DEVELOPMENT OF A CHEMICAL MUTAGENESIS STRATEGY TO GENERATE SUPPRESSOR MUTATIONS OF SPORE COAT PERMEABILITY IN *Clostridioides Difficile*

A Thesis

by

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Submitted to the Graduate and Professional School of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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August 2023

Major Subject: Microbiology

ABSTRACT

Obligate anaerobe *Clostridioides difficile* relies on the formation of spores to survive in aerobic environments. Despite the role of *C. difficile* spores in transmission of the pathogen there remains much unknown about the sporulation and germination pathways of *Clostridioides difficile*. Outer spore layers are essential for interaction with the environment and spore resistance to decontaminants. The outer spore layers of *C. difficile* spores are complex and our understanding of them lacking due to a low number of orthologs shared with model gram positive spore forming bacteria species like *Bacillus subtilis*. Mutations in the outer spore layer genes *cdeC* and *cotL* render spores susceptible to lysozyme-triggered germination due to defects in spore coat formation. The aim of this study was to develop a genetic screen to identify determinants of spore coat formation in *C. difficile* spores. Therefore, we first defined conditions for screening for resistance to lysozyme-triggered germination in *C. difficile cdeC*::CT mutant spores. We observed that heat is required to kill lysozyme-germinated *cdeC*::CT spores. Next, we tested whether a genetic screen of Ethyl methanesulfonate (EMS) mutagenized *cdeC*::CT was able to identify suppressor mutants with restored wild type resistance to lysozyme-triggered germination. Our results demonstrate that our screen was not sensitive enough to identify suppressor mutants. Since our screen was unsuccessful, we then tested if there were optimal heat and mutagenesis conditions that might be utilized in future screens. Overall, these results indicate that the same genetic screen in a mutant background with a more sensitive phenotype of lysozyme-triggered germination, such as *cotL*, may identify suppressor mutants of spore coat permeability.

ACKNOWLEDGEMENTS

I would like to thank my committee chair Dr. Paredes-Sabja, along with my committee members, Dr. Siegele and Dr. Zeng, for their interest, support, and guidance throughout the duration of this research.

I would also like to thank my friends and colleagues at Texas A&M University along with the department faculty and staff, especially Jennifer Bradford and Marina Maale for their amazing support.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a thesis committee consisting of Professors Daniel Paredes-Sabja and Deborah Siegele of the Department of Microbiology and Professor Lanying Zeng of the Department of Biochemistry and Biophysics.

Funding Sources

Graduate study was supported by start-up funds for Dr. Daniel Paredes-Sabja's lab at Texas A&M University.

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1. INTRODUCTION

Clostridioides difficile (*C. difficile*) is a gram-positive obligate anaerobe and a major cause of nosocomial antibiotic related diarrhea. *C. difficile* is treated using antibiotics, has a high rate of recurrence at around 20-30%, and is transmitted via spore contamination in the environment or direct contact with spores [15, 20]. The spore morphotype is essential to transmission as *C. difficile* vegetative cells are unable to survive in aerobic environments [10, 15]. There is a lack of knowledge of the mechanisms of spore assembly in *C. difficile* as it differs greatly from the model spore forming organism *Bacillus subtilis* [15]. *Bacillus subtilis* (*B. subtilis)* the model organism of spore forming gram positive bacteria lacks the outer most spore layer, the exosporium, and only 25% of *B. subtilis* spore coat proteins have homologs in *C. difficile* [1, 8, 18, 19]. *C. difficile* spore assembly occurs in two cell compartments, the forespore and mother cell. Expression of assembly machinery is controlled separately under different sigma factors. In the forespore, sigma factors F and G control spore assembly of the inner layers of the spore. In the mother cell, sigma factors E and K control assembly of the outer spore layers [17, 18].

There are seven layers of a *C. difficile* spore [15, 17]. The innermost layer is the spore core which contains DNA, RNA, enzymes and calcium dipicolinic acid (Ca-DPA). The inner membrane is the second most inner layer of the spore which contains germinant receptors and has low permeability to small molecules. The third layer is the germ cell wall which becomes the cell wall of the vegetative cell after germination. The fourth layer is the peptidoglycan (PG) cortex. Next is the outer membrane which is required for spore formation and although it does not present a permeability barrier it is important for spore resistance [17]. The second most outer layer is the spore coat which consists of multiple layers of proteins and plays an essential role in spore resistance. The outermost layer of the spore is the exosporium which consists of multiple layers of

proteins. The exosporium is electron dense, has hair like projections, and is in direct contact with the environment [16].

