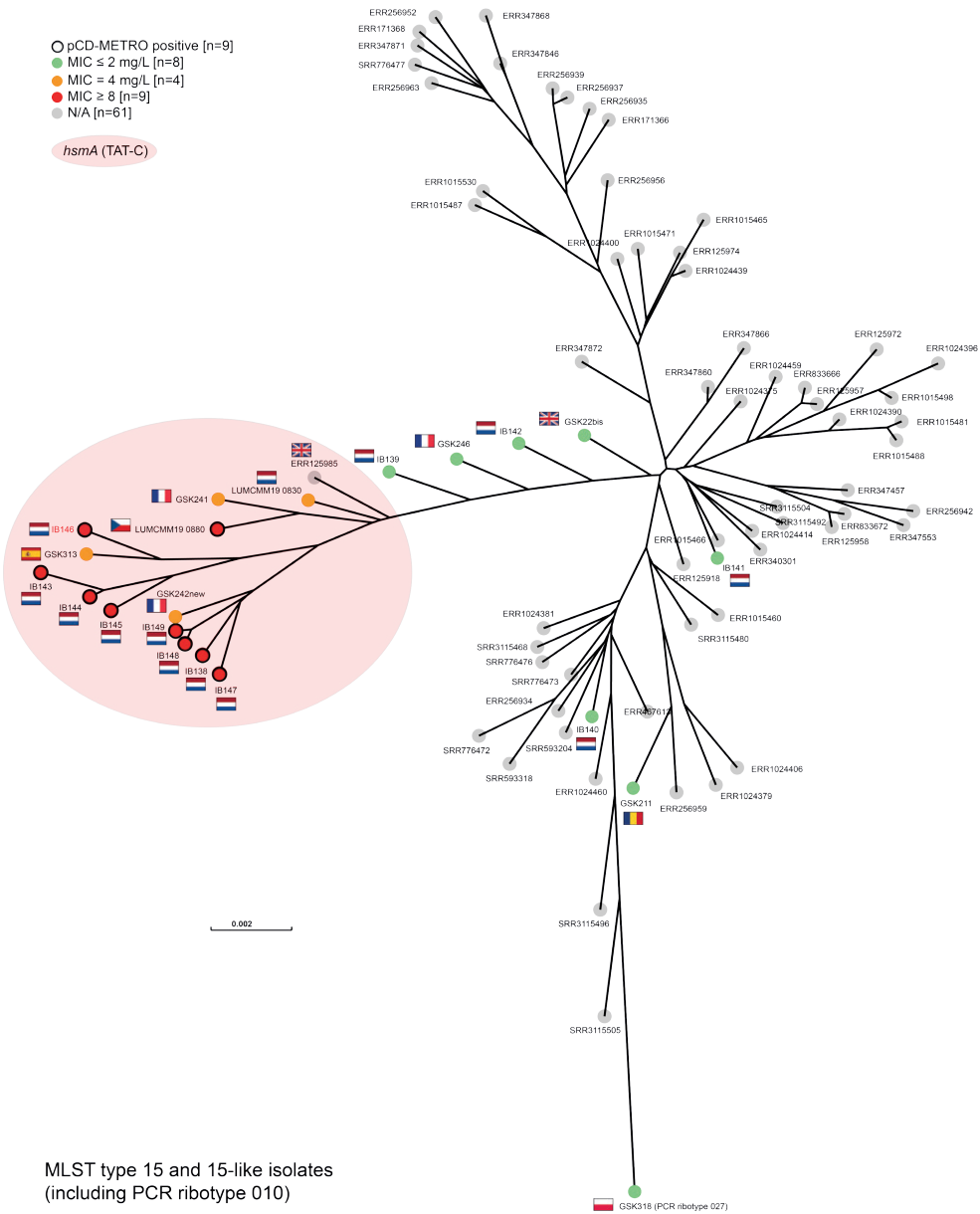


Figure 4. The C-terminal deletion in *hsmA* is associated with a lineage of ST15 and ST15-like isolates. When available, metronidazole susceptibility data and pCD-METRO carriage are indicated as illustrated in the legend. Country flags prior to strain name indicate the strain origin, when known. Strain names (235) or SRA accession numbers are included. Distances in tree are shown in logarithmic scale.

Discussion

In this study we describe a collection of clinical *C. difficile* isolates that demonstrate a heme-dependent increase in MIC to metronidazole and make the observation that four strains determined to be resistant to metronidazole required heme supplementation for this phenotype. Additionally, we show that a C-terminal deletion at position 372 in *hsmA* correlates to metronidazole resistance in RT010 isolates.

The observation that the MICs for certain antibiotics vary depending on the type of medium used has been well documented in other organisms, but little to no data is available for *C. difficile* (178, 235). One of the best known examples is the effect of divalent calcium on daptomycin susceptibility of a variety of organisms but other examples have been documented as well (320). For instance, *Escherichia coli* was sensitive to bleomycin in LB broth, but was resistant to this antibiotic in glucose minimal medium, though the mechanism behind this difference remains unclear (321). Similar results were obtained for *Moellerella wisconsensis* and *Proteus* spp. for fosfomycin resistance when comparing MICs on IsoSensitest broth and (cation-adjusted) Mueller Hinton broth (322, 323). Additionally, medium-dependent activity of gentamicin sulfate against enterococci has also been encountered, showing medium-dependent activity of antibiotics against bacteria is not a phenomenon restricted to Gram-negative organisms (324).

In this study we observed that almost all strains showed an increase in metronidazole MIC when grown on blood agar compared to BHI (**Figure 1, Table 2**). We note the limitation that the COMBACTE-CDI collection we analyzed here does not encompass an unbiased collection of PCR ribotypes, and in particular clade 2 strains (RT016, RT027, RT176, RT181, RT198) appear to be overrepresented. We found that heme supplemented to the blood agar plates was the causative determinant for this phenotype (**Figure 2, Table 2**), presumably through the ability of heme to detoxify the nitro-radicals generated by metronidazole activation (325). As lethal concentrations of antimicrobials are thought to generate toxic radicals by altering cellular metabolism, we expected to find heme-dependent alterations in antibiotic susceptibility for other antibiotics than metronidazole (255, 326). However, no heme-dependent reduction in vancomycin susceptibility was found in our study, suggesting that the effect of heme shows specificity for metronidazole under the conditions tested.

Elegant work by Knippel *et al.* has demonstrated that reduced metronidazole susceptibility upon heme supplementation in R20291 was largely mediated by the *hsmRA* operon, leading to the question whether presence/absence or sequence variants of these genes underlie heme-dependent resistance in other RTs (257). Based on the present dataset, however, we were unable to identify specific sequence variants of this operon (or in the *hatRT* operon also involved in heme detoxification) that could explain why the vast majority of strains are less susceptible to metronidazole upon heme supplementation. The genes appear to be (near-) universally conserved amongst different *C. difficile* types (data not shown) and the same signature is found in strains that do or do not respond to heme supplementation, and those that do or do not qualify as resistant (**Table 1, Table 2, Supplemental Table 1**). These results imply that other factors than the genome sequence of the *hatRT* and *hsmRA* operons can contribute to heme-dependent reduction in metronidazole susceptibility.

Nevertheless, we did identify a C-terminal deletion in *hsmA* that correlated to heme-dependent metronidazole resistance in RT010 (ST15) isolates (**Figure 3, Figure 4**). Isolates without this deletion did become less susceptible to metronidazole in presence of heme, but did not exceed the EUCAST criterium for resistance to this antibiotic (87). We validated our findings in the collection of clinical isolates used in the pCD-METRO study (235). At present, there is no structural information on the HsmA protein, though homology between the protein and heme-containing cytochromes has been noted (257). HsmA has been postulated to act through sequestration of heme, but the effect of the altered C-terminal sequence on the affinity for heme remains to be elucidated.

The COMBACTE-CDI collection analyzed here includes a limited number of strains per RT (**Table 1**). It will be interesting to see whether targeted analyses of larger collections of specific ribotypes will reveal additional sequence variants of *hsmAR* associated with reduced susceptibility or resistance to metronidazole.

Our data hint at a possible cumulative effect of chromosomal and extrachromosomal determinants in metronidazole resistance as strains carrying the pCD-METRO plasmid are dispersed over the resistant ST15/ST15-like (RT010) lineage characterised by the TAT-C *hsmA* signature (**Figure 4**). Strains that possess both the C-terminal adenine deletion in *hsmA* and the pCD-METRO plasmid have a higher metronidazole MIC (MIC $\geq$ 8 mg/L vs 4 mg/L in the pCD-METRO negative

RT010s). As no pCD-METRO positive RT010 isolate containing the TATAC signature sequence was present in this collection, we do not know if pCD-METRO carriage without the deletion can still result in an MIC of ≥ 8 mg/L, though this appears to be the case in RT020 and RT027 (235). Irrespective of the effect on metronidazole MICs, pCD-METRO carriage is associated with the 1-bp deletion in *hsmA* in RT010 isolates. Though this might result from a selection bias (by preferentially characterizing isolates with higher MICs), it is conceivable that the deletion facilitates pCD-METRO carriage in some way.

