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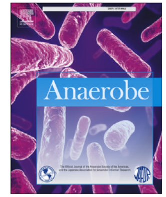
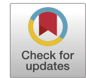


Clostridioides difficile (including epidemiology)

Clostridioides difficile (including epidemiology)

Visualization of fidaxomicin association with the exosporium layer of *Clostridioides difficile* spores



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A B S T R A C T

Background: Fidaxomicin has novel pharmacologic effects on *C. difficile* spore formation including outgrowth inhibition and persistent spore attachment. However, the mechanism of fidaxomicin attachment on spores has not undergone rigorous microscopic studies.
Materials & methods: Fidaxomicin attachment to *C. difficile* spores of three distinct ribotypes and *C. difficile* mutant spores with inactivation of exosporium or spore-coat protein-coding genes were visualized using confocal microscopy with a fidaxomicin-bodipy compound (green fluorescence). The pharmacologic effect of the fidaxomicin-bodipy compound was determined. Confocal microscopy experiments included direct effect on *C. difficile* wild-type and mutant spores, effect of exosporium removal, and direct attachment to a comparator spore forming organism, *Bacillus subtilis*.
Results: The fidaxomicin-bodipy compound MIC was 1 mg/L compared to 0.06 mg/L for unlabeled fidaxomicin, a 16-fold increase. Using confocal microscopy, the intracellular localization of fidaxomicin into vegetative *C. difficile* cells was observed consistent with its RNA polymerase mechanism of action and inhibited spore outgrowth. The fidaxomicin-bodipy compound was visualized outside of the core of *C. difficile* spores with no co-localization with the membrane staining dye FM4-64. Exosporium removal reduced fidaxomicin-bodipy association with *C. difficile* spores. Reduced fidaxomicin-bodipy was observed in *C. difficile* mutant spores for the spore surface proteins CdeC and CotE.
Conclusion: This study visualized a direct attachment of fidaxomicin to *C. difficile* spores that was diminished with mutants of specific exosporium and spore coat proteins. These data provide advanced insight regarding the anti-spore properties of fidaxomicin.
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1. Introduction

Clostridioides difficile is a spore forming, anaerobic bacterium and the most common cause of death due to infectious gastroenteritis in the North America and Europe, and associated with significant morbidity and economic burden [1–3]. Pathogenesis of *C. difficile* infection (CDI) begins by ingestion and germination of spores, which are common in the environment [4,5]. The spore external main layers consist of an outer coat exosporium followed by the spore coat and outer membrane encoded by spore coat and exosporium genes [6–8]. Persistence of spores is associated with recurrent CDI, which occurs in about 25% of patients treated with oral vancomycin or metronidazole [9,10]. Fidaxomicin, a macrocyclic antibiotic compound with a limited antibacterial activity against the gut microflora demonstrated reduced CDI recurrence rates compared to vancomycin [11]. Fidaxomicin has also been

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shown to have effects on *C. difficile* including inhibition of spore production [12] and spore outgrowth [13]. Co-incubation of fidaxomicin with *C. difficile* spores of several different ribotypes demonstrated persistence of fidaxomicin on spores despite several different washing techniques [14]. However, visualization of the attachment has not been performed and the underlying mechanisms of attachment are unknown. A fidaxomicin-bodipy compound with green fluorescence allowed further characterization of this pharmacologic property. Thus, the purpose of this study was to describe and visualize the direct association of fidaxomicin with *C. difficile* spores and determine the spore structural layer area of attachment.

2. Results

2.1. Characterization of fidaxomicin-bodipy properties

Using *C. difficile* R20291, the MIC of the fidaxomicin-bodipy compound was 1 mg/L compared to 0.06 mg/L for unlabeled fidaxomicin, a 16-fold increase. Using confocal microscopy, intracellular localization of fidaxomicin into vegetative *C. difficile* cells was observed (Fig. 1). The fidaxomicin-bodipy compound was also able to inhibit spore outgrowth (Figure S1) validating the findings of Chilton et al. [14]. Spore concentrations treated with high concentration of fidaxomicin-bodipy were unchanged while increased vegetative cells (10^6–10^8 CFU) were observed in control (non-treated) spores. These properties (MIC, localization and spore outgrowth inhibition) confirmed that fidaxomicin-bodipy was an active compound comparable to standard fidaxomicin.

