

# Fidaxomicin Inhibits Spore Production in *Clostridium difficile*

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Fidaxomicin (FDX) is a novel antimicrobial agent with narrow-spectrum and potent bactericidal activity against *Clostridium difficile*. In recent clinical trials, FDX was superior to vancomycin in preventing recurrences of *C. difficile* infection. A possible mechanism of reducing recurrence may be through an inhibitory effect on sporulation. The effect of FDX and its major metabolite, OP-1118, on *C. difficile* growth and sporulation kinetics was compared with that of vancomycin, metronidazole, and rifaximin. Drugs at subminimum inhibitory concentrations (sub-MICs) were added to cells at an early stationary phase of growth; this was followed by collection of cells at various intervals for quantitation of total viable cell and heat-resistant spore counts on taurocholate-containing media. The effect of the drugs at 2–2.5× MIC on the expression of sporulation genes in *C. difficile* was also compared using quantitative reverse-transcriptase polymerase chain reaction. Both FDX and OP-1118 (1/4× MIC) inhibited sporulation when added to early-stationary-phase cells in *C. difficile* strains, including the epidemic NAP1/BI/027 strain. In contrast, vancomycin, metronidazole, and rifaximin (at similar sub-MICs) did not inhibit sporulation. The number of spores following treatment with comparator drugs increased to the same level as the no-drug control treatment. Expression of mother cell-specific (*spoIIID*) and forespore-specific (*spoIIR*) sporulation genes also was inhibited by FDX and OP-1118 but not significantly by vancomycin. Both FDX and OP-1118 (unlike vancomycin, rifaximin, and metronidazole) effectively inhibited sporulation by *C. difficile*. The inhibitory effect of FDX on *C. difficile* sporulation may contribute to its superior performance in sustaining clinical response and reducing recurrences and may also be beneficial in decreasing shedding and transmission of this pathogen.

*Clostridium difficile* is a spore-forming anaerobic bacterium that has become a major cause of infectious diarrhea in healthcare settings and in the community [1]. Several major outbreaks have occurred in North America and Europe, including a recent outbreak in July 2011 in several hospitals in Ontario, Canada [2–5]. Although the production of potent toxins is responsible for symptoms of the disease, endospore formation is also an important factor that contributes to disease

transmission and recurrence. Spores can survive, germinate, and proliferate in the gut following exposure to antibiotic treatment that suppresses the intestinal microbiota [6–9]. Spores shed in fecal matter are extremely difficult to eradicate; they can persist within hospitals and healthcare facilities for extended periods, leading to new or recurrent infections [10–13].

*Clostridium difficile* has been treated primarily with vancomycin, metronidazole, or both, and these drugs have been available for the past 30 years. However, these treatment options have significant limitations. Importantly, following treatment, *C. difficile* infections (CDIs) recur in approximately 25% of individuals treated with either vancomycin or metronidazole, and some patients experience multiple recurrences [1, 14].

Fidaxomicin (FDX) is a novel narrow-spectrum antibacterial agent that was recently approved for the treatment of *C. difficile*-associated diarrhea [15–18]. In recent clinical trials with >1100 subjects with CDI,

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FDX was shown to be superior to vancomycin in providing sustained clinical response (response without recurrence of disease) at 25 days following therapy [14]. Fidaxomicin inhibits bacterial RNA polymerase activity at a very early stage of transcription, distinguishing its mode of action from those of other transcription initiation inhibitors, such as members of the rifamycin family [19]. Because FDX blocks gene transcription rather than cell-wall synthesis (the target of vancomycin) and because FDX does not act through the production of DNA-damaging free radicals (putative mode of action of metronidazole) [20], it potentially could inhibit the expression of genes related to sporulation. In this study, we examined the effect of FDX and its major metabolite (OP-1118) on sporulation kinetics and expression of sporulation genes.