Our findings suggest that the heme-dependent reduction in metronidazole susceptibility is common in *C. difficile* (**Table 2**). Though heme levels can be elevated at the host-pathogen interface during CDI, (317) pathogenicity does not seem a requirement for the heme-dependent reduction in MIC as it is also found in non-toxigenic strains such as those belonging to RT010. For the same reason, it is unlikely that extensive and continued use of metronidazole provided the selective pressure for the acquisition and/or persistence of this phenomenon during evolution (83, 84, 294). In the majority of the strains the MIC will likely not be raised over the EUCAST cut-off (2 mg/L) for resistance, (87) but they clearly do become less susceptible to metronidazole. Due to absorption in the small intestine and sequestration- or inactivation of the microbiota, levels of metronidazole at the end of the colon are potentially low as determined by concentrations found in fecal material (276, 295, 297, 298). It is therefore quite possible that a moderate increase in metronidazole MIC could in fact facilitate growth of *C. difficile* in patients treated with metronidazole. Whether or not heme-dependent reduced susceptibility plays a role in treatment failure (that does not appear to correlate with metronidazole resistance) remains to be established (15, 235).

An important implication of our findings is that a well-described testing method in diagnostic antimicrobial susceptibility testing is of the utmost importance. The type of media and supplementation used can influence the MIC of certain antibiotics as we have demonstrated for heme supplementation and metronidazole susceptibility. It is our experience that small interlaboratory differences in standard operating procedures exist despite conforming to CLSI standards, which may explain the differences in MICs sometimes found between institutions. As medium-dependent differences in antimicrobial susceptibility may be both antibiotic- and organism-specific, this argues for a standardized method per genus rather than a standard testing method for all anaerobic organisms.

In conclusion, we have demonstrated that heme is the causative agent of medium-dependent reduction in metronidazole susceptibility in clinical *C. difficile* isolates of different ribotypes, but does not influence vancomycin susceptibility. Additionally, we have found a deletion in the C-terminal part of *hsmA* which correlates to metronidazole resistance in RT010 isolates.

Materials and Methods

Strain characterization

The strains analyzed here were collected as part of the COMBACTE-CDI study. Strains were characterized by PCR ribotyping following the *C. difficile* Ribotyping Network of England and Northern Ireland protocol and tested for metronidazole susceptibility in agar dilution experiments at the Healthcare Associated Infection group of the University of Leeds, United Kingdom (271, 327). Confirmation of PCR ribotype and additional susceptibility testing was performed at the Netherlands Reference Laboratory of *C. difficile*, hosted at the Leiden University Medical Center, The Netherlands (13). Use of the strains for the present study was approved by the Management Board of the COMBACTE-CDI Consortium. Strains were further characterized by whole genome sequencing (see below).

Metronidazole minimal inhibitory concentrations were determined using the agar dilution method according to CLSI guidelines on either Wilkins-Chalgren agar (in Leeds) or Brucella Blood Agar (BBA) (in Leiden) (86). Although no formal breakpoints have been defined, we used the EUCAST epidemiological cutoff of 2 mg/L to define metronidazole resistance in *C. difficile* in this study (87).

Determining medium-dependent metronidazole resistance

Agar plates on which *C. difficile* was grown were always incubated anaerobically at 37°C in a Don Whitley A55 workstation (10% CO₂, 10% H₂ and 80% N₂ atmosphere). For determining medium-dependent metronidazole resistance, *C. difficile* strains were first grown on reduced Brain Heart Infusion (BHI) agar plates supplemented with 0,5% yeast extract (Sigma-Aldrich) and *Clostridium Difficile Selective Supplements* (CDSS, Oxoid) for 24-48 hours. From these plates bacterial suspensions corresponding to 2.0 McFarland turbidity were made in PBS. These suspensions were then applied on BBA plates (bioMérieux) or on BHI agar supplemented with

0.5% yeast extract and when applicable with 1 µg/ml vitamin K (Sigma-Aldrich), 5% sheep blood (Thermo Fisher Scientific) and/or 5 µg/ml hemin (Sigma-Aldrich). Metronidazole or vancomycin E-tests (bioMérieux) were then applied and growth was evaluated after 48 hours of anaerobic incubation. Pictures were taken with an Interscience Scan 500 Automatic colony counter. Figures were prepared using ggPlotteR (<http://huygens.science.uva.nl/ggPlotteR/>) and Adobe Illustrator CC.

Whole genome sequencing and signature analysis

As part of the COMBACTE-CDI project, DNA extracts of the selected isolates were processed for whole genome sequencing at GSK Bio, as per their standard operating procedures. Briefly, total genomic DNA's were quantitated using Quant-iT dsDNA High-Sensitivity Assay Kit (Life technologies) and SYNERGY H1 microplate reader. Sequencing libraries were prepared from 1 ng of DNA using the Nextera XT DNA Library Prep kit (Illumina) and libraries concentrations were normalized using bead normalization as described by the manufacturer. Sequencing was performed on the Illumina MiSeq platform with MiSeq v3 600-cycles kit or on the Illumina NextSeq 500 platform with NextSeq 550 High-Output v 2.5 300-cycles kit. FASTQ files passed the quality control checks and strain identification confirmation (*C. difficile*), as performed in the BIOMERIEUX EPISEQ® CS beta platform (<https://www.biomerieux-episeq.com/>). Sequence data for this study is publicly available under BioProject number PRJNA679767 (sample accessions: SAMN16845917-16845948).

For the SNP analysis, reads were mapped to the *hsmA*, *hsmR*, *hatT* and *hatR* genes obtained from the reference sequence of strain R20291 (GenBank entry FN545816.1). Mapping of reads to the reference sequences was done with Bowtie2 tool (version 2.3.4, options: --local --qc-filter) and results of this mapping were analyzed using Samtools (version 1.7, default options) and depth of the reads coverage was calculated using IGV tool (version 2.3.98, options: -w 1) (301, 328, 329). Average depth calculated for all positions of the analyzed isolates is shown in **Supplemental Table 1**. Values of depth of coverage obtained for each position (nucleotides or indels) for all samples were filtered using a minimum coverage of 5 reads for each position. Mapping results for all positions were compared between all strains. Positions where at least one strain has a mutation or variation compared to the corresponding reference sequence were included in the generation of the genetic signature of *hsmA*, *hsmR*, *hatT* and *hatR*.

Phylogenetic analysis

ST15 and 15-like publicly available whole-genome sequencing data (**Supplemental Table 2**) were selected based on a previous analysis, and corresponding FASTQ files were downloaded from the SRA (n=85) (284). wgSNP analysis was performed on the Enterobase platform with a selected minimum frequency threshold of 0.1 (318, 319). Three entries were excluded from the analysis, as they did not pass the quality control of the Enterobase platform (**Supplemental Table 2**) (319). A wgSNP maximum likelihood tree was generated with RaxML and tree branches were represented in a log scale for clarity (330). Figure was further prepared for publication using Adobe Illustrator CC.

Acknowledgements

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Transparency declaration

The COMBACTE-CDI consortium includes partners GSK, Pfizer, DaVolterra, AstraZeneca, Sanofi Pasteur and bioMérieux. The companies did not have a role in the design and execution of the experiments for this study but provided the WGS of the isolates (GSK) and allowed examination of the FASTQ files (bioMérieux). Conceived the study: IMB, EJK, JF, WKS. Performed experiments: IMB, CH, IMJGBS, BS, EC, JF. Analyzed data: IMB, SN, IAS, VV, KD, JF, WKS. Drafted manuscript: IMB, WKS. All authors edited and approved the final version of the manuscript. Parts of the data in this manuscript have previously been presented at the virtual 7th International *C. difficile* Symposium.



6

GENERAL DISCUSSION

Antimicrobial resistance (AMR) is a contemporary problem that results in approximately 700.000 deaths per year globally (331). As modern health care systems are reliant on antimicrobial use, this number is predicted to rise to 10 million by 2050 if no action is taken, and will be accompanied by an enormous reduction in the Gross Domestic Product (GDP) (66). The general principle behind antimicrobial resistance generation is that antimicrobial use will select for resistant bacteria by killing susceptible ones, allowing propagation of the resistant strains. Additionally, antimicrobials can facilitate de novo resistance generation by providing an environment in which particular changes resulting in reduced susceptibility (for instance uptake of extrachromosomal elements or mutagenesis) lead to an increase in fitness (332, 333). Widespread and excessive antimicrobial use can thus contribute to the abundance of resistant bacteria in different settings. These resistant bacteria in humans, animals, healthcare settings and other environments such as water, soil and food (so called 'resistomes'), contribute to further transmission of resistant bacteria (334). The problem of AMR is therefore not restricted to human medicine, but should be regarded in the context of 'One Health': the approach in which human health is regarded as closely related to animal health and the environment (335).

Clostridioides difficile is an organism with intrinsic and acquired resistance to various antimicrobials. Infection with this bacterium is associated with a substantial economic burden (20, 45). Antimicrobial use disrupts the healthy intestinal microflora normally providing colonization resistance against *C. difficile* and in this manner predisposes individuals to a *C. difficile* infection (CDI) (23). *C. difficile* is traditionally regarded as a nosocomial pathogen since hospitalized patients frequently use antibiotics, have significant comorbidities and stay in an environment with spread- and transmission of spores (20). However, an increasing number of CDIs appear to be unrelated to hospitals and antimicrobial use, and instead can be traced back to the community: Community-Associated CDI (CA-CDI) (56). The highly resilient endospores produced by *C. difficile* are ubiquitous and it is possible humans are asymptotically colonized by *C. difficile* (60). PCR ribotype 078 (RT078) isolates are associated with CA-CDI and are isolated from both humans and farm animals, suggesting animal reservoirs have a role in the spread of *C. difficile* (65). However, it is also important to increase our understanding of the molecular mechanics behind antimicrobial resistance in *C. difficile*, of which aspects are explored in this thesis.