2.2. Fidaxomicin-bodipy association with C. difficile spore exosporium outer layer

The fidaxomicin-bodipy compound (green) was visualized surrounding the *C. difficile* spore membrane (red) on the external side of the spore only with no co-localization with the membrane staining with FM4-64 (Fig. 2). Similar patterns were observed with all three *C. difficile* ribotypes tested (027, 012 and 078).

To assess whether this association was specific to the outer layer of the spore, the exosporium of the spores was depleted using two separate methods either sarkosyl or trypsin. The *C. difficile* spores were treated with fidaxomicin-bodipy prior to removal of the exosporium using the sarkosyl or trypsin solution (Fig. 3). Removal of the exosporium almost completed eliminated the fidaxomicin-bodipy attachment to the spore surface.

2.3. Fidaxomicin-bodipy association with B. subtilis spores

To investigate the specificity of fidaxomicin-bodipy association to the spore outer layer the attachment of the fidaxomicin-bodipy compound with *B. subtilis* spores was investigated (Figure S2). Compared to *C. difficile*, there was no observable interaction of fidaxomicin-bodipy with the *B. subtilis* spore outer layer.

2.4. Saturation curve of fidaxomicin-bodipy bound to C. difficile 630 ΔermB spores

To begin dissecting the exosporium proteins involved in binding of fidaxomicin-bodipsy to *C. difficile* spores, the saturation of the fluorescence intensity at various concentrations of fidaxomicin-bodipy and exposure times was determined. *C. difficile* spores were incubated with a range of fidaxomicin-bodipy concentrations (5–100 μg/ml) and images taken at different exposure times (10–500 ms). Analysis of the fluorescent signal of individual spores revealed that the fluorescent intensity of *fidaxomicin-bodipy* bound to *C. difficile* spores was concentration and exposure-time dependent (Figure S3). Plotting the fluorescence signal revealed that an fidaxomicin-bodipy concentration and exposure time that would allow adequate detection of an increase and/or decrease in fluorescence intensity upon incubation fidaxomicin-bodipy to *C. difficile* spores was 50 μg/ml and 50 ms of exposure time.

2.5. Effect of inactivation of exosporium protein-coding genes in fidaxomicin-bodipy binding to C. difficile spores

To preliminary identify exosporium fidaxomicin-bodipy-binding proteins, mutant strains of exosporium encoding-genes with ClosTron insertional inactivation were used. Inactivation the exosporium collagen-like proteins encoded by *bclA1* and *bclA2* had no relevant change in fidaxomicin-bodipy binding to *C. diffcile* spores. Notably, *C. difficile* spores of a bclA3 mutant strain had a ~3-fold increase in immunofluorescence associated to fidaxomixin-bodipy binding (Fig. 4). In the absence of the cysteine rich protein, CdeC, a significant decrease in fidaxomicin-bodipy was evidenced. By contrast, absence of CdeM, led to a significant increase in fidaxomicin-bodipy binding to *C. difficile* spores. These results

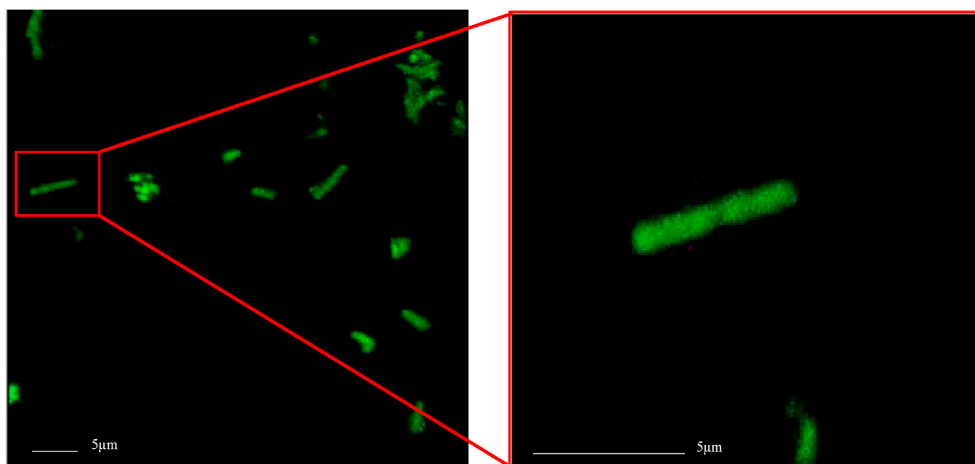


Fig. 1. Fidaxomicin-bodipy (green) is internalized into vegetative *C. difficile* R20291 cells after 24h incubation at sub-MIC concentration (0,5 μg/mL) 63X magnification, confocal imaging.

