

Both Fidaxomicin and Vancomycin Inhibit Outgrowth of *Clostridium difficile* Spores

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Fidaxomicin (FDX) is approved to treat *Clostridium difficile*-associated diarrhea and is superior to vancomycin in providing a sustained clinical response (cure without recurrence in the subsequent 25 days). The mechanism(s) behind the low recurrence rate of FDX-treated patients could be multifactorial. Here, we tested effects of FDX, its metabolite OP-1118, and vancomycin on spore germination and determined that none affected the initiation of spore germination but all inhibited outgrowth of vegetative cells from germinated spores.

Clostridium difficile, a Gram-positive, spore-forming, obligate anaerobe, causes intestinal infections, usually in people who have recently completed antibiotic therapies for unrelated conditions (1). Antibiotics cause alterations in the normally protective

colonic microbiota, creating a niche for *C. difficile* to colonize (2, 3). To cause disease, *C. difficile* spores, which are unaffected by inciting antibiotics, must germinate to vegetative, or actively growing, bacteria in the anaerobic environment of the colon in order to produce the toxins that are responsible for the primary disease symptoms (4–6). Therefore, *C. difficile* spores, which are highly resistant to chemical disinfectants and antibiotics, are the source of infection. Although the exact mechanism and receptors involved in *C. difficile* spore germination are not clearly defined, both taurocholic acid and glycine have been identified as factors that synergistically stimulate germination of spores into virulent vegetative cells that secrete potent toxins (7, 8).

Vancomycin and metronidazole are commonly prescribed to treat *C. difficile* infections (CDI) (1). However, patients treated with vancomycin or metronidazole frequently relapse with *C. difficile* disease (1). Recently, fidaxomicin (FDX) was approved in the United States, Europe, and Canada as an alternative for the treatment of CDI. During phase 3 clinical trials, FDX was shown to be superior to vancomycin in sustaining clinical response without recurrence for up to 25 days following treatment (9, 10).

Multiple factors may lie behind the reduced rate of relapsing CDI in FDX-treated patients. Both FDX and its main metabolite OP-1118 have been shown to strongly inhibit *C. difficile* spore formation (11). FDX also has a reduced impact on the normally protective colonic microbiota (12, 13). In this study, we evaluated whether FDX might block germination of *C. difficile* spores.

To test the effect of FDX on *C. difficile* spore germination, we purified spores from *C. difficile* strains CD196 (14) and UK1 (15), as described previously (16). To provide a quantitative measure of the effects of FDX, OP-1118, and vancomycin on *C. difficile* spore germination, we analyzed the kinetics of the initiation of spore germination. By measuring the maximum rate of spore germination under different conditions, we are able to determine an apparent K_m , defined as the concentration that provides a half-max-

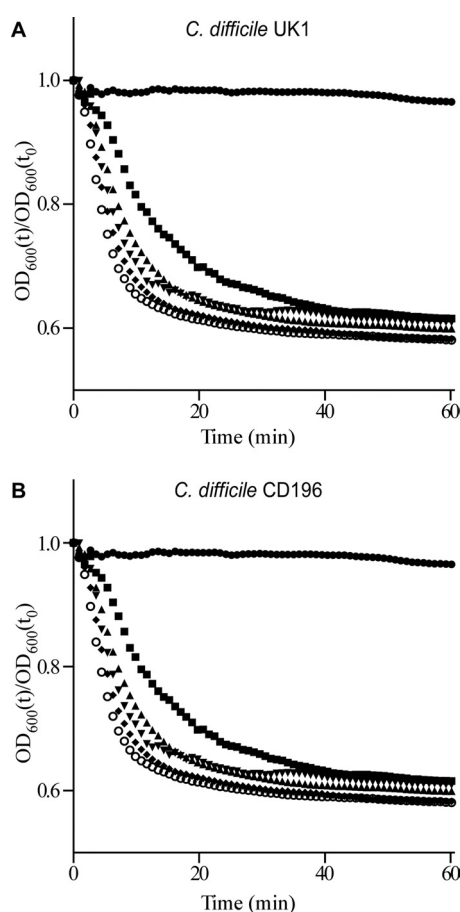


FIG 1 Analyzing the initiation of spore germination in *C. difficile* UK1 and *C. difficile* CD196. Purified *C. difficile* UK1 spores (A) and *C. difficile* CD196 spores (B) were suspended in BHIS medium alone (●) or in BHIS medium supplemented with 2 mM TA (■) or 5 mM TA (▲) or 10 mM TA (▼) or 20 mM TA (◆) or 50 mM TA (○). The initiation of germination was followed at A_{600} , and values were normalized to t_0 . The experiment was performed in triplicate, and quantified values of the apparent K_m for TA are listed in Table 1.