The role of σ^B in the pleiotropic effects following DNA polymerase inhibition

As disruption of the healthy intestinal microflora is a risk factor for CDI, it is important to use a narrow-spectrum antibiotic in order to disturb the resident microbiota as little as possible. Currently, the only narrow-spectrum antibiotic used in treatment of CDI is fidaxomicin. The novel antibiotic ibezapolstat (formerly known and referred to as ACX-362E) is currently tested in phase 2 clinical trials. Ibezapolstat inhibits the DNA polymerase III α subunit, which is required for replication in Gram-positive organisms with low [G+C]-content (105). Ibezapolstat is therefore presumed inactive against many bacteria part of the gut microbiota. Results from the phase 1 clinical trial indeed demonstrated that, compared to vancomycin, ibezapolstat treatment resulted in a distinct microbiome profile with an increase in Actinobacteria. (105). Preliminary results of the phase 2A trial indicated that all CDI patients treated with ibezapolstat met the primary- and secondary endpoint of clinical cure at the end of the study, and sustained clinical cure of no recurrence in the 28 following days (acurxpharma.com). In **Chapter 2** we have demonstrated that exposure of 630 Δ erm with ibezapolstat leads to a gene-dosage shift. This shift is characterized by an increase in copy number of genes in close proximity to the origin of replication (*oriC*) resulting from multifork replication (**Chapter 2, Figure 3**). As multifork replication is a process occurring during fast, exponential growth to accommodate short doubling times, this effect is amplified when the replisome responsible for DNA replication is stalled (336). Indeed, in *Bacillus subtilis* it was shown that replisome inhibition did not abolish, but potentially even stimulated replication (187). We have shown that DNA polymerase inhibition increases the copy number as well as the expression of genes in close proximity to the *oriC*.

One of the genes whose copy number and expression was increased as a result of ibezapolstat treatment, was the alternative sigma factor B (σ^B) which regulates the general stress response in many Gram-positive organisms (337). The general stress response is important for the ability of the organism to cope with various environmental stresses and nutrient availability (200). Close proximity of a regulator gene to the *oriC* may reflect its importance during early replication or replication inhibition, and we hypothesized that a similar genomic organization could be a conserved response to replication stress (199). There is indeed evidence that genome organization is not random. When the laboratory adapted 630 Δ erm strain was sequenced, differences in genome sequence were found compared to its parental strain 630 (190). An additional rRNA/tRNA cluster was found in close proximity to *oriC* in the laboratory strain,

resulting in an extra copy of ribosomal RNA (*rrn*) operons. Fast growing organisms more frequently contain a higher number of *rrn* operons, and an origin-proximal location results in more rapid availability of ribosomes during early replication to cope with increased protein demand during fast growth (338, 339). Though costly, constant growth under nutrient-rich conditions, which are the conditions often used in the laboratory, may allow acquisition of extra *rrn* operons and consequently improve fitness (340). This example illustrates how genome organization can lead to direct fitness effects and selection. The origin-proximal existence of a transcriptional regulator is therefore an interesting observation and could imply early activation of σ^B during replication inhibition is beneficial to *C. difficile* growth, despite very costly expression (**Chapter 3, Figure 2**) (169). To test if σ^B activity could change ibezapolstat susceptibility in *C. difficile*, a disk diffusion test, a growth curve in liquid culture or agar dilution could be performed on wildtype 630 Δ *erm* and IB56 (630 Δ *erm* Δ *sigB*). Although both 630 Δ *erm* and IB56 grew similarly in the presence of subinhibitory concentrations of ibezapolstat in liquid culture (data not shown), we have not looked at the effect of adding this antibiotic at the levels of the MIC or above. It is possible σ^B does not provide a fitness advantage during replication inhibition and is not involved in reduced ibezapolstat susceptibility, in which case no differences between wildtype 630 Δ *erm* and IB56 will be found in the previously proposed experiments. Instead, σ^B could be beneficial to the bacteria during normal (fast) growth, which is in itself a stressful condition and may have led to the origin proximal location of this sigma factor.

Approximately 13% of the 630 Δ *erm* genome was shown to be differentially regulated by ibezapolstat exposure (**Chapter 2, Figure 2**). This differential regulation is effectuated by a direct effect of the gene-dosage shift but is also mediated by indirect effects, such as the increase in expression of transcriptional regulators. During our work on the gene-dosage effect of ibezapolstat treatment in 630 Δ *erm*, a study was published describing the putative *C. difficile* σ^B regulon. A large overlap was seen between genes demonstrating an increase in expression after ibezapolstat treatment and those positively regulated by σ^B , even after we significantly refined the regulon (**Chapter 3**). We showed that removal of σ^B reduced or abolished the increase in gene expression following ibezapolstat treatment. This result demonstrated that DNA polymerase inhibition increased the expression of σ^B and some of its target genes as a result, contributing to the large number of detected differentially regulated genes. Situations that perturb replication, such as DNA replication inhibition, are believed to result in single stranded DNA (ssDNA), potentially activating the SOS response regulated by LexA/RecA (217,

341). Similarly, the heat shock response regulated by HrcA/CtsR could also be activated by replication inhibition. Although none of the regulators of these stress responses, including *sigB*, were found to be differentially regulated after 5 hours of subinhibitory ibezapolstat treatment based on the RNA-seq experiments, differential regulation of the *sigB* operon after DNA polymerase inhibition could be demonstrated using luciferase reporters (**Chapter 2, Figure 5**). As a result it could be possible that both the SOS response and the heat shock response could result in differential regulation of genes following ibezapolstat treatment, as was seen for σ^B (213, 214). It is unclear if DNA polymerase inhibition increases σ^B expression as a result of the gene dosage shift or due to direct activation of σ^B , but it is likely both effects contribute to this transcriptional response. Marker frequency analysis (MFA) demonstrated that the relative increase of origin-proximal genes compared to terminus-proximal genes could be as high as a 10-fold (**Chapter 2, Figure 4**). Although the fold changes were averaged, the results obtained from the RNA-seq also demonstrated a clear correlation between the change in amount of RNA transcripts and genome location of the gene (**Chapter 2, Figure 3**). These results imply the increase in σ^B may be primarily caused by the gene dosage shift resulting from ibezapolstat treatment. Genetically modifying strain 630 Δ *erm* by moving *sigB* from its origin-proximal location to one closer to the terminus (*terC*) and repeating the experiment and transcriptome analysis (**Chapter 2**) will provide more information on whether this is indeed the case. However, all these results apply to a transcriptional level and do not provide information on any posttranslational modifications resulting from ibezapolstat treatment. Using a quantitative proteomics approach such as SILAC (Stable Isotope Labeling by Amino acids in Cell culture), more information can be acquired on the changes in protein levels following DNA replication inhibition.

DNA replication inhibition and mutagenesis mediated by the general stress response

DNA polymerase inhibition and the role of the general stress response in this process remains poorly understood in *C. difficile*. Several studies have demonstrated a link between mutagenesis resulting from DNA damage and the general stress response, although the SOS response is also involved in this process, as will be illustrated below. It has been shown in *E. coli* that overexpression of the SOS response by the general stress response leads to overexpression of translesion synthesis (TLS) (error-prone) polymerases and, in combination with existing DNA damage, to mutagenesis, potentially facilitating antimicrobial resistance (342-347). In *C. difficile* the SOS response was demonstrated to control biofilm- and toxin production and

sporulation in addition to DNA repair and could be induced by the fluoroquinolone levofloxacin (152). It is not yet known if the SOS response (possibly in combination with the general stress response) is also associated with error-prone DNA repair and mutagenesis in *C. difficile*. The capacity for antibiotic resistance generation by DNA damage, the SOS response and the general stress response has been shown recently in *E. coli*. Ciprofloxacin, a fluoroquinolone class of antibiotic and DNA-gyrase inhibitor, stalls the bacterial replication fork and, similarly to ibezapolstat, increases the copy number of origin-proximal genes (188). It is known that sub-inhibitory concentrations of ciprofloxacin can induce *de novo* mutations resulting in resistance generation in *E. coli* (348). This ability of ciprofloxacin to induce novel mutations leading to antibiotic resistance was found to be mediated by the general stress response sigma factor σ^S (RpoS), the equivalent of σ^B in various gram-negative organisms (337). Ciprofloxacin use resulted in double-stranded breaks (DSBs) which activated the SOS response in *E. coli* (349). The activated SOS response in turn inhibited cellular respiration and disturbed the electron transport chain (ETC), which resulted in an increase in radical oxygen species (ROS). These radical species induced σ^S and initiated mutagenic break repair (MBR) by activating error-prone DNA polymerases, resulting in mutations in *rpoB* and *ampD* that generated resistance to rifampicin and ampicillin, respectively (**Figure 1A**) (349). Activation of σ^S was only seen in a fraction of cells, leading to a bet hedging population of so called ‘gambler’ cells that risked acquiring debilitating mutations for a chance of becoming more resistant to different antibiotics. General stress response mediated mutagenesis was also observed after β -lactam addition in *E. coli* (350).