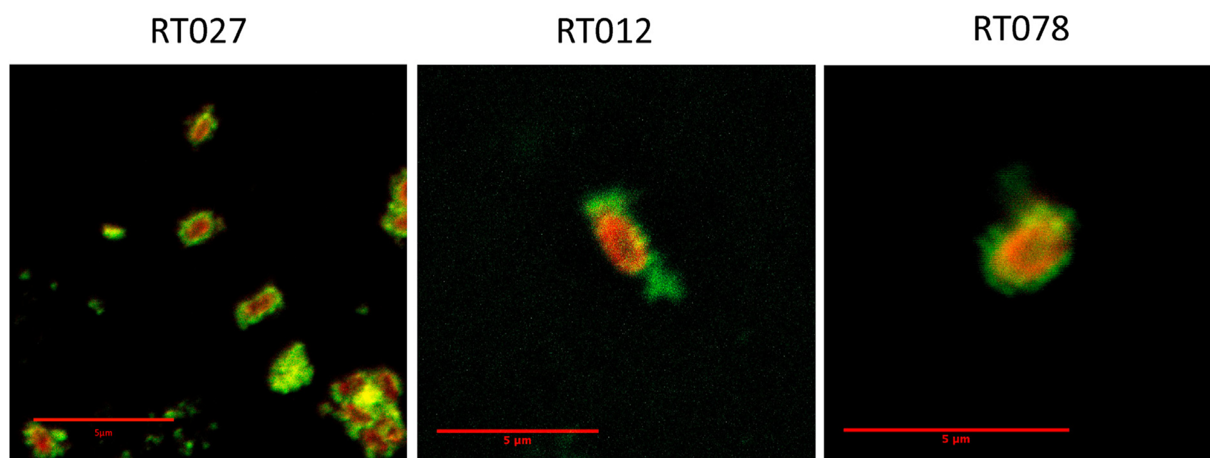


Fig. 2. Fidaxomicin-bodipy is associated with *C. difficile* spore outer layer from three different ribotypes (027, 012, 078). Green: fidaxomicin-bodipy, red: *C. difficile* spore (FM4-64), 63X magnification, confocal imaging.