The outer spore layers have essential functions for resistance to decontaminants and molecules larger than about 14kDa [16]. *cdeC* is a 1218 base pair gene that codes for a protein found to be essential for proper spore coat and exosporium assembly and is localized in the exosporium of fully formed spores [2, 3]. Mutants of *cdeC* were constructed and found to have reduced resistance to heat, ethanol, and lysozyme [2, 3]. Treatment with lysozyme causes a large portion of *cdeC* mutant spores to germinate when compared to wild type spores. *cotL* is a 399 base pair long gene that encodes a spore coat protein expressed in the mother cell during spore formation. *cotL* deletion mutants show similarly high levels of permeability to lysozyme [1].

Suppressor mutation screening can be used as a strategy for identifying genes of interest in a pathway. Random mutagenesis will increase the likelihood of suppressor mutations in the population. Ethyl methanesulfonate (EMS), a DNA alkylating agent, produces single point mutations and has been used to induce mutations in *C. difficile* [21]. Another method of generating random mutations in bacteria is through the construction of transposon libraries. Transposons are mobile DNA sequences capable of changing location within a genome [13]. These mobile sequences can be used as a form of random mutagenesis where the transposon is inserted from a plasmid into the genome. Plasmids containing transposons with selective markers and inducible instability can generate pools of mutants in which each mutant contains a single transposon insertion [4].

pRPF215 is a plasmid used to construct transposon insertion libraries in *C. difficile*. The pertinent elements of the plasmid are a *mariner* transposon of the *ermB* erythromycin resistance gene and the *Himar1* transposase gene under control of the P_{tet} promoter. The P_{tetR} promoter is

oriented towards the plasmid origin of replication and causes tetracycline-dependent plasmid instability [5]. This design allows for induction of a transposition event and disruption of plasmid replication simultaneously, as well as a method for selecting successful transposon insertion mutations using erythromycin.

2. METHODS

TABLE 1. Bacterial strains, primers, and plasmids used

Name	Relevant characteristic	Source
C. difficile		
630Δ*ermB*	An erythromycin sensitive derivative of *C. difficile* strain 630	[9]
630Δ*ermB cdeC*::CT	630Δ*ermB* carrying *cdeC* with ClosTron insertion	[3]
R20291 CM196	Ribotype 027, epidemically relevant *C. difficile* strain with deletion causing increased sporulation	[12]
R20291 CM196 *cdeC*::CT	R20291 CM196 carrying *cdeC* with ClosTron insertion	[2]
R20291 Δ*pyrE*/*pyrE* Δ*cdeC*	R20291 CM196 carrying *cdeC* deletion and *pyrE* complementation	Not published
E. Coli		
CA434	A kanamycin and tetracyline resistant conjugation strain containing donor plasmid pR702	[7]
CA434 (pYN4-*cotL*)	A kanamycin and tetracyline resistant conjugation strain containing donor plasmid pR702 and the pMTL-YN4 *cotL* mutator plasmid	This work
CA434 (pRPF215)	A kanamycin and tetracyline resistant conjugation strain containing donor plasmid pR702 and pRPF215	This work
Plasmids		
pMTL-YN4	Pseudo suicide vector containing: Clostridium perfringens *catP* resistant cassette, Clostridium sporogenes *pyrE*; Carries unaltered *colE1* origin of replication; *traJ* encoding transfer function of the RP4 *oriT* region; *RepA* and *Orf2*, the replication region of the Clostridium botulinum plasmid pBP1; and AscI/Sbf1 sites for the cloning of the right-hand homology arm/left-hand homology arm cassette.	[6, 15]
pMTL-YN4 *cotL* mutator plasmid	pMTL-YN4 plasmid containing homology arms for *cotL* deletion	This work
pRPF215	Plasmid containing a *mariner* transposon with erythromycin cassette with *Himar1* transposase and inducible instability in the presence of anhydrotetracycline	[5]
Primers		
P62	5'-GAATTTACTTAGCCACCGGTGTTTCGGG-3' used to detect ClosTron insertion in *cdeC*	[3]
P63	5'-TTTCTTCCTACTATATCTCCTAATGGGTCTAAATCG-3' used to detect ClosTron insertion in *cdeC*	[3]
P1088	5'-CGATCGGGCCCCCTGCAGGCCAAATCCACTTGCTAGAAGCTTAG-3' used to amplify the left homology arm of the *cotL* mutator plasmid	This work
P1089	5'-AAATTAGATACAGAGAAGTCTAACCCTCATGAGTAGATAAAAATAAAC-3' used to amplify the left homology arm of the *cotL* mutator plasmid	This work

TABLE 1 Continued. Bacterial strains, primers, and plasmids used

Name	Relevant characteristic	Source
P1090	5'-ATGAGGGTTAGACTTCTCTGTATCTAATTTTTTCATAAAAAAACCCC-3' used to amplify the right homology arm of the *cotL* mutator plasmid	This work
P1091	5'-CTAAGGATTCAGAACGGCGCGCCGCCTGTGTTCTTATCTTCTACTAATTCATC-3' used to amplify the right homology arm of the *cotL* mutator plasmid	This work