Interestingly, σ^B was shown to be similarly stochastically expressed in *C. difficile* (169). Our refined σ^B regulon also demonstrated that σ^B positively regulated *recA* and *uvrBAC* endonucleases involved in the SOS response and DNA repair (**Chapter 3**), implying that σ^B could enhance the SOS response (152, 217, 351). Additionally, the SOS-inducible subunit of the PolIII complex, DnaE, was demonstrated to be error-prone in, for instance, *Streptococcus pyogenes* (352-354). Although DnaE lacks proofreading activity, it was found to be essential for DNA replication in *B. subtilis*. DnaE appears to be involved in a ‘handoff’ mechanism *in vitro*, in which the RNA primers in lagging strand synthesis were briefly extended by this part of the PolIII complex, after which more extensive elongation by PolC followed (355). Time-lapse microscopic imaging experiments confirmed the brief association of DnaE with the replisome *in vivo*, supporting the results obtained *in vitro* (356). Despite this short interaction time, DnaE was reported to be

involved in mutagenesis in *B. subtilis* (353). Based on our regulon, DnaE was positively regulated by σ^B in *C. difficile*. DnaE was shown to be a part of the SOS response in other organisms, but based on *in silico* analysis this gene was not regulated by LexA in *C. difficile* (217, 352, 354). However, the results described above do indicate a certain degree of convergence for the SOS response and the general stress response, as *uvrBAC* are activated early in the SOS response, but were also clearly regulated by σ^B in *C. difficile* (**Chapter 2, Figure 6** and **Chapter 3, Figure 4**) (217, 351). It is not impossible that replication disturbed by DNA polymerase inhibition activates or reinforces the SOS response in a σ^B dependent manner. Based on this information, an interesting question to answer is whether σ^B could be similarly involved in mutagenesis after ibezapolstat exposure or other forms of DNA replication inhibition (**figure 1B**). Although no resistance to this antibiotic was found in *C. difficile*, it is possible resistance to other types of antibiotics could be induced as has been reported in *E. coli* (232, 350). It should be mentioned that no increase in dwelling time of DnaE in association with the replisome was found in *B. subtilis* after DNA polymerase inhibition by HPUra (a DNA polymerase inhibitor), but perhaps this may not be relevant for mutagenesis (if it is indeed mediated by DnaE), or other error prone polymerases may still be involved in mutagenesis resulting from DNA damage and the general- and SOS stress response (356). Ames tests have been used to evaluate the mutagenic (carcinogenic) potential of compounds by incubating auxotrophic bacterial strains with the desired compound and determining if this auxotrophy is lost. A similar approach can be used to determine if ibezapolstat treatment is involved in σ^B dependent mutagenesis in *C. difficile* (**Figure 2**). In this assay, wildtype 630 Δ *erm* and IB56 (Δ *sigB*) can be incubated with ibezapolstat (or other compounds such as metronidazole, mitomycin C and hydrogen peroxide), after which they are plated on agar medium containing an antibiotic (for instance rifampicin). In parallel these two strains will be plated without having been exposed to ibezapolstat or another compound to determine the 'background' mutation frequency. Comparing the number of (resistant) colonies that emerge will indicate if the tested compound increases the mutation frequency (in a σ^B dependent manner) in *C. difficile*. Genes known to be often involved in resistance to these antibiotics can be sequenced in these resistant colonies to determine what mutations have occurred, or this could be determined by whole genome sequencing analysis (WGS). It should be mentioned that these kind of mutagenic processes are believed to be mediated by ROS which may not be formed under anaerobic conditions (357). Similarly, ampicillin treated *E. coli* developed resistance to norfloxacin while this process was inhibited by the presence of radical scavengers such as thiourea and strict anaerobic conditions (358).

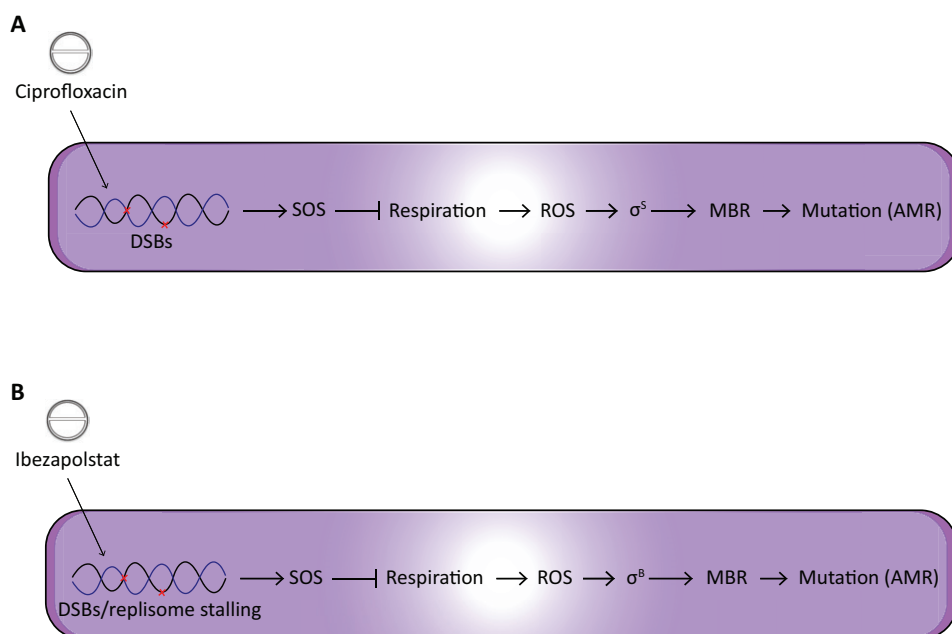


Figure 1. Mechanisms for antibiotic-induced resistance generation. A) Ciprofloxacin induced mutagenesis leading to antibiotic resistance, mediated by the SOS- and general stress response (σ^S) through mutagenic break repair (MBR) as was shown in *E. coli*. **B)** Hypothetical situation of ibezapolstat induced mutagenesis in *C. difficile*, mediated by the SOS and general stress response (σ^B) through mutagenic break repair. DSBs: Double-stranded breaks; SOS: SOS response; ROS: Radical Oxygen Species; MBR: Mutagenic break repair; AMR: Antimicrobial resistance. Image adapted from (349).

However, many central metabolic processes and electron transport chains differ in facultative anaerobes from strictly anaerobic organisms and not many studies are conducted on the latter type of organism. It would therefore still be novel and of interest to further study these stress-response based mutagenic processes in *C. difficile*.

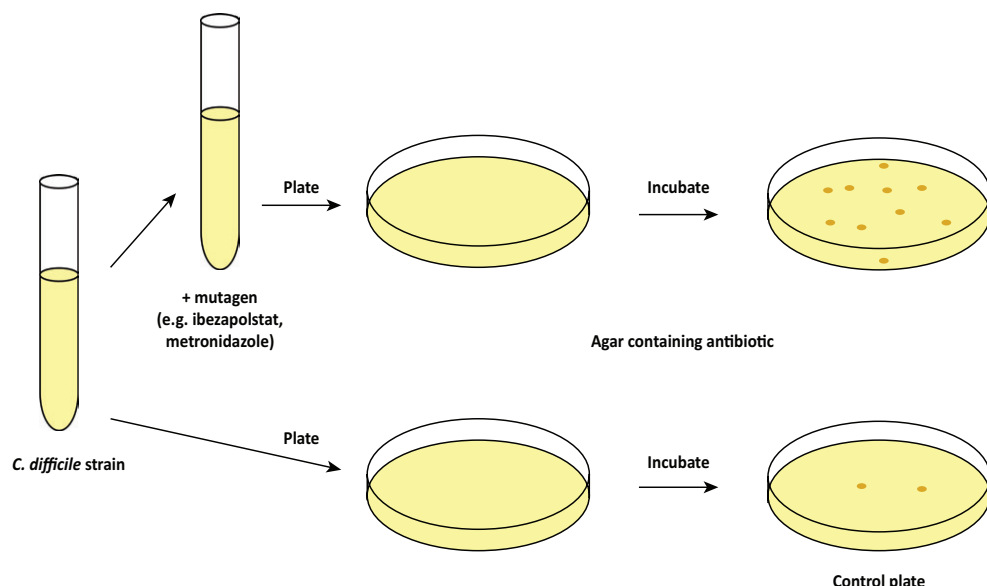


Figure 2. Adaptation of the ‘Ames test’ to determine the (σ^B -mediated) mutagenic potential of certain compounds and antimicrobials in *C. difficile*.

Antibiotics disturb cellular downstream processes which may lead to σ^B activation

The general stress response in *C. difficile* is involved in protection against physiological- (host) and inflammatory responses such as ROS, reactive nitrogen species (RNS), nitric oxide (NO) and antimicrobial peptides (20, 172). It has also proven crucial for survival under oxygen tension, an observation reinforced by our results indicating approximately a third of the entire σ^B regulon is designed to regulate genes putatively involved in oxidative and nitrosative stress responses such as thioredoxin reductases (**Chapter 3**) (161). A role for the general stress response and antimicrobial resistance was demonstrated in other organisms, and for reduced susceptibility to rifampicin and mitomycin C in *C. difficile* (160, 170, 230, 231). Preliminary results hinted at a relationship between σ^B and metronidazole resistance (data not shown) which was the premise in which we wanted to study the role of the general stress response and antibiotic resistance in *C. difficile*. Our work was complicated by the toxicity of σ^B leading to loss of the plasmid used for *in trans* overexpression of this sigma factor (**Chapter 3, Figure 3**).