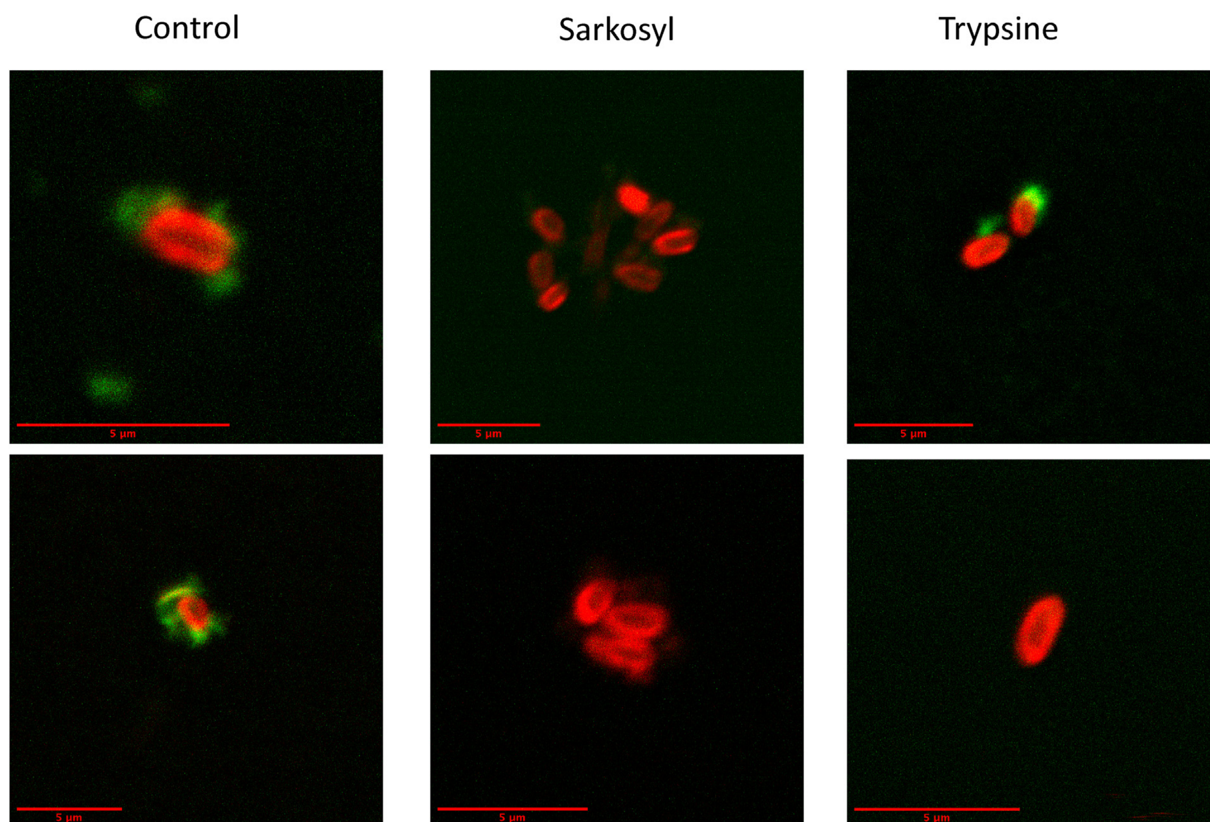
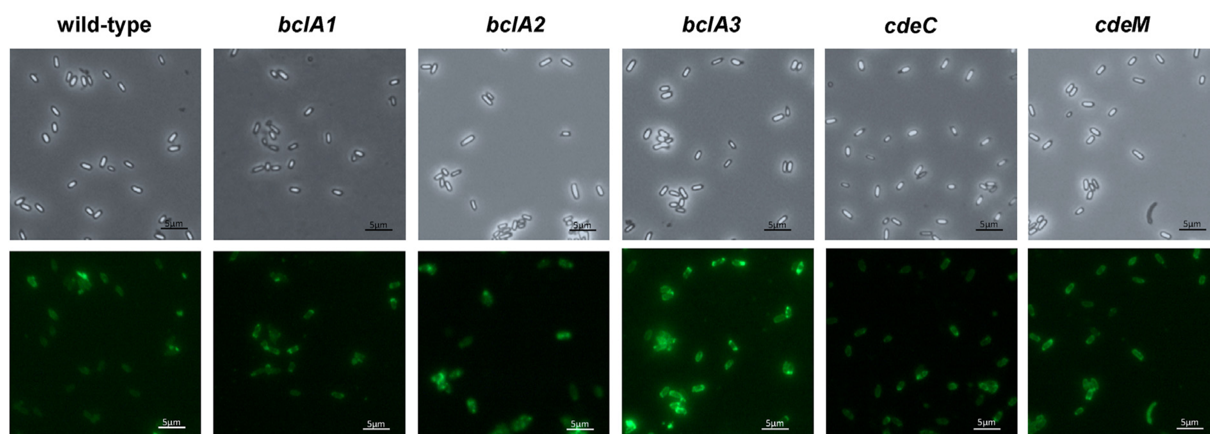


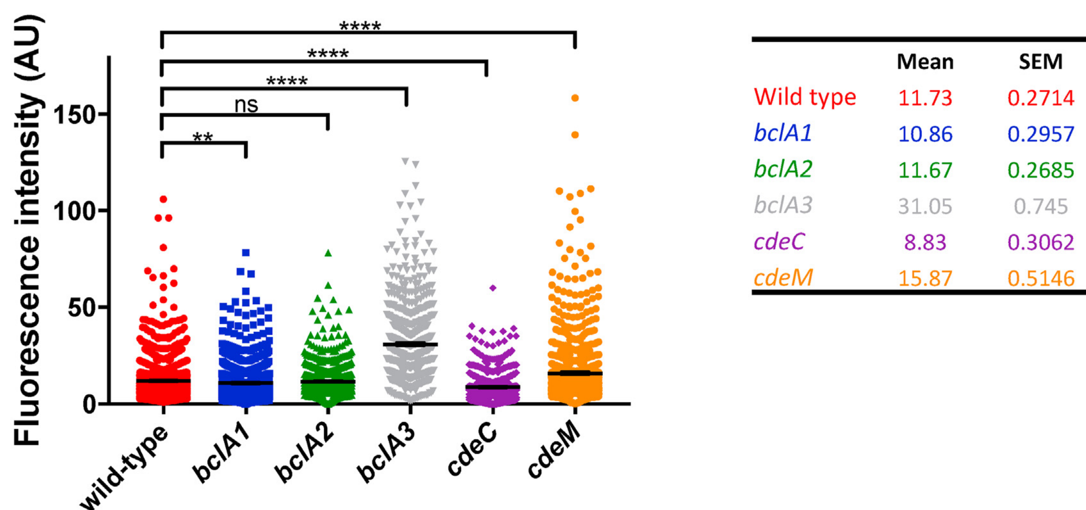
Fig. 3. Fidaxomicin-bodipy association with *C. difficile* spore after exosporium removal on strain R20291 (RT027). Green: fidaxomicin-bodipy, Red: *C. difficile* spore (FM4-64), 63X magnification, confocal imaging. Each row represents separate experiments.