## METHODS

### Bacterial Strains

*Clostridium difficile* UK-14, a restriction endonuclease analysis (REA) group BI (BI) strain isolated during an epidemic *C. difficile* outbreak in England (Meridian Biosciences strain number SM8-6865), was kindly provided by Dr Dale Gerding. Strain UK-1, a North American pulsed-field gel electrophoresis type 1 (NAP1)/BI/polymerase chain reaction (PCR) ribotype 027 (027) strain from the Stoke-Mandeville Hospital outbreak, also was obtained from Dr Gerding. Strain CD196 (considered to be the closest nonepidemic ancestor of the 027 strains [21]) was obtained from Dr Michel Popoff. *Clostridium difficile* American Type Culture Collection (ATCC) 43255 (VPI 10463) was obtained from the ATCC. The UK-14 and ATCC 43255 strains were used to study the effect of drugs on sporulation kinetics. Both strains were stored at  $-80^{\circ}\text{C}$  in Brucella broth supplemented with hemin at  $5\ \mu\text{g}/\text{mL}$  and vitamin  $\text{K}_1$  at  $10\ \mu\text{g}/\text{mL}$  (SBB) containing 10% glycerol. The isolates were subcultured on 5% blood-agar plates under anaerobic conditions (80% nitrogen, 10% carbon dioxide, and 10% hydrogen) before testing.

Strains UK-1 and CD196 were used to study the effect of drugs on the expression of sporulation genes. They were stored at  $-80^{\circ}\text{C}$  in brain heart infusion (BHI) medium containing 15% glycerol. The strains were isolated on BHI agar supplemented with 5% yeast extract and 0.1% L-cysteine (BHIS). A single colony was used to inoculate 3% bacto-tryptose, 2% yeast extract, and 0.1% thioglycolate, pH 7.4 (TY) medium for overnight cultures at  $37^{\circ}\text{C}$  under anaerobic conditions.

### Antimicrobial Agents

Vancomycin, rifaximin, and metronidazole (all obtained from Sigma-Aldrich) were prepared as 10-mg/mL stock solutions in water, methanol, and dimethyl sulfoxide (DMSO), respectively. Similar stock concentrations of FDX and OP-1118 were

prepared by dissolving the compounds in DMSO. All drugs were diluted further to an appropriate concentration in growth media before use for minimum inhibitory concentration (MIC) determination and for their effects on sporulation.

### MIC Determination

Minimum inhibitory concentrations were determined under settings designed to match the planned conditions of the sporulation and messenger RNA (mRNA) expression experiments. The Clinical Laboratory Standard Institute microbroth (rather than agar) dilution method with slight modification was used for MIC determination vs ATCC 43255 and UK-14 strains [22, 23]. Lysed blood, which obscures the color of media, was omitted from the culture media (without affecting the growth of *C. difficile*) so that the redox indicator, resazurin, could be used to monitor the anaerobicity of the chamber. Briefly, microtiter plates with serially diluted drugs were equilibrated in an anaerobic glove box for a minimum of 3 hours. *Clostridium difficile* ( $10^5$  colony-forming units [CFUs]) was added to each well. Following a 48-hour incubation at  $35^{\circ}\text{C}$ , the plates were examined for growth. The MIC was defined as the drug concentration at which no growth or the most significant reduction of growth was observed.

To determine MICs for strains CD196 and UK-1, susceptibility tests were performed in BHIS broth as follows. *Clostridium difficile* strains were grown to an optical density at 600 nm ( $\text{OD}_{600}$ ) of 0.5 (exponential growth phase) and then diluted to give an inoculum of approximately  $5 \times 10^5$  CFUs/mL in 2 mL of fresh medium supplemented with a range of concentrations of FDX, OP-1118, or vancomycin. Cultures were incubated under anaerobic conditions for 18 hours at  $37^{\circ}\text{C}$ . The MIC was defined as the lowest concentration of antibiotic that prevented visible turbidity after 18 hours.

### Growth and Sporulation Kinetics

Overnight cultures of *C. difficile* strains ATCC 43255 and UK-14 grown on blood agar plates (Hardy Diagnostics) were suspended in SBB to an  $\text{OD}_{600}$  of approximately 0.4. The suspension was diluted further in SBB (1:100) and incubated at  $37^{\circ}\text{C}$  in an anaerobic chamber until the cells reached an early stationary phase of growth (approximately 10 hours for the UK-14 strain and approximately 24 hours for the ATCC 43255 strain). Sub-MICs of drugs were then added to the cells, and cells were monitored for a period of approximately 1 week. Samples were withdrawn at several different times for quantitation of heat-resistant spores (survivors of incubation at  $60^{\circ}\text{C}$  for 20 minutes) and total CFUs by serially diluting each sample (in water for spore CFUs or SBB for total CFUs) and plating each in duplicate on BHIS agar supplemented with 0.1% taurocholate.