In an attempt to work around this toxicity the plate-based luciferase assay was designed to establish an indirect relation between activation of the general stress response and antibiotic exposure. In this assay we could measure σ^B dependent promoter activation of genes in its

regulon and analyze if antibiotic exposure would therefore alter activation of the general stress response. We found this to be the case for a subset of σ^B target genes after metronidazole exposure, which could be explained by the fact certain genes in the regulon appear to be regulated by other factors as well (**Chapter 2**, and **Chapter 3**). Additionally, although CD2963 expression appeared to be strictly dependent on σ^B , no clear activation attributable to metronidazole could be detected. As CD2963 is a L,D-transpeptidase it is possible that σ^B strongly induces transcription of this gene for cell wall synthesis to facilitate growth under nutrient-rich conditions. In such a situation high background levels will cloud the chemiluminescent signal of activation attributable to metronidazole exposure. However, for CD0350 (encoding a putative hydrolase involved in oxidative stress) we observed σ^B dependent activation induced by the presence of all compounds tested, which included antibiotics with a different mechanism of action (**Chapter 3, Figure 6**). Although we expected metronidazole and hydrogen peroxide to activate σ^B due to their role in redox and Fenton reactions, respectively, it was surprising to observe this effect for the other tested antibiotics as well (124, 130). These are believed to target processes unrelated to DNA damage or oxidative and nitrosative stress responses, but instead act on the cell wall (vancomycin), DNA replication (ibezapolstat), or translation (lincomycin). Furthermore, despite clear activation of σ^B dependent genes, no effect on the minimal inhibitory concentration (MIC) was seen between wildtype 630 Δ *erm* and the *sigB* knockout for metronidazole when tested by E-test (data not shown). In contrast, a moderate difference in metronidazole susceptibility between 630 Δ *erm* and the *sigB* mutant was found when tested with a disk diffusion assay (359). Although there is apparent overlap between the transcriptional response following metronidazole treatment and σ^B activation, these results imply the effect of σ^B on metronidazole susceptibility is minor and strongly dependent on the resolution of the assay used.

Interesting results have been published and- although far from conclusive- may provide part of an explanation on how toxic compounds with different mechanism of action could activate σ^B . Over the years a dividedness has followed the so-called ‘ROS’ debate, in which bactericidal antibiotics are believed to generate ROS and/or hydroxyl radicals (360). These deleterious radical species are believed to disrupt cellular processes such as depleting NADH, destabilizing iron-sulfur clusters and causing oxidative damage, and that these species strongly contribute to antibiotic lethality (253-256, 358, 360, 361). Relationships between antibiotic susceptibility and ROS production have been demonstrated in *Staphylococcus aureus*, *Enterococcus faecalis*,

Pseudomonas aeruginosa and *E. coli* (362). Despite the great number of studies demonstrating a relationship between antibiotics and oxidative stress responses, this claim has been questioned. One study performed by Liu *et al.* did not find ROS formation after exposing *E. coli* to antibiotics, and antibiotic lethality persisted even under anaerobic conditions (357). Similarly, another study reported no differences in antibiotic killing under aerobic- or anaerobic conditions even in the presence of a radical quencher (363). Part of these results are believed to be explained by the slowed respiration and subsequent inhibition of reactive species formation under anaerobic conditions. Supplementation with nitrate, frequently used as a terminal electron acceptor under anaerobic conditions, facilitated killing by various antibiotics under anoxia and imply reactive nitrogen species, just like ROS, contribute to antibiotic lethality (253). The results obtained from these studies all imply that usage of different antibiotics ultimately result in the formation of radical species, be they oxygen- or nitrogen based or otherwise, but may not be the sole reason for antibiotic lethality. Based on the σ^B regulon and its seemingly extensive role in dealing with these kind of stresses, it seems likely these radical species could also be involved in σ^B activation. Additionally, interesting ideas about the proximal effects (the conventional 'mechanism of action') and ultimate effects (physical or genetic destabilization leading to cell death) of antibiotics have been discussed (364). There is a growing body of evidence that antibiotics with different proximate effects converge in disrupting downstream processes, inducing cell damage and destroying critical processes required for metabolism and cellular- and genomic integrity, limiting the ability of bacteria to cope with antibiotic exposure, the ultimate effect (**Figure 3**) (364). Although the primary antibiotic target is important in determining how- and which of these downstream processes are disrupted, it appears the latter is what truly leads to cell death. It is likely that a variety of these so called 'death processes' induced by the ultimate antibiotic effects also lead to σ^B activation (365). The implications of potential activation of the general stress response under these conditions warrants further study.

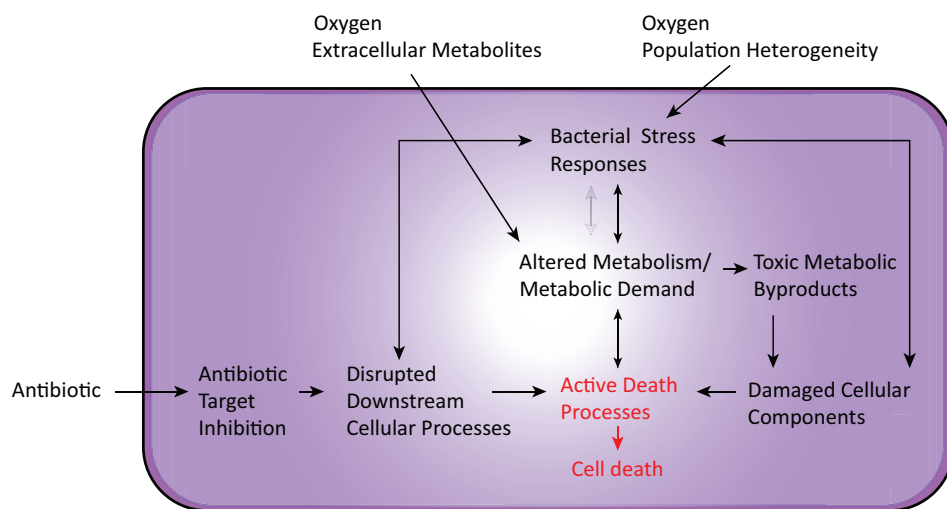


Figure 3. Downstream effects of antibiotic exposure. Antibiotics exert downstream cellular effects after target inhibition inducing active death processes. The resulting damage could activate stress responses which may facilitate further disruption. Environmental factors such as nutrient availability and population heterogeneity can influence various aspects of these interrelated processes. Image adapted from (365).

Heme (ab)use in *C. difficile*

We have observed that heme supplementation reduced metronidazole susceptibility and sometimes lead to resistance in *C. difficile* (Chapter 4). Although radical species are important in synergistic killing, it is not the ultimate effect of each and every kind of antibiotic (361, 364). No significant effect on heme supplementation and vancomycin susceptibility was seen in *C. difficile*, while we did see this for metronidazole (Chapter 5, Figure 2). As the ultimate effects of metronidazole lethality are believed to be mediated by nitro-radical formation, the putative *in vivo* function of heme may explain how it could act protectively in these conditions. Heme is an iron-containing protoporphyrin with homology to the cytochrome b561 family (366). It is known to be toxic to numerous organisms such as *S. aureus*, *Bacillus anthracis*, *Listeria monocytogenes* and *E. faecalis* as illustrated by the presence of detoxification system *hatRT* and *hsmRA* orthologs such as *hrtAB* and *hssRS* (367-370). *C. difficile* R20291 *hatR*, *hatT*, *hsmR* or *hsmA* ClosTron mutants were significantly more susceptible to heme toxicity than the wildtype strain (257, 317). Heme toxicity is thought to originate from nonspecific redox cycling occurring between heme and oxygen, and by destabilization of the cell membrane (369, 371). Despite the apparent necessity for heme detoxification systems in organisms able to infiltrate

vertebrate hosts, heme can be utilized for processes beneficial to bacteria as well. In respiring bacteria b561 family cytochromes such as heme can act as carriers of electrons originating from catabolized carbon sources in order to generate a proton-motive force (372, 373). No respiration occurs in *C. difficile*, but it is able to utilize heme as a nutrient source under iron-depleted conditions (374). Additionally, the observation that *hsmA* clostron mutants generated more radical oxygen species (ROS) when treated with heme compared to its untreated control, implies *hsmA* bound heme may be used as an electron sink comparable to the function of cytochrome b561 (257). It is possible heme can be used to ‘catch’ the damaging nitro-radicals formed by metronidazole activation, but that vancomycin mediated toxicity originates from other disturbed downstream processes not compensated for by heme.