suggest that CdeC might be a target candidate for fidaxomicn binding.

2.6. Effect of inactivation of spore-coat protein-coding genes in in fidaxomicin-bodipy binding to C. difficile spores

Spore coat proteins are conceived to be underlying the exosporium layer of *C. difficile* spores [15]; however, most of these proteins are accessible to small molecules with molecular masses lower than 13 kDa, such as fidaxomicin-bodipy. No significant difference in fidaxomicin-bodipy binding to *C. difficile* spores was observed in spores lacking CotB and CotCB compared to the parental wild-type strain (Fig. 5); however a significant increase in fidaxomixin-bodipy binding was evidenced in spores of a *cotA* mutant. By contrast, in *C. difficile* spores lacking CotE, showed a 1.3-fold decrease in fidaxomicin-bodipy binding. These results suggest while CotE could be a potential target for fidaxomixin-bodipy-binding, the absence of CotA might leads to a disruption of the spore-surface integrity and consequent increase in binding sites.





	Mean	SEM
Wild type	11.73	0.2714
bclA1	10.86	0.2957
bclA2	11.67	0.2685
bclA3	31.05	0.745
cdeC	8.83	0.3062
cdeM	15.87	0.5146

Fig. 4. Effect of inactivation of exosporium protein-coding genes in fidaxomicin-bodipy binding to *C. difficile* spores. A) Representative images of spores from strains with inactivated exosporium proteins (BclA1, BclA2, BclA3, CdeC and CdeM) by ClosTron insertional inactivation and spores from the parental strain (wild type). Images were taken at 100X and scale bar represents 5 μm. B) Comparison of the relative fluorescent intensity from inactivated mutants in *bclA1*, *bclA2*, *bclA3*, *cdeC* and *cdeM*, against wild type. At least 100 random spores were analyzed in triplicate, and relative fluorescent intensity was estimated using ImageJ. Kruskal-Wallis test was done to test for significance. Statistical significance was defined as $p < 0.01$ (**), or $p < 0.0001$ (****).

3. Discussion

C. difficile is the most common healthcare associated pathogen in the USA [16]. CDI is characterized by a high recurrence rate of 25% or higher [17]. Treatment with fidaxomicin has been shown to reduce the incidence of CDI recurrence by approximately 50% [18]. The anti-recurrence properties have been attributed to less microbiome disruption compared to vancomycin [11]. However, recent data suggesting effect of fidaxomicin on spores indicate a potential other alternative anti-recurrence effect [12,14]. Chilton et al. demonstrated that fidaxomicin persistence on *C. difficile* spores preventive vegetative growth and subsequent toxin production [14]. This study validated these findings and also provides, for the first time a direct visualization of the attachment of fidaxomicin on *C. difficile* spores. Using *C. difficile* ribotypes with different exosporium architecture, we demonstrated that a fidaxomicin-bodipy compound was pharmacologically comparable to non-labelled fidaxomicin and that the fidaxomicin-bodipy compound bound to the external layer of the *C. difficile* spore without co-location with membrane staining. The fidaxomicin-bodipy compound was almost completely removed if the exosporium layer was removed. The fidaxomicin-bodipy compound was not able to associate with spores of *B. subtilis*. Using *C. difficile* mutant strains with inactivation of exosporium or spore-coat protein-coding genes, several candidates genes were identified that abrogated the association. Strengths of this study include validation of the fidaxomicin-bodipy as a robust tool to study and visualize fidaxomicin direct interaction with *C. difficile* spore outer layer and phenotypic visualization of the direct interaction between fidaxomicin and *C. difficile* spores.

These results demonstrate that fidaxomicin directly attaches to the exosporium of *C. difficile* spores. Fidaxomicin-bodipy surrounded but did not colocalize with the FM4-64 staining, a non-specific marker for bacterial membranes. Exosporium removal post fidaxomicin-bodipy treatment completely removed the association. The absence of fidaxomicin-bodipy interaction with the *B. subtilis* spore outer layer despite its high hydrophobicity also suggest a specificity for *C. difficile* spore surface components.