2.1 Growth media

C. difficile cultures were routinely grown under anaerobic conditions in 3.7% Brain Heart Infusion broth supplemented with 0.5% yeast extract and 0.1% cysteine (BHIS) or on 1.5% agar BHIS plates with 0.1% sodium taurocholate (ST) as needed to induce germination. Erythromycin and lincomycin antibiotics were added to final concentrations of 10 μg/mL and 20 μg/mL respectively as needed to select for ClosTron *cdeC*::CT mutants. BHIS CCT plates contained cefoxitin (16 μg/mL), D-cycloserine (250 μg/mL), and thiamphenicol (15 μg/mL). Spores were grown on 1.5% agar 70:30 sporulation media plates containing (per liter): 63 g peptone (Bacto 211 677), 3.5 g Protease peptone (Difco 211 684), 11.1 g BHI (Difco 211 059), 1.5 g yeast extract (Difco 212 750), 1.06 g Tris, and 0.7g $(NH_4)SO_4$. *E. coli* cultures were grown in Luria-Bertani (LB) broth containing 1% bacto-tryptone, 0.5% yeast extract, and 0.5% NaCl or on 1.5% agar LB plates. Conjugations included passages on *C. difficile* minimal medium (CDMM) (15) which contained (per liter): 10g casamino acids, 0.5 g L-tryptophan, 0.5g L-cysteine, 5 g disodium phosphate, 5 g sodium bicarbonate, 0.9 g monopotassium phosphate. 0.9 g sodium chloride, 10 g D-glucose, 0.04 g ammonium sulfate, 0.026 g calcium chloride dihydrate, 0.02 g magnesium chloride hexahydrate, 0.01 g manganese chloride hexahydrate, 0.001 g cobalt (II) chloride hexahydrate, 0.004 g ferrous sulfate heptahydrate, 0.001g D-biotin, 0.001g calcium D-

pantothenate, and 0.001g pyridoxine. Chemicals were purchased from Sigma. Five-fluorotic acid (FOA) and uracil were added to CDMM when indicated at final concentrations of 1 mg/mL and 5 μg/mL respectively.

2.2 PCR verification of *cdeC*::CT mutants

Genomic DNA from *cdeC*::CT mutants in 630 Δ*ermB* and R20291 backgrounds was isolated. The gene *cdeC* was PCR amplified using Taq DNA polymerase (ThermoFisher) and the primer set P62 and P63 shown in Table 1. Wild type *cdeC* produced the expected amplicon of 857 bp and *cdeC* with the ClosTron intron produced the expected amplicon of approximately 2.7 kb.

2.3 Spore harvesting and purification

Spores were produced by first streaking BHIS agar plates with the appropriate *C. difficile* strain and incubating the plates overnight anaerobically at 37°C. A single healthy colony was then picked to inoculate a 10mL BHIS tube and then incubated overnight anaerobically at 37°C. The overnight cultures were either directly plated on 70:30 agar plates by pipetting 250 μL to each plate or diluted by pipetting 7 μL of the overnight culture into a new 10 mL BHIS tube then plating 100 μL of the diluted culture onto 70:30 agar plates. Plates were incubated anaerobically for 3-5 days at 37°C. After incubation plates were removed from the anaerobic chamber. Spores were harvested by scraping the growth from plates into 1.5 mL microcentrifuge tubes containing 1 mL sterile water. Microcentrifuge tubes containing the scraped growth were incubated at 4°C overnight in order to lyse remaining mother cells and release spores. Spores were then pelleted by centrifugation at 14000 rpm for 5 minutes and washed with sterile water 5 times. Washed spore suspensions were centrifuged at 14000 rpm for 20 minutes through 60% (w/v) filtered

sucrose in water to separate spores from cell debris. Purified spores were then washed in 1mL sterile water 3 times to remove any remaining sucrose. Spore suspensions were quantified by diluting 1:100 in sterile water and pipetting 10 µL of the spore suspension onto a Neubaur chamber, counting the number of spores in 5 squares of the chamber and multiplying the average number of spores per square by 2.5 x 10^7. Spore suspensions were adjusted to a stock concentration of 5 x 10^9 spores/mL.

2.4 Series of assays for the characterization of a lysozyme-triggered germination phenotype in *cdeC*::CT mutants

The first assay was conducted using spores from strains in the 630 laboratory strain background. Five µL of 630Δ*ermB* wildtype and *cdeC*::CT spores (5 x 10^9 spores/mL) were suspended in 95 µL with the following final concentrations: 0 mg/mL, 1 mg/mL, 2.5 mg/mL, 5 mg/mL, and 10 mg/mL of lysozyme then incubated at 37℃ for 8 or 24 hours aerobically. After incubation solutions were serially diluted and spot plated on BHIS agar plates containing 0.1% ST to induce germination. Plates were incubated anaerobically overnight at 37℃ and