### Quantitative Reverse-Transcription PCR Analysis of Sporulation Gene Expression

Overnight cultures of *C. difficile* grown in TY were used to inoculate fresh TY medium at OD<sub>600</sub> of approximately 0.05. Drugs (FDX at 2× MIC or OP-1118 and vancomycin at 2.5× MIC) were added to the cultures either at the onset of the stationary phase ( $T_0$ ) or 2 hours after  $T_0$  ( $T_2$ ). RNA was prepared from bacterial cells harvested at  $T_0$ ,  $T_2$ , and 4 hours after  $T_0$  ( $T_4$ ) as previously described [24, 25]. RNA was quantified by absorbance using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). Primers for quantitative PCR (qPCR) were designed using the online PrimerQuest tool from Integrated DNA Technologies [26], and amplification efficiencies for each primer set were determined prior to use. Synthesis of complementary DNA (cDNA) was performed on 500 ng of RNA using random hexamer primers and the QuantiTect Reverse Transcription Kit (QIAGEN) according to the manufacturer's recommendations. To control for chromosomal DNA contamination, mock cDNA synthesis reactions containing no reverse transcriptase were used as negative controls in subsequent amplifications. Complementary DNA samples were diluted 4-fold and used as templates for qPCR of *rpoC* (primers oLB122 [CTAGCTGCTCCTATGTCTCACATC] and oLB123 [CCAGTCTCTCCTGGATCAACTA]), 16s ribosomal RNA (rRNA) (primers oLB189 [GATTTACTTCGGTAAAGAGCGG] and oLB190 [CCTTACCAACTAGCTAATCAGACG]), *spoIIR* (primers oLB191 [CTTCCAGCTGGTGAATATAAAGC] and oLB192 [CATAGTGGAGGGAACATAACACA C]), and *spoIIID* (primers oLB193 [GAGATCTCATATAGAGGAAAGGGC] and oLB194 [AACTTCTTTGGCAAGGGATG]) using Roche SYBR Green I PCR mix and a Roche Light-Cycler 480 II thermocycler. Reactions were performed in a final volume of 20 μL using 4 μL of diluted cDNA and primers at 1-μM final concentration. Amplification included 45 cycles of the following steps: 10 seconds at 95°C, 10 seconds at 53°C, and 15 seconds at 72°C. Reactions were performed in triplicate using cDNA synthesized from each of a minimum of 2 biological replicates, and results are presented as mean and standard error of the mean values of the data obtained. Results were calculated using the  $2^{-\Delta\Delta Ct}$  method [27],

in which the amount of target mRNA is normalized to that of an internal control transcript (*rpoC* or 16s rRNA).

## RESULTS

To ensure the use of sub-MIC drug levels, which would not lead to bactericidal activity during kinetics studies, MICs were first determined using the microbroth dilution method. The MIC data for each strain are summarized in Table 1.

### Effect of Drugs on Kinetics of Growth and Sporulation

To isolate the impact of drugs on sporulation and avoid inhibitory effects on vegetative growth, kinetic studies were performed with the addition of drugs at an early stationary phase, when spore formation was beginning.