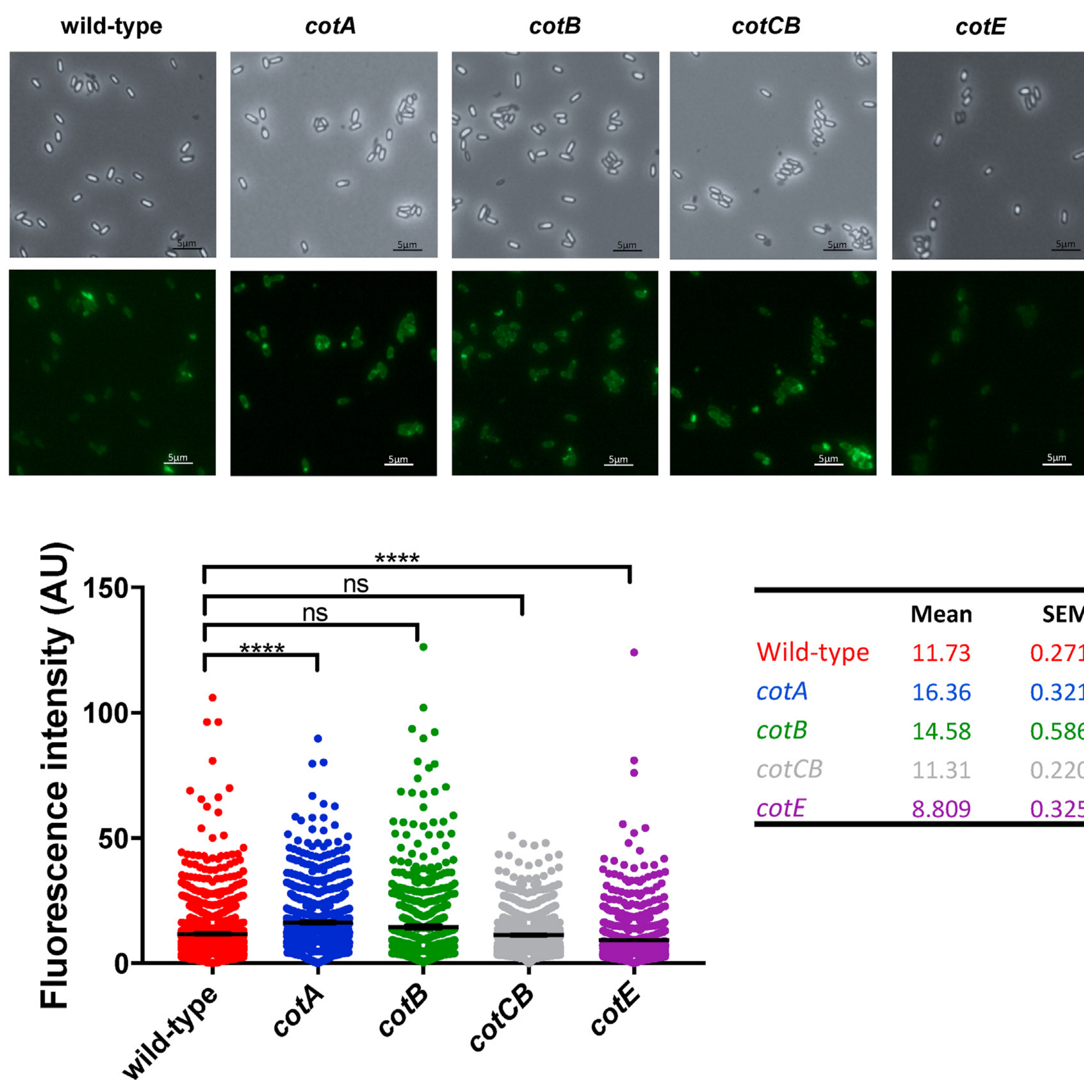


Fig. 5. Effect of inactivation of spore-coat protein-coding genes in in fidaxomicin-bodipy binding to *C. difficile* spores. A) Representative images of spores from strains with inactivated coat proteins (CotA, CotB, CotCB and CotE) by ClosTron insertional inactivation and spores from the parental strain (wild type). Images were taken at 100X and scale bar represents 5 μm. B) Comparison of the relative fluorescent intensity from inactivated mutants in *cotA*, *cotB*, *cotCB* and *cotE*, against wild type. At least 100 random spores were analyzed in triplicate, and relative fluorescent intensity was estimated using ImageJ. Kruskal-Wallis test was done to test for significance. Statistical significance was defined as $p < 0.0001$ (****).