Received 3 August 2012 Returned for modification 25 September 2012

Accepted 5 November 2012

Published ahead of print 12 November 2012

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doi:10.1128/AAC.01611-12

TABLE 1 Apparent K_m values of *C. difficile* spores for taurocholic acid

Condition	K_m value (mM \pm SD) for strain ^a :	
	<i>C. difficile</i> UK1	<i>C. difficile</i> CD196
No drug	1.97 \pm 0.13	2.16 \pm 0.31
0.25 μ g/ml FDX	1.93 \pm 0.30	2.56 \pm 1.10
2.5 μ g/ml OP-1118	1.89 \pm 0.51	3.49 \pm 0.97
2.5 μ g/ml vancomycin	2.9 \pm 0.49	2.89 \pm 0.36

^a Values represent the averages of the results of three independent experiments and are listed in millimolar \pm 1 standard deviation from the mean. Differences are not statistically significant (Student's *t* test).

imal germination rate, for taurocholic acid (TA), a *C. difficile* spore germinant. This strategy has been used to test the effects of different compounds on *C. difficile* spore germination (16–19), *C. sordellii* spore germination (18, 20), and *Bacillus anthracis* spore germination (21). If the drugs were to affect the initiation of spore germination, a change in apparent K_m should be detected.

C. difficile UK1 and *C. difficile* CD196 spores were suspended in

BHIS (brain heart infusion–5 g/liter yeast extract–0.1% L-cysteine) medium alone or in BHIS medium supplemented with 2 mM TA or 5 mM TA or 10 mM TA or 20 mM TA or 50 mM TA, and germination was assayed at room temperature by following changes in A_{600} using a PerkinElmer Lambda 25 spectrophotometer. As expected, *C. difficile* UK1 spores (Fig. 1A) and *C. difficile* CD196 spores (Fig. 1B) did not germinate in BHIS medium alone; TA is required for *in vitro* *C. difficile* spore germination (7, 8). Moreover, we observed a concentration-dependent, TA-dependent increase in the rate of *C. difficile* spore germination. To analyze the effects of FDX, OP-1118, and vancomycin on *C. difficile* spore germination, we added 0.25 μ g/ml FDX (2 \times MIC) or 2.5 μ g/ml OP-1118 (2.5 \times MIC) or 2.5 μ g/ml vancomycin (2.5 \times MIC) and measured the kinetics of *C. difficile* spore germination. Germination in the above conditions was followed in triplicate samples; a representative sample is shown in Fig. 1. The apparent K_m values for TA in the presence or absence of the tested drugs, as determined from the data in Fig. 1 and two additional replicates, are shown in Table 1. The addition of FDX, OP-1118, or vanco-