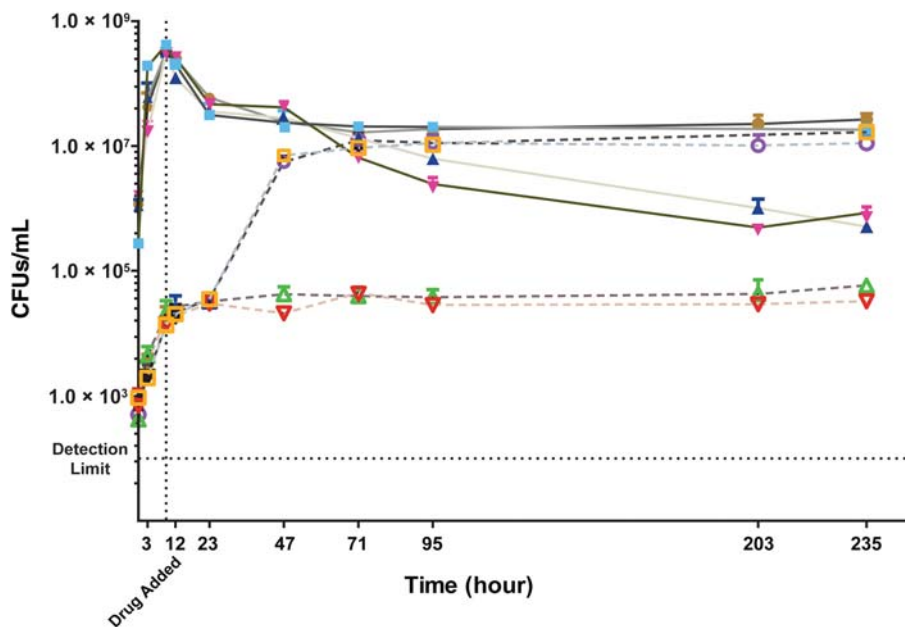
Sporulation kinetics varied considerably between the 2 strains. The epidemic UK-14 strain sporulated very early and efficiently, with heat-resistant spore counts as high as  $10^3$  by approximately 3 hours of incubation and reaching  $10^7$  CFUs/mL by approximately 72 hours (Figure 1). In contrast, *C. difficile* ATCC 43255 sporulated much less efficiently, reaching a maximum of approximately  $10^5$  CFUs/mL (Figure 2). It is notable that both strains also demonstrated instability in their sporulation phenotype; in some runs, the ATCC strain failed to sporulate at all and the number of heat-resistant spores in the UK-14 strain reached only  $10^5$  CFUs/mL. Despite the inhibitory activity of FDX in all sporulation runs, experimental runs in which the strains failed to sporulate sufficiently were not included in the average values shown in the figures.

Following the addition of FDX or OP-1118 (both at 1/4× MIC) to stationary-phase cells of *C. difficile* strains ATCC 43255 and UK-14, spore production was halted completely (Figures 1 and 2). Although neither FDX nor OP-1118 eliminated preexisting spores, both prevented the production of additional spores. This suppression was dose dependent for OP-1118; 2 days following exposure to OP-1118 at 1/8× MIC, the spore count had increased by 1 log and remained at that level (which nevertheless was still 1 log lower than that of the no-drug control) through the end of the experiment, approximately 7 days (data not

**Table 1. Minimum Inhibitory Concentrations for Fidaxomicin, OP-1118, Vancomycin, Metronidazole, and Rifaximin**

<i>Clostridium difficile</i> Strain	MIC (μg/mL)				
	Fidaxomicin	OP-1118	Vancomycin	Metronidazole	Rifaximin
ATCC 43255	0.125	1	0.5	ND	ND
UK-14	0.5	4	0.5	2	0.004
CD196	0.125	1	1	ND	ND
UK-1	0.125	1	1	ND	ND

Abbreviations: ATCC, American Type Culture Collection; MIC, minimum inhibitory concentration; ND, not determined.

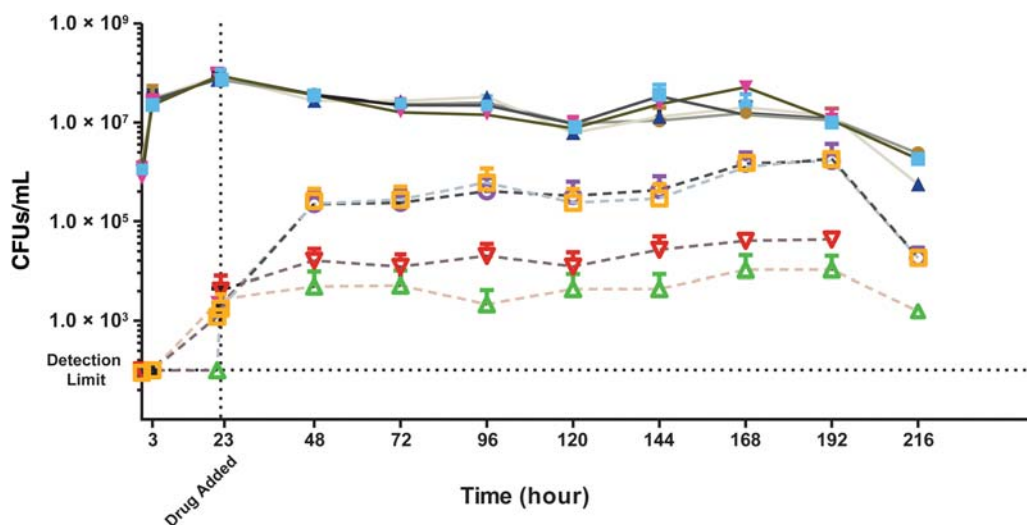


**Figure 1.** Development of total viable counts (filled symbols with solid lines) and heat-resistant spores (open symbols with dashed lines) in *Clostridium difficile* UK-14 strain: in the absence of drugs (●, ○), in the presence of fidaxomicin (FDX) (▲, △), in the presence of OP-1118 (▼, ▽), and in the presence of vancomycin (■, □). The data represent the averages of 6 independent runs for no drug control, 4 independent runs for FDX, 2 independent runs for OP-1118, and 1 run for vancomycin. Error bars represent the standard error of the mean. Abbreviation: CFUs, colony-forming units.

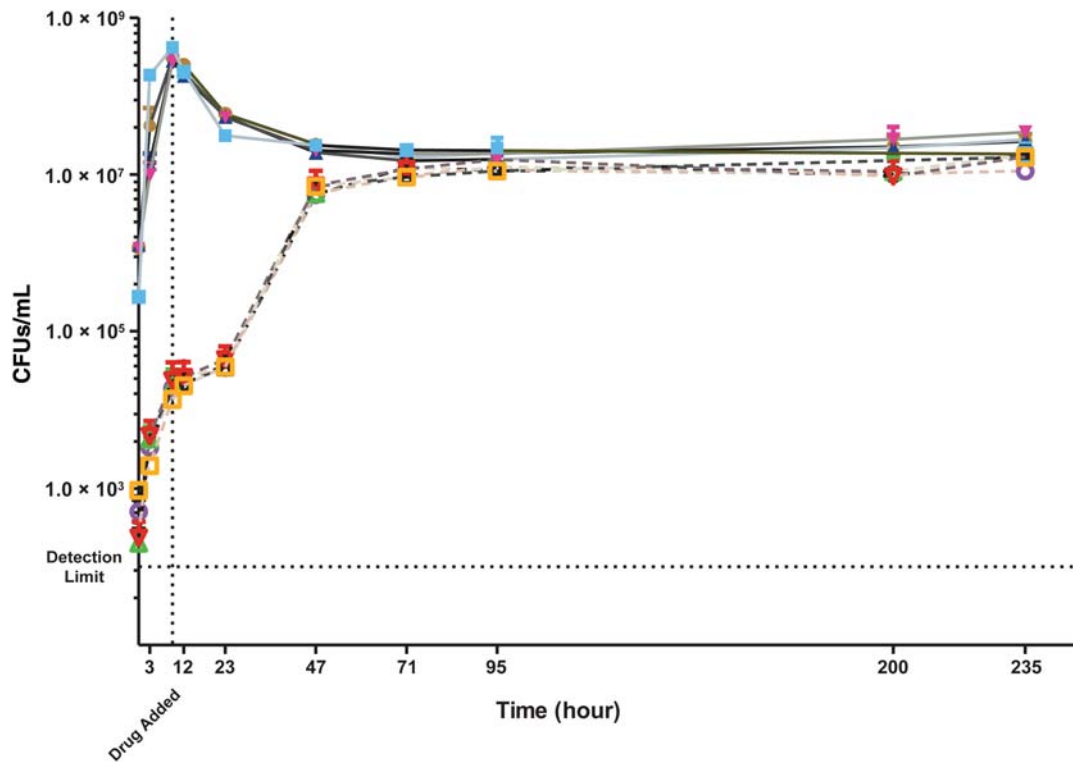
shown). Fidaxomicin at  $1/4\times$  or  $1/8\times$  MIC completely inhibited spore formation (data for  $1/8\times$  MIC not shown).

In contrast to FDX and OP-1118, the comparator drug vancomycin (tested at similar sub-MICs) had no effect on

sporulation in the UK-14 or ATCC 43255 strains (Figures 1–3). The spore count following exposure to vancomycin was very similar to that of the no-drug control throughout the duration of the experiments even if the drug was added at the



**Figure 2.** Development of total viable counts (filled symbols with solid lines) and heat-resistant spores (open symbols with dashed lines) in *Clostridium difficile* American Type Culture Collection 43255 strain: in the absence of drugs (●, ○), in the presence of fidaxomicin (FDX) (▲, △), in the presence of OP-1118 (▼, ▽), and in the presence of vancomycin (■, □). The data represent the averages of 3 independent runs for FDX, 4 independent runs for vancomycin, and 4 independent runs for OP-1118. Error bars represent the standard error of the mean. Abbreviation: CFUs, colony-forming units.



**Figure 3.** Development of total viable counts (filled symbols with solid lines) and heat-resistant spores (open symbols with dashed lines) in *Clostridium difficile* UK-14 strain: in the absence of drugs (●, ○), in the presence of metronidazole (▲, △), in the presence of rifaximin (▼, ▽), and in the presence of vancomycin (■, □). The data represent averages of 6 independent runs for no control drug, 4 independent runs for metronidazole, 3 independent runs for rifaximin, and 1 run for vancomycin. The error bars represent the standard error of the mean. Abbreviation: CFUs, colony-forming units.

late log phase of growth (data not shown). Similar to the case for vancomycin, the other comparators, metronidazole and rifaximin, also failed to inhibit spore formation of strain UK-14; again, the number of spores increased at the same rate as for the control cells that were not exposed to any drug (Figure 3).

### Effect of Drugs on Expression of Sporulation Genes

To assess the effect of drug treatment on sporulation gene expression, we measured the accumulation of 2 sporulation-specific mRNAs in cells treated with drugs at the onset of stationary phase,  $T_0$ , in 2 strains (UK-1 and CD196). As shown in Table 2, accumulation of *spoIIR* mRNA, coding for an early ( $\sigma^F$ -dependent) forespore-specific gene product, increased substantially between  $T_0$  and  $T_4$  in the absence of drugs in strains UK-1 and CD196. That accumulation was almost completely prevented by the addition of FDX (0.25  $\mu\text{g}/\text{mL}$ ) or OP-1118 (2.5  $\mu\text{g}/\text{mL}$ ) at  $T_0$ . Similarly, accumulation of *spoIIID* mRNA, encoding a midstage mother-specific ( $\sigma^E$ -dependent) protein, was strongly inhibited by FDX and OP-1118 added at  $T_0$  (Table 2). In contrast, the addition of vancomycin at  $T_0$  had little inhibitory effect, if any, on subsequent accumulation of *spoIIR* or *spoIIID* mRNAs (Table 2). Statistical analysis

using the Student *t* test did not demonstrate a difference between vancomycin and the control group ( $P = .19$  for  $T_2$  samples and  $P = .33$  for  $T_4$  samples in Table 2). Owing to small sample size (control [ $n = 10$ ] and vancomycin [ $n = 4$ ] at  $T_2$  and vancomycin [ $n = 2$ ] at  $T_4$ ), the power was low.

When FDX or OP-1118 was added to sporulating cells of strain CD196 at  $T_2$ , the levels of *spoIIR* and *spoIIID* mRNAs not only failed to increase within the next 2 hours but also decreased to a low level (Table 3). This result suggests that FDX and OP-1118 block synthesis of the sporulation mRNAs, and that the mRNAs are unstable and disappear in the absence of continued synthesis. The addition of vancomycin at  $T_2$  had a relatively small effect on further accumulation of *spoIIR* and *spoIIID* mRNAs (Table 3).

## DISCUSSION

Endospore formation is a complex process in which 2 subcellular compartments cooperate to carry out the cellular morphogenesis that leads to the production of the metabolically dormant and environmentally resistant spore. The detailed mechanism of spore formation has been studied in great detail

**Table 2. Quantitative Reverse-Transcriptase Polymerase Chain Reaction Analysis of *spolIR* and *spolIID* Expression Following Fidaxomicin, OP-1118, or Vancomycin Treatment**

Time	Control (no drugs), Mean (±SD)	Fidaxomicin, Mean (±SD)	OP-1118, Mean (±SD)	Vancomycin, Mean (±SD)
Strain UK-1: expression ratio <i>spolIR/rpoC</i>				
$T_0$	1 (0.68–1.45)	...	...	...
$T_2$	11.92 (7.99–17.78)	0.39 (0.16–0.93)	0.78 (0.39–1.57)	11.75 (6.64–20.78)
$T_4$	15.53 (11.81–20.43)	0.47 (0.16–1.34)	1.71 (0.98–2.99)	12.16 (8.27–17.86)
Strain UK-1: expression ratio <i>spolIID/rpoC</i>				
$T_0$	1 (0.56–1.78)	...	...	...
$T_2$	31.33 (19.6–50.08)	0.29 (0.11–0.75)	0.43 (0.13–0.87)	12.19 (4.08–36.40)
$T_4$	42.11 (29.13–60.89)	0.17 (0.09–0.31)	0.24 (0.13–0.45)	25.95 (13.48–49.94)
Strain CD196: expression ratio <i>spolIR/rpoC</i>				
$T_0$	1 (0.59–1.67)	...	...	...
$T_2$	4.44 (3.55–5.54)	0.35 (0.22–0.56)	0.60 (0.19–1.87)	...
$T_4$	7.26 (2.94–17.88)	0.34 (0.21–0.54)	2.34 (—)	...
Strain CD196: expression ratio <i>spolIID/rpoC</i>				
$T_0$	1 (0.58–1.71)	...	...	...
$T_2$	3.95 (3.11–5.01)	0.18 (0.09–0.32)	0.20 (0.04–0.99)	...
$T_4$	16.30 (5.17–51.42)	0.01 (0.04–0.24)	0.03 (—)	...

*Clostridium difficile* strains UK-1 and CD196 were grown in 3% bacto-tryptose, 2% yeast extract, and 0.1% thioglycolate, pH 7.4 medium to the onset of the stationary phase ( $T_0$ ). Each culture was then divided into aliquots that received no drug, fidaxomicin (0.25 µg/mL), OP-1118 (2.5 µg/mL), or vancomycin (2.5 µg/mL), as indicated. RNA was harvested 2 hours ( $T_2$ ) and 4 hours ( $T_4$ ) after the addition of drugs, complementary DNA was synthesized using random primers, and quantitative polymerase chain reaction (PCR) was performed using gene-specific primers for *spolIR* or *spolIID*. Results were normalized to an internal control gene (*rpoC*) and are presented as the ratio of each transcript level relative to wild-type and no-drug controls. Quantitative PCR was done in triplicate for each sample. Mean and standard deviation values reflect results from at least 3 biological replicates, except for the 2 biological replicates for the  $T_4$  samples after vancomycin addition with strain UK-1 and the single  $T_4$  samples for OP-1118 with strain CD196.

Abbreviation: SD, standard deviation.

for *Bacillus subtilis*. Much less is known about *C. difficile* sporulation, but many of the most important regulatory factors, including the RNA polymerase sigma factors that direct compartment-specific gene expression, are highly conserved in the

2 species. When a bacterial spore is formed and shed into the environment, it is very difficult to eradicate with common antiseptics owing to the resistance of the spore to heat, strong chemicals, and radiation. Shedding of *C. difficile* spores by

**Table 3. Quantitative Reverse-Transcriptase Polymerase Chain Reaction Analysis of *spolIR* and *spolIID* Expression Following Drug Addition at  $T_2$** 

Time	Control (no drugs), Mean (±SD)	Fidaxomicin, Mean (±SD)	OP-1118, Mean (±SD)	Vancomycin, Mean (±SD)
Strain CD196: expression ratio <i>spolIR/16s rRNA</i>				
$T_0$	1 (0.59–1.68)	...	...	...
$T_2$	1.11 (0.66–2.77)	...	...	...
$T_4$	2.4 (1.92–3.00)	0.50 (0.26–0.95)	0.28 (0.22–0.37)	1.74 (1.03–2.95)
Strain CD196: expression ratio <i>spolIID/16s rRNA</i>				
$T_0$	1 (0.58–1.71)	...	...	...
$T_2$	7.03 (1.93–25.62)	...	...	...
$T_4$	25.75 (16.61–41.02)	2.51 (0.81–7.77)	1.03 (0.72–1.49)	25.99 (13.90–48.60)

*Clostridium difficile* strain CD196 was grown in 3% bacto-tryptose, 2% yeast extract, and 0.1% thioglycolate, pH 7.4 medium to the second hour ( $T_2$ ) after the onset of the stationary phase ( $T_0$ ). At that time, the culture was divided into aliquots that received no drug, fidaxomicin (0.25 µg/mL), OP-1118 (2.5 µg/mL), or vancomycin (2.5 µg/mL), as indicated. RNA was harvested from untreated or drug-treated cells 2 hours later (ie, at  $T_4$ ), complementary DNA was synthesized, and quantitative polymerase chain reaction was performed using gene-specific primers for *spolIR* or *spolIID*. Results were normalized to an internal control gene (*16s rRNA*) and are presented as the ratio of each transcript level relative to wild-type and no-drug controls. Mean and standard deviation from the mean values reflect results from at least 2 biological replicates.

Abbreviation: SD, standard deviation.

infected patients in the hospital environment is thought to be a major source of new and recurrent infections. Therefore, any treatment that leads to inhibition of spore formation in patients with CDI can potentially lower the transmission and recurrence of infection. Although standard treatments with vancomycin and metronidazole inhibit the growth of vegetative cells, their effects on spore formation have not been examined previously. In this study, we report that unlike vancomycin or metronidazole, FDX and its major metabolite, OP-1118, are very effective in inhibiting spore formation by *C. difficile*, even in strains that have high sporulation efficiency. These data support previous clinical findings by Louie et al [28], who reported significantly lower spore counts in stool samples of FDX-treated subjects compared with those of vancomycin-treated subjects.

In our analysis, we examined only the formation of heat-resistant CFUs; the spores were not quantified by microscopy. Microscopic analysis appeared to be unnecessary for the UK-14 strain because within a few days, almost all CFUs were heat resistant. There have been conflicting reports of the sporulation efficiency of the ATCC 43255 strain [29, 30]. Consistent with studies by Burns et al [30], the numbers of heat-resistant spores we obtained were low at 24 hours and increased after 48 hours. However, the maximum spore titer was variable and in no case reached the level of  $10^7$  spores/mL reported by that group. Under our laboratory conditions, in some cases, the number of heat-resistant spores produced by the ATCC strain was <100 CFUs/mL. It is notable that different media were used in both laboratories; whereas Burns et al [30] cultivated the strains in BHIS, we used SBB and omitted cysteine so that we could use the supernatants for toxin titer determinations in another study. Alternatively, the discrepancies reported for spore counts may be due to the presence of mixed phenotypes within the strain and selection for phenotype with low sporulation efficiency under our laboratory conditions. Such mixed sporulation phenotypes, with segregation of mutant clones that do not sporulate efficiently, have been reported previously and may explain discrepancies between laboratories and within runs in our laboratory [31]. We also observed similar phenotype instability with the UK-14 strain in some runs. Such observations are in support of better experimental design, as noted previously [32], to ensure reproducibility of sporulation proficiencies in *C. difficile*.

Our data indicate that when FDX and OP-1118 at sub-MICs are added to *C. difficile* cells in the early stationary phase, cell viability is not affected, but the sporulation process is arrested. Furthermore, we demonstrated that such arrest can be attributed to potent inhibition of transcription of at least some and probably all sporulation genes. Fidaxomicin and OP-1118 even block further accumulation of mRNAs that had begun to accumulate before addition of the drugs. Thus, FDX

or OP-1118 can prevent completion of the sporulation process in the majority of cells, even when added after the process has been initiated. In contrast, vancomycin and metronidazole were ineffective at blocking sporulation when added to stationary-phase cells, and vancomycin had no consistent or pronounced effect on sporulation gene expression when added at either  $T_0$  or  $T_2$ . Although in some cases the mean expression ratio in the vancomycin-treated arm was reduced compared with control, this difference did not appear significant, as indicated by the widely overlapping intervals between the vancomycin-treated and control groups. These results fit with the mechanisms of action of the drugs. That is, FDX and OP-1118 inhibit transcription, a process that must occur continuously during growth and sporulation for the cell to produce a spore. Vancomycin and metronidazole inhibit processes specific to growing cells. Hence, their inability to block sporulation in stationary-phase cells might have been anticipated. However, the case of rifaximin requires additional explanation. The target of rifaximin, as for FDX and OP-1118, is the bacterial RNA polymerase. That the addition of rifaximin to stationary-phase cells had no effect on subsequent spore formation may indicate that the drug is not taken up by stationary-phase cells efficiently enough to block stationary-phase gene expression. Alternatively, rifaximin may have selective effects on the different forms of RNA polymerase that are active in growing and sporulating cells. Interestingly, Hawirko et al [33] found that rifampin was more effective at blocking sporulation of *Clostridium botulinum* when added during exponential growth than during the stationary phase.

In summary, the results presented here show that FDX and OP-1118 are potent inhibitors of *C. difficile* spore formation and are effective even when added after the differentiation process has begun. This property may contribute to the greater effectiveness of FDX compared with vancomycin in reducing recurrence of CDI.

## Notes

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