Indeed, *B. subtilis* exosporium presence is controversial and few of its coat proteins have orthologs in *C. difficile* (20 in *Clostridia* genomes on about 70 in *B. subtilis)* [19,20]. *B. subtilis* outer spore coat layer is hydrophobic similar to *C. difficile* [21]. Thus, it is likely that other properties of *C. difficile* spores rather than spore hydrophicity may predict attachment of fidaxomicin.

Mutant experiments suggest that CdeC and CotE might be potential targets for fidaxomicin and spore associations; however, further experiments will be required to confirm these interactions. It is noteworthy that the absence of CotA, CdeM and to a greater extent, BclA3, led to an increase in the levels of fidaxomicin-bodiopy, which suggest that these proteins affect the exosporium and/or spore coat integrity leading to the exposure of additional fidaxomicin-binding sites in the spore surface. This variability in the fidaxomicin loaded in the exosporium layer could be due to the balance of proteins during the assembly of the exosporium. Previous reports from our group suggest that the correct assembly of the exosporium depends on the abundance of different exosporium proteins [7]. For instance, CdeM recruits CdeB, CotA and CdeC which in turns recruits CdeA and CotB; while BclA1, BclA2 and BclA3 require both CdeM and CdeC to be allocated on the exosporium surface [7,22].

These data taken together with previous studies support the hypothesis that persistent association of fidaxomicin with *C. difficile* spores could be part of the anti-CDI recurrence mechanism of action. Although considered a narrow-spectrum anti-*C. difficile* antimicrobial, two recent clinical trials of other narrow spectrum anti-*C. difficile* antibiotics (surotomycin [23] and cadazolid [24]) did not demonstrate a consistent anti-recurrence effect. Likewise, in the fidaxomicin clinical trials, patients infected with *C. difficile* ribotype 027 strains had higher rates of CDI recurrence compared to patients infected with other ribotypes [18]. Although we included one

ribotype 027 strain in our study, future epidemiologic studies will be required to better understand the clinical significance of these findings. This study has several limitations. We used static, *in vitro* models to study and visualize the association between *C. difficile* spores and fidaxomcin. Future, dynamic flow and animal models will be required to confirm these results on whether this spore attachment affects onset of CDI. The longevity of this persistence and addition of variables associated with intestinal infections should be tested in future *in vitro* model. Clinical studies will be required to test degree of attachment based on ribotype of exosporium properties along with an assessment of rates of recurrent CDI.

In conclusion, this study visualized a direct attachment of fidaxomicin to *C. difficile* spores. These data provide advanced insight regarding the anti-spore properties of this compound.

4. Material and methods

4.1. Bacterial strains and culture media

Three *C. difficile* ribotypes known to produce exosporium with different ultrastructures were used to assess attachment of fidaxomicin-bodipy to *C. difficile* spores (CD630: ribotype 012; R20291: ribotype 027; and clinical strain MT225: ribotype 078) [6]. To determine the potential binding site of fidaxomicin-bodipy, the compound was exposed to several *C. difficile* mutant spores with inactivation of exosporium or spore-coat protein-coding genes (Supplemental table 1). To determine the specificity of the fidaxomicin-bodipy compound to *C. difficile* sores, *Bacillus subtilis* ATCC 23857 was used.

C. difficile was grown at 37 °C under anaerobic conditions in an anaerobic chamber in BHIS medium: 3.7% weight vol^{-1} brain heart infusion broth (BD, USA) supplemented with 0.5% weight vol^{-1} yeast extract (BD, USA) and 0.1% weight vol^{-1} l-cysteine (Merck, USA) or on BHIS agar plates.

4.2. Antibiotics and MIC determination

A green fluorescent conjugate of fidaxomicin, fidaxomicin-bodipy was constructed and provided by Merck along with standard fidaxomicin. Both compounds were re-suspended in ethanol and MICs were determined by broth microdilution in BHI medium as previously described [25,26].