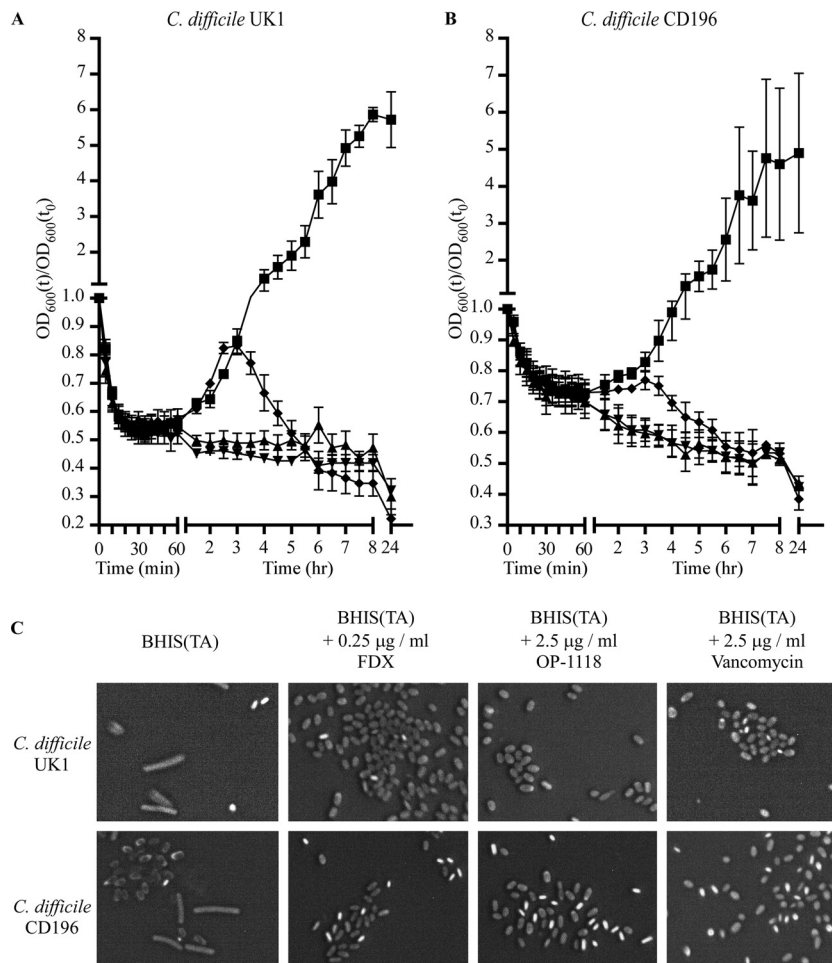


FIG 2 Measuring outgrowth of *C. difficile* spores during antibiotic exposure. Purified *C. difficile* UK1 spores (A) and *C. difficile* CD196 spores (B) were suspended in BHIS medium supplemented with 2 mM TA alone (■) or with 0.25 μ g/ml FDX (\blacktriangle) or 2.5 μ g/ml OP-1118 (\blacktriangledown) or 2.5 μ g/ml vancomycin (\blacklozenge). A_{600} was followed at 5-min intervals for 60 min and then every 30 min for another 7 h and finally determined at 24 h. Germination and outgrowth were followed at 37°C in an anaerobic environment and normalized to t_0 . Data points represent averages of the results of 3 independent experiments, and error bars represent 1 standard deviation from the mean and, at times, are smaller than the data point itself. (C) Samples were taken at the 150-min time point (above) and analyzed by phase-contrast microscopy. Dormant spores appear bright; germinated spores appear dark. Magnification: \times 1,000. Contrast was adjusted using Canvas X and applied equally to all images.

mycin had no effect on the apparent K_m for TA, which was similar to the previously reported value (16). Moreover, none of these compounds induced germination, suggesting that these antibiotics do not either positively or negatively affect the initiation of spore germination. Similar findings on spore germination were observed with *C. difficile* strain UK14 (15) with concentrations that are suprainhibitory for vegetative growth: 200× the MIC of FDX and 25× the MIC of OP-1118 (data not shown).

Since these drugs do not affect the initiation of spore germination, we tested whether later stages of germination would be affected. We assayed outgrowth by following changes in A_{600} in an anaerobic environment (10% hydrogen, 5% CO₂, 85% nitrogen) over a 24-h period. *C. difficile* UK1 spores (Fig. 2A) or *C. difficile* CD196 spores (Fig. 2B) were suspended in BHIS medium alone or BHIS medium supplemented with 2 mM TA [BHIS(TA)] or BHIS(TA) supplemented with 0.25 μg/ml FDX or 2.5 μg/ml OP-1118 or 2.5 μg/ml vancomycin. Spores suspended in BHIS medium alone did not germinate during the duration of the experiment (data not shown). Spores suspended in BHIS(TA) initially showed reduced absorbance, indicating spore germination, but showed an increase in the A_{600} at approximately 2 h, and this increase continued for the duration of the experiment. We did not detect outgrowth (i.e., the rise in A_{600} subsequent to the initial drop) when *C. difficile* UK1 spores or *C. difficile* CD196 spores were suspended in BHIS(TA) with FDX or OP-1118. However, when outgrowth was measured in the presence of vancomycin, we observed an increase followed by a decrease in A_{600} , suggesting that outgrowth had begun but was then inhibited by vancomycin. We confirmed by phase-contrast microscopy that the increase in absorbance observed for *C. difficile* UK1 (Fig. 2A) and *C. difficile* CD196 (Fig. 2B) was correlated with the disappearance of phase-bright spores and appearance of phase-dark spores and vegetative cells in the suspension (Fig. 2C). Spores incubated with any of the antibiotics transitioned from the phase-bright to the phase-dark state, but no vegetative cells appeared when antibiotics were present (Fig. 2C). These results suggest that FDX, OP-1118, and vancomycin inhibit outgrowth of vegetative cells from *C. difficile* spores, though FDX and OP-1118 may inhibit outgrowth at an earlier stage than does vancomycin, because spores germinated in the presence of FDX or OP-1118 did not show any increase in optical density (OD) during germination. This result is in agreement with the different mechanisms of action of the two drugs and supports previous observations that compounds which inhibit cell wall synthesis inhibit later stages of outgrowth (22). In contrast, FDX, which targets RNA polymerase and therefore inhibits production of RNA and proteins, completely inhibited outgrowth throughout the duration of the experiments (23).

In addition to inhibition of spore formation, inhibiting the conversion of *C. difficile* spores to actively growing vegetative cells could be a powerful way to prevent relapsing CDI. We found that FDX, OP-1118, and vancomycin do not affect the initiation of *C. difficile* spore germination but do inhibit outgrowth of a vegetative cell from the germinating spore. This action would also prevent the synthesis of toxins and any downstream effects. Thus, FDX, OP-1118, and vancomycin inhibit some of the earliest stages in *C. difficile* pathogenesis but the effects of FDX alone on *C. difficile* spore germination cannot explain the reduced frequency of relapsing CDI. Use of a narrow-spectrum antibiotic that inhibits spore formation and also prevents the outgrowth of germinated spores could be especially powerful, since it would allow most of

the microbiota time to recover and reestablish colonization resistance.

ACKNOWLEDGMENTS

We thank A. L. Sonenshein for critical comments during preparation of the manuscript.

This project was funded through an Optimer Pharmaceuticals, Inc., funded contract.

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