Based on the effects on the host resulting from CDI it could indeed be beneficial for *C. difficile* to carry genes involved in heme detoxification. Damage to the gut epithelium from toxin exposure can lead to blood leaking into the intestinal lumen and after hemolysis may result in free heme (375, 376). Severe *C. difficile* infections can indeed result in bloody diarrhea and C57BL/6 mice infected with the RT027 strain R20291 demonstrated accumulation of hemoglobin in the cecum during CDI (20, 317). For this reason, carriage of the *hatRT* and *hsmRA* operons for heme detoxification may be found frequently in *C. difficile*. Indeed, performing a BLAST search of these operons against 5336 assembled *C. difficile* genomes available at the NCBI database demonstrated that approximately 60% of all available isolates contained a chromosomal sequence with significant homology to the R20291 *hatRT* operon, and approximately 80% demonstrated this for *hsmRA* (data not shown). If the widespread availability of heme detoxification systems in *C. difficile* is indeed associated with the frequent encounter of blood in the intestinal lumen from toxin production, it is puzzling to see these operons being present in nontoxigenic isolates as well. Additionally, heme-mediated resistance to metronidazole by a C-terminal deletion in *hsmA* was also detected in nontoxigenic RT010 isolates. Several explanations for this may exist. First, as 10% of the genome of *C. difficile* is made of mobile genetic elements (MGEs) it may be possible that the pathogenicity loci containing the various toxin genes were lost after acquisition of the *hatRT* and/or *hsmRA*-like operons (27, 35, 51, 199). Performing *in silico* analysis on the evolutionary ‘age’ of RT010 isolates in relation to toxinogenic strains would give a good indication if this could indeed have occurred. Nontoxigenic *C. difficile* isolates are often isolated from dogs and metronidazole is frequently used for treatment of CDI and *Giardia* spp. infections in these pets, and extensively used to

treat enteric diseases and protozoal infections in animals in veterinary medicine (377, 378). It is therefore possible that sufficient metronidazole pressure contributed to retention of heme detoxification mechanisms as these may facilitate heme-mediated metronidazole resistance. Second, it may be possible that colonization by nontoxigenic strains occurs frequently in conjunction with an infection caused by a toxigenic strain. Nontoxigenic strains are believed to not cause diarrhea but are still sometimes isolated from patients suffering from diarrhea. In routine diagnostics usually only one- or a few colonies are picked for laboratory typing, and it is possible the symptoms are caused by a toxigenic strain missed at that point in time. It may be worthwhile to investigate if patients with CDI indeed are also frequently colonized by nontoxigenic strains, by picking and typing multiple colonies during (routine) diagnostics.

It is not yet clear why the deletion in *hsmA* leads to metronidazole resistance in the tested *C. difficile* isolates. The deletion at position 372 in *hsmA* results in a frameshift, adding 6 additional amino acids to the C-terminus in comparison to wildtype protein. Introducing this *hsmA* deletion in the chromosome of 630 Δ *erm* by Allelic-Coupled Exchange (ACE) and testing metronidazole susceptibility in this mutant and the wildtype, would confirm if this deletion is indeed responsible for an increase in metronidazole MIC or associated with reduced heme toxicity. Alternatively, *hsmA* could be knocked out and wildtype and mutant *hsmA* could be overexpressed *in trans*. Testing metronidazole and heme susceptibility in these mutants would provide valuable information on the role of *hsmA* in *C. difficile*. Wildtype and mutant *hsmA* could be overexpressed in *E. coli* with and without heme addition, and *in vitro* spectrophotometric assays as described in Knippel *et al.* would then indicate if heme binding affinity would increase as a result of this deletion (257).

***hsmA* and pCD-METRO in metronidazole resistance**

The deletion in *hsmA* leading to a frameshift and subsequent extension of the C-terminus was found in sequence type 15- (ST15, RT010) and a ST15-like isolate and lead to heme-dependent metronidazole resistance in the RT010s (**Chapter 5, Figure 3**). Based on the phylogenetic analysis (**Chapter 5, Figure 4**) it appears this deletion was introduced at a certain point in time in a specific divergent clade of ST15 and ST15-like isolates. It was surprising to see that all strains carrying the metronidazole resistance conferring plasmid pCD-METRO (**Chapter 5**) also carried the deletion in *hsmA*. pCD-METRO carriage was demonstrably involved in metronidazole resistance, as introduction of pCD-METRO^{shuttle} in the metronidazole susceptible

630 Δ *erm* caused this strain to become resistant to this antibiotic (MIC ≥ 8 as determined by agar dilution). pCD-METRO is also found in multiple PCR ribotypes that lack the *hsmA* deletion ((235) and unpublished observations). Furthermore, pCD-METRO carriage was associated with higher MICs and unconditional (heme-independent), stable metronidazole resistance. As the deletion in *hsmA* also caused metronidazole resistance (MIC=4) in RT010 isolates lacking pCD-METRO, carrying both of these determinants seems at least partially redundant. Despite this apparent redundancy, pCD-METRO carriage did not occur in ST15- and ST15-like strains with ‘wildtype’ *hsmA*, which is puzzling. As mentioned previously, this deletion in *hsmA* was found in strains often isolated from dogs, which are frequently exposed to metronidazole (377, 378). Based on our data (**Chapter 4**) it is likely that pCD-METRO is transferred to *C. difficile* under metronidazole pressure, at least in human patients. Perhaps the deletion in *hsmA* occurs most rapidly under metronidazole pressure, and a further increase in MIC resulting from subsequent pCD-METRO acquisition could still be beneficial to the bacterium (379). Alternatively, it is possible that the deletion in *hsmA* predisposes isolates for pCD-METRO uptake in an as of yet unknown manner. More information on this process could be provided by further enlarging the collection of metronidazole resistant isolates in order to see if there truly are no pCD-METRO positive RT010 isolates that do not carry the deletion in *hsmA*, and to study the evolutionary diversity between these isolates. As we currently understand very little about the transfer and origins of pCD-METRO, further elucidating the occurrence of these events will also contribute to our understanding in these processes.

The origin of pCD-METRO

Above we mention that we still understand little about the origins of the plasmid (**Chapter 4**). The plasmid conferring metronidazole resistance in *C. difficile*, pCD-METRO, was found in clinical isolates of different ribotypes, including RT010, RT020 and RT027, from various (European) countries. On multiple occasions, we have found potential transfer by comparing longitudinally obtained susceptible and resistant isolates, including – recently – a new PCR ribotype, RT005 (data from the National Reference Laboratory for *Clostridioides difficile* at the Leiden University Medical Center, not shown). Our data imply that this plasmid can be acquired by horizontal gene transfer (HGT) from an unknown donor organism. However, several conditions need to be met in order for HGT to occur. Sufficient donor cells carrying the transferable determinant are required for HGT to be adaptive. This is difficult in a mobile environment, but could be facilitated in biofilms due to closer proximity of donor and recipient cells (380). There is

evidence that subinhibitory concentrations of metronidazole can increase biofilm formation in *C. difficile*. The RT010 strain carrying pCD-METRO did not exhibit increased biofilm production after exposure with subinhibitory concentrations of metronidazole, whereas this increase was observed in the other (metronidazole sensitive) RT010 isolates (381). This implies metronidazole susceptibility is required for increased biofilm formation in the presence of metronidazole in these strains. An acquired extrachromosomal element needs to be sufficiently beneficial to the recipient to compensate for the energetic costs associated with competence, as well as for the risk of taking up selfish genetic elements (382). Additionally, our data seems to imply that this plasmid was acquired under metronidazole pressure, as after failed metronidazole treatment a resistant, pCD-METRO positive clonal isolate was detected in two unrelated patients suffering from *C. difficile infection* (CDI).

To date we have no experimental proof for which donor organism could have been involved in the transfer of pCD-METRO to *C. difficile*. Performing BLAST analysis of the pCD-METRO nucleotide sequence on the nucleotide collection (nr/nt) at the NCBI did not provide relevant results. Repeating this BLAST analysis using amino acid sequences demonstrated some homology for the putative truncated nitroimidazole reductase (*nim*) gene with those found in *Bacteroides fragilis*, whereas parts of the replicon could be detected in *Campylobacter jejuni* (data not shown). As our results imply that pCD-METRO might be common in the donor, it is possible that this species has not been cultured yet or a pCD-METRO containing strain of the organism has not yet been whole genome sequenced. Very recent data indicates that the plasmid may have been found in metagenomic data, but as this was unbinned it is not possible to trace which organism this was (Quinten Ducarmon, personal communication) (383). A partial hit was found for the metallohydrolase and Tn5 ORFs and this hit was binned as *Phascolarctobacterium* sp CAG:207 (unknown *Phascolarctobacterium*) (383, 384). *Phascolarctobacterium faecium* is a Gram-negative obligate anaerobe that utilises succinate, can be isolated from feces and appears to be a commensal in humans (385). A complete pCD-METRO sequence was recovered from another metagenomic dataset, but could not be assigned to a particular genus or species (383). In an attempt to elucidate which organism contained (part of) pCD-METRO in these metagenomic datasets, it could be informative to perform Pacific Biosciences (PacBio) long and high accuracy circular consensus sequencing (CCS). This was shown to improve taxonomic binning and genome reconstruction as compared to Illumina HiSeq and would help identify the pCD-METRO donor organism (386). Alternatively, Hi-C sequencing could be performed. This

technique utilizes cross-linking of molecules in close proximity, for instance using formaldehyde, to identify intra- and inter-chromosomal associations (387, 388). This method has been successfully applied in associating plasmids with the host chromosome (389, 390). Performing Hi-C on patient fecal material containing pCD-METRO in which *C. difficile* is likely not the sole host, would allow us to determine the original donor by analyzing the chromosomal sequence cross-linked to pCD-METRO. This experiment was not performed at the time due to lack of available stored fecal material, as well as suboptimal fecal storage conditions potentially affecting microbial community profiles (391).

The mechanism of pCD-METRO mediated metronidazole resistance

Another current outstanding question is how pCD-METRO confers metronidazole resistance in *C. difficile*. Potential mechanisms in which *C. difficile* becomes resistant to metronidazole is by reductive inactivation of metronidazole, drug efflux, alterations in pyruvate-ferredoxin oxidoreductase (PFOR) activity or adaptation by various stress responses (124). The most likely candidate to mediate one of the aforementioned effects present on the plasmid was open reading frame (ORF) 6. This ORF demonstrated homology to *nimB*, sometimes detected in metronidazole resistant *B. fragilis* (134). However, *in trans* overexpression of this gene did not confer metronidazole resistance in 630Δ*erm*. No changes in MIC were observed when ORF6 was expressed together with the upstream putative metallohydrolase under the control of either an inducible or its native promotor (**Chapter 4**). The *nimB* sequence as encoded by ORF6 is truncated, however, and based on structural predictions, obtained using phyre2, it is likely the active site of this *nim* protein is missing. Based on these results, a different gene or combinations of genes are possibly responsible for the metronidazole resistant phenotype. In an attempt to analyze this further we attempted to perform QuickChange site-directed mutagenesis to introduce a premature stop-codon into every ORF to sequentially disrupt each gene present on pCD-METRO^{shuttle}. We were successfully able to introduce a premature stop codon into ORF1 (a hypothetical protein) but this disruption did not influence metronidazole resistance conferred by pCD-METRO^{shuttle} (data not shown). No mutations could be introduced in the other ORFs, primarily due to unwanted secondary mutations in the replicon (ORF5) of the plasmid, potentially due to its toxicity in *E. coli*. Alternative strategies would be to clone each ORF or parts of the plasmid under an inducible promoter and conjugate these into *C. difficile* and test metronidazole susceptibility using an E-test. Using pRPF185-based vectors with both *ermB* and *catP* resistance markers will allow us to make gene combinations on two separate

plasmids. A transposon mutagenesis based approach could be used to generate disruptions in the different ORFs on pCD-METRO^{shuttle} to analyze the effect of the resulting mutant plasmid on metronidazole resistance (392). Additionally, thermal proteome profiling (TPP) could be used to find which of the proteins encoded by pCD-METRO bind to metronidazole and could be involved in its inactivation. This technique is based on the principle that proteins denature and become insoluble when heated, and these properties will change when proteins are bound to a ligand, in this case metronidazole (393). An experimental setup that has been described as 2D-TPP uses liquid chromatography mass spectrometry (LC-MS/MS) and computational approaches to identify proteins with significantly altered thermal stability, which is an indication they interact with the added ligand (394). Performing 2D-TPP on *C. difficile* carrying or not carrying pCD-METRO and adding various concentrations of metronidazole, may indicate which of the proteins encoded by pCD-METRO have a different thermal profile. This would imply these proteins interact with metronidazole and could be involved in its inactivation.

In conclusion, the work described in this thesis has demonstrated how antibiotic use and the general stress response act in concert, exerting both direct targeted and aspecific secondary effects. Although the consequences of these observations are unknown in *C. difficile*, this discussion outlines how ultimate antibiotic effects relate together to these processes. Additionally, the work presented in this thesis described two novel mechanisms conferring metronidazole resistance in *C. difficile*, one of which was acquired from an as of yet unknown donor organism. Altogether this information will facilitate our understanding of antibiotic resistance in *C. difficile* and the role of antimicrobial resistance determinants in the resistome (from a One Health perspective), and contribute to the development of novel therapeutics.



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LIST OF PUBLICATIONS

(in chronological order)

Van Eijk E*, **Boekhoud IM***, Kuijper EJ, Bos-Sanders IMJG, Wright G, Smits WK. Genome Location Dictates the Transcriptional Response to PolC Inhibition in *Clostridioides difficile*. Antimicrobial Agents and Chemotherapy. 2019. 63:e01363-18. doi: 10.1128/AAC.01363-18.

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Boekhoud IM, Hornung BVH, Sevilla E, Harmanus C, Bos-Sanders IMJG, Terveer EM, Bolea R, Corver J, Kuijper EJ, Smits WK. Plasmid-mediated metronidazole resistance in *Clostridioides difficile*. Nature Communications. 2020. 11:598. doi: 10.1038/s41467-020-14382-1

Boekhoud IM, Michel AM, Corver J, Jahn D, Smits WK. Redefining the *Clostridioides difficile* σ^B Regulon: σ^B Activates Genes Involved in Detoxifying Radicals That Can Result from the Exposure to Antimicrobials and Hydrogen Peroxide. 2020. mSphere 5. doi: 10.1128/mSphere.00728-20

Boekhoud IM, Sidorov I, Nooij S, Harmanus C, Bos-Sanders IMJG, Viprey V, Spittal B, Clark E, Davies K, Freeman J, Kuijper EJ, Smits WK. Heme is crucial for medium-dependent metronidazole resistance in clinical isolates of *C. difficile*. bioRxiv. 2020. doi:10.1101/2020.11.18.388959. Manuscript in press at the Journal of Antimicrobial Chemotherapy



DUTCH SUMMARY / NEDERLANDSE SAMENVATTING

De darmbacterie *Clostridioides difficile*, ook wel bekend als *Clostridium difficile*, is overal in het milieu aanwezig. Vanwege het strikt anaerobe karakter van dit organisme is de bacterie niet in staat om in aanwezigheid van zuurstof te groeien. Als aanpassing op deze zuurstofintolerantie is *C. difficile* in staat om (endo)sporen te vormen die zeer resistent zijn tegen o.a. zuurstof, antibiotica, hitte en chemicaliën zoals ontsmettingsmiddelen, maar die niet in staat zijn te groeien alvorens de omgeving gunstiger voor het organisme is. Aangetroffen sporen kunnen de gastheer oraal binnentreden en in de darm ontkiemen tot vegetatieve cellen, wat kan leiden tot asymptomatische kolonisatie of een symptomatische infectie. Zodra kolonisatie gepaard gaat met symptomen van een darmontsteking, wordt er gesproken van een *Clostridioides difficile* infectie (CDI), wat kan resulteren in (milde) diarree, enterocolitis of een levensbedreigende pseudomembraneuze colitis (ernstige darmontsteking). Deze symptomen worden veroorzaakt door verscheidene toxines uitgescheiden door *C. difficile* die schade toebrengen aan het darmepitheel. Normaliter draagt een divers en stabiel microbioom van de darm (alle daarin aanwezige micro-organismen) bij aan bescherming tegen *C. difficile* kolonisatie en infectie. Pas wanneer de darmflora wordt verstoord door bijvoorbeeld antibioticumgebruik of significant van compositie verandert door stijgende leeftijd of onderliggende aandoeningen, kan ingestie van sporen tot kolonisatie en CDI leiden. Antibioticumgebruik, hoge leeftijd en onderliggend lijden zijn hierdoor significante risicofactoren voor CDI. Mede hierdoor is CDI de belangrijkste oorzaak voor nosocomiale (ziekenhuis) diarree en is ziekenhuisverblijf een additionele risicofactor voor de ontwikkeling van deze infectie. De extra maatregelen voor infectiepreventie en de relatief lange opnameduur bij CDI patiënten zorgen ervoor dat de kosten geassocieerd met deze infectie aanzienlijk zijn, variërend van 5- tot 6 miljard per jaar in de Verenigde Staten en 3 miljard in de Europese Unie. De laatste jaren is er ook een toename in CDI die niet teruggevoerd kan worden op de gezondheidszorg. Deze patiënten voldoen ook niet aan het typische profiel van antibioticumgebruik en onderliggend lijden. Hoewel de reden hiervoor niet bekend is, wordt *C. difficile* ook veel aangetroffen in het milieu en bij dieren. Het wordt daarom ook wel beschouwd als een typisch 'One Health' pathogeen, waarbij One Health er van uitgaat dat de gezondheid van de mens afhankelijk is van dieren en het milieu.

Hoewel antibioticumgebruik het risico op CDI kan verhogen, is antimicrobiële therapie de standaardbehandeling voor deze infectie. Het antibioticum metronidazol is lange tijd gebruikt voor de behandeling van milde infecties, maar vanwege gebleken inferioriteit aan vancomycine (ook een antibioticum) zijn de richtlijnen voor CDI behandeling reeds aangepast in Amerika en

zullen de Europese richtlijnen ongetwijfeld spoedig volgen. Desondanks wordt metronidazol over de hele wereld nog veelvuldig gebruikt voor de behandeling van CDI en andere infecties. Voor infecties met ernstiger beloop wordt doorgaans vancomycine gebruikt, en voor infecties met een hoge kans op een recidiverende (terugkerende) infectie wordt fidaxomicine geadviseerd. In de praktijk wordt meestal voor vancomycine gekozen vanwege de hoge kosten van een behandeling met fidaxomicine. Hoewel *C. difficile* resistent kan zijn tegen zeer veel verschillende soorten antibiotica, komt resistentie tegen metronidazol, vancomycine en fidaxomicine nog niet veel voor. Antibiotica slaan echter niet bij alle patiënten aan en recidiverende infecties komen relatief vaak voor. Fecale microbiota transplantatie (FMT), het transplanteren van de ontlasting van een gezonde donor naar een patiënt met een ernstige recidiverende CDI, is zeer effectief gebleken in het behandelen van recidieven, maar de gevolgen hiervan op lange termijn zijn onbekend. Er zijn verschillende waarschuwingen afgegeven door de Amerikaanse *Food and Drug Administration* (FDA) omtrent het gebruik van FMT omdat er een kans bestaat dat ongewenste (antibioticumresistente en/of pathogene) bacteriën van de donor worden doorgegeven aan de ontvanger en een infectie veroorzaken. Ook bestaat er op deze manier de kans dat genen die zorgen voor antibioticumresistentie worden doorgegeven aan gevoelige bacteriën. Meer onderzoek naar de neveneffecten van FMT in vergelijkbare processen is noodzakelijk. Om deze redenen is het belangrijk onderzoek te verrichten om ons begrip van antimicrobiële resistentie (AMR) in *C. difficile* beter te begrijpen, opdat deze kennis kan bijdragen in de ontwikkeling en/of verbetering van nieuwe therapieën alsook de ontwikkeling van nieuwe antibiotica.

Het vermogen van bacteriën om zich aan te passen aan de omgeving is een belangrijke overlevingsstrategie. In de levenscyclus van *C. difficile* komen de cellen in aanraking met schadelijke concentraties zuurstof, pH waarden, temperatuurwisselingen, osmotische druk en antimicrobiële peptiden geproduceerd door nabijgelegen bacteriën of de gastheer. Door zogeheten stress-responsen zijn bacteriën in staat de transcriptie van hun genen te veranderen (het bijbehorende 'regulon'), en deze veranderingen kunnen bijdragen aan de overleving. Twee belangrijke stress-responsen zijn de SOS respons, welke voornamelijk bij DNA schade wordt geactiveerd, en de algemene stress-respons die gereguleerd wordt door de alternatieve sigma factor B (σ^B). Het is bekend dat de algemene stress-respons belangrijk is voor de tolerantie voor lage concentraties zuurstof in *C. difficile*, maar lijkt ook een rol te spelen bij de gevoeligheid voor de antimicrobiële middelen rifampicine en mitomycine C. Daar mitomycine C DNA schade

veroorzaakt lijkt er een verband te zijn tussen de DNA schade, de algemene- en de SOS stress responsen.

Een nieuw antibioticum, ibezapolstat (ook wel ACX-362E genoemd) dat sinds kort in klinische ontwikkeling is, remt DNA replicatie door een DNA polymerase te inhiberen. Lage concentraties van dit antibioticum leidde ertoe dat er relatief meer kopieën gemaakt werden van genen (en dientengevolge ook transcripten van deze genen) die zich dichtbij het startpunt van replicatie bevonden ten opzichte van die genen die verder van dit replicatiepunt af lagen (hoofdstuk 2). Het gen coderend voor σ^B bevindt zich dichtbij het replicatie startpunt en werd dus geïnduceerd door lage concentraties ibezapolstat, hetgeen resulteerde in het aanzetten van σ^B -afhankelijke genen. Het is mogelijk dat deze respons bijdraagt aan het aanpassingsvermogen van *C. difficile* tegen DNA replicatie inhibitie.

Om de algemene stress respons in *C. difficile* beter te karakteriseren is het regulon (alle genen die door σ^B worden geactiveerd of gedeactiveerd) van σ^B bepaald (hoofdstuk 3). Hieruit is gebleken dat een significant gedeelte van de algemene stressrespons gericht is op het afhandelen van schadelijke zuurstof- en stikstofradicalen. Omdat het antibioticum metronidazol geacht wordt bacteriën te doden door middel van stikstofradicalen lijkt er een verband te bestaan tussen de algemene stress respons en de wijze waarop metronidazol cellen doodt. Aangezien ongereguleerde activiteit van σ^B toxisch was voor *C. difficile*, is er door middel van constructen met een promotor, gekoppeld aan een expressiecassette (reporterconstructen), aangetoond dat verschillende antibiotica (metronidazol, ibezapolstat, vancomycine en lincomycine) en waterstofperoxide transcriptie van een aantal σ^B gereguleerde genen activeerden. Op basis van deze resultaten bestaat het vermoeden dat antibiotica, maar ook andere stoffen die lijden tot dood van cellen, het metabolisme en de homeostase dermate beïnvloeden dat σ^B wordt geactiveerd of dat er hierdoor radicalen worden geproduceerd die eveneens tot activatie van σ^B leiden.

In dit proefschrift zijn twee nieuwe ontdekkingen gedaan omtrent metronidazolresistentie in *C. difficile* (hoofdstuk 4 en 5). Uit een patiënt die leed aan recidiverende *C. difficile* infecties werden twee stammen geïsoleerd van hetzelfde type waarvan er één gevoelig was voor metronidazol en één resistent tegen metronidazol, ondanks dat er maar 1 puntmutatie in het chromosoom aantoonbaar was (hoofdstuk 4). Verder onderzoek toonde aan dat de

metronidazol-resistente stam een plasmide bevatte dat wij pCD-METRO hebben genoemd. Introductie van dit plasmide in een metronidazol gevoelige laboratoriumstam zorgde ervoor dat deze metronidazol resistent werd. Screening van een grote, wereldwijde collectie klinische *C. difficile* stammen op de aanwezigheid van dit plasmide liet blijken dat pCD-METRO in vrijwel alle stammen aanwezig was met een stabiele metronidazol-resistentie en dus een belangrijke oorzaak van deze resistentie in *C. difficile* is.

In dit onderzoek naar pCD-METRO was er één metronidazol-resistente stam die het plasmide niet bevatte, maar wel minder hoge- en bovendien medium-afhankelijke- resistentie liet zien (hoofdstuk 5). Deze stam was resistent tegen metronidazol wanneer deze werd opgekweekt op medium waar vitamine K, heem en schapenbloed was verwerkt, maar was gevoelig wanneer de stam groeide op een voedingsbodem zoals deze veel gebruikt wordt in het moleculair biologisch laboratorium. Gedurende de COMBACTE-CDI studie, een 3-jaar durende Europese studie met als doel om de epidemiologie en klinische impact van CDI te karakteriseren, waren er stammen verzameld die resistent- of verminderd gevoelig bleken voor metronidazol. Nader onderzoek toonde aan dat de metronidazol-resistente stammen in deze collectie niet het pCD-METRO plasmide bevatten. Wel werd duidelijk dat deze stammen medium-afhankelijke resistentie vertoonden wat terug te leiden was naar de aanwezigheid van heem. Nagenoeg alle stammen lieten eenzelfde heem-afhankelijke verhoging van de Minimaal Inhiberende Concentratie (MIC) zien, ook al leidde dit niet per definitie tot resistentie. Wij hebben aangetoond dat stammen van een bepaald type van *C. difficile* met heem-afhankelijke metronidazol-resistentie een mutatie (deletie) hebben in het gen *hsmA*, waarvan de transcriptie verandert in de aanwezigheid van heem. Deze deletie was verrassend genoeg ook aanwezig in de isolaten die het pCD-METRO plasmide bevatten. Fylogenetische vergelijkingen lieten zien dat de stammen met de deletie een aparte groep vormen, waarbij een deel van de stammen waarschijnlijk later ook het plasmide heeft opgenomen. Hoewel het niet bekend is hoe heem in alle andere stammen tot een verhoging van de MIC voor metronidazol leidt, heeft dit onderzoek het belang van *hsmA* en heem afhankelijke metronidazolresistentie in RT010 stammen aangetoond.

In conclusie heeft het werk in dit proefschrift een verband gelegd tussen remming van de DNA polymerase en de algemene stress-respons in *C. difficile*. Deze algemene stress respons is in dit werk beter gekarakteriseerd en we hebben een relatie met antimicrobiële resistentie en σ^B aangetoond. Tevens beschrijft het werk in dit proefschrift twee nieuwe mechanismen

die tot metronidazolresistentie in *C. difficile* leiden: we hebben het allereerste plasmide dat antibioticumresistentie geeft in *C. difficile* beschreven en we hebben aangetoond dat heem leidt tot verminderde metronidazolgevoeligheid in verschillende klinische isolaten.



CURRICULUM VITAE

Ilse Michelle Boekhoud was born on March 13th, 1991 in Dordrecht, the Netherlands. After completing her secondary education at 'C.S.G. Develsteincollege' in Zwijndrecht in 2010, she started studying Biomedical Sciences at the Leiden University Medical Center (LUMC). During her Bachelor studies she finished the minor Brain & Cognition. After completing an internship on diabetic nephropathy under supervision of Dr. E. de Heer and Dr. J. J. Baelde at the Department of Pathology at the LUMC, she received her Bachelor of Science in 2013. She continued with a Biomedical Sciences research Master following the 'Immunity, Infection and Tolerance' (IIT) track graduate program, during which she finished an internship studying cancer stem cells and low proteasome activity at the Department of Medical Oncology at the Erasmus MC, under supervision of prof. dr. J.E.M.A. Debets. After realizing that infectious diseases were her main scientific interest, she then completed an internship at the LUMC Department of Medical Microbiology. Supervised by R. Knaap and dr. ir. M. Kikkert, she studied the Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) nsp2 protease and used structure-guided mutagenesis to disrupt its function in immune evasion. After obtaining her Master's degree in 2016 she remained at the Department of Medical Microbiology, where she started her PhD research on antimicrobial resistance in *Clostridioides difficile* under supervision of Dr. W. K. Smits and Prof. Dr. E.J. Kuiper in the section Experimental Bacteriology. As a part of the 2019 Clostpath conference, she was part of the organizing committee for the pre-Clostpath Young Scientist Meeting. During her PhD she also worked abroad for short periods of time in the laboratories of Prof. Dr. D. Jahn at Braunschweig University in Germany and Dr. R. Fagan at the University of Sheffield in the United Kingdom. She considers these experiences as having been valuable and worthwhile for her PhD research as well as for her personal development. Starting November 2020 she works as a scientific employee at the Commissie Genetische Modificatie (COGEM), located at the Rijksinstituut voor Volksgezondheid en Milieu (RIVM) in Bilthoven, the Netherlands.