4.3. Spore preparation

For visualization studies with CD630, R20291, or MT255, each strain was plated on blood agar and incubated at 37 °C under anaerobic conditions for 5 days. The growth from the plates was then scraped out with ice-cold sterile water and incubated overnight at 4 °C. The next day the samples were transferred to microcentrifuge tubes on the top of a 10 mL bed volume of 50% sucrose in water and centrifuged for 20 min at 3200×g. The spore pellet was then washed 5 times with and resuspended in sterile water. Spore concentration was measured by plating 100 μl of 10-fold serial dilutions on blood agar.

For visualization studies with the *C. difficile* mutant spores, spore preparation was done as previously described [7]. Briefly, 100 μL of 1:1,000 dilution of an overnight culture in BHIS was plated in 70:30 agar plates. Plates were incubated for 7 days at 37 °C under anaerobic conditions in anaerobic chamber Bactron III-2 (Shellab USA). Then plates were removed from the chamber, and colonies were scraped out with ice-cold sterile milliQ water. Then the sporulated culture was washed five times with ice-cold milliQ water in micro-centrifuge at 18,400×g for 5 min each. To separate spores, the sporulated culture was loaded in 45% weight vol^{-1} autoclaved Nycodenz (Axell USA) solution and centrifugated at 18,400×g for 40 min to separate the spores from cellular debris. Spore pellet was recovered and washed 5 times at 18,400×g for 5 min with ice-cold sterile milliQ water to remove Nycodenz. Spores were counted in Neubauer chamber, and volume adjusts at 5×10^9 spores mL^{-1} and stored at −80 °C.

4.4. Spore association assay and quantification

C. difficile spores of approximately 10^{6-7} CFU/mL were treated with fidaxomicin-bodipy 100 μg/mL for 1h at room temperature in 100 μl PBS along with untreated controls. The samples were washed five times in PBS centrifugation for 5 min at 15,000 rpm, with transfer to a new tube between washes 3 and 4 [14]. Total viable count and spore enumeration (after alcohol shock) were quantified after 48h incubation in BHIS at 37 °C in the anaerobic chamber by plating serial dilutions on blood agar plates incubated anaerobically for 48 h at 37 °C.

4.5. Exosporium removal

To remove the *C. difficile* exosporium, approximately 10^{6-7} spores were treated with fidaxomicin-bodipy 100 μg/mL for 1h at room temperature in 100 μl PBS along with untreated controls and then incubated for 2h at 37 °C and 200 rpm in 30 μL of either trypsin or sarkosyl solution [27]. The sarkosyl solution consisted in 1% sarkosyl (or N-lauroylsarcosine sodium salt, Sigma-Aldrich), 0,3 mg/mL proteinase K (from DNA extraction kit, Qiagen), 25 mM PBS (pH 7,4). As an alternative treatment, 5 μg of trypsin were used in 25 mM PBS (pH 7,4). Sarkosyl and tryspin treated spores were washed 5 times in sterile distilled water and stored at −80 °C prior to use.

4.6. Sample preparation for microscopy

Bacterial vegetative cells or spores were fixed in 1.6% paraformaldehyde for 1h at room temperature and washed with distilled water. The bacterial inner and outer membrane was stained with 10 μg/mL FM4-64 (ThermoFisher Scientific) for another hour, the samples were washed and kept at 4 °C until further analysis. The cells were examined by confocal microscopy (Leica SP8). Fidaxomicin-bodipy excitation/emission spectrum was set at ~461–489/501-549-nm; FM4-64 at ~515/640 nm.

Fidoximicin-bodipy (FDX-BDP) binding assay to C. difficile mutant spores. C. difficile spores of approximately 10^{6-7} CFU/mL were used for the assay. Fidoximicin-bodipy (FDX-BDP) was used at 100 μg/mL, 50 μg/mL, 25 μg/mL, 10 μg/mL and 5 μg/mL in 20 μL PBS sterile and incubated for 1 h at room temperature to determine optimal concentration of fidaxomicin-bodipy to be used for downstream experiments. To remove the unbound FDX-BDP, the spores were washed by centrifugation at 14,000 rpm for 10 min 5 times with sterile water, and then 3 μL were mounted in pads of 1% agarose. Visualization was done at 100x with immersion oil using a BX53 Olympus fluorescence microscope. Microscopy experiments with mutant *C. difficile* spores were treated with 50 μg/mL of fidaxomicin-bodipy for 1 h at room temperature and micrographs were taken at 50 ms time of exposure. Images were analyzed using ImageJ.

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Declaration of competing interest

KW Garey: Research support from Merck & Co.
All other authors: None to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.anaerobe.2021.102352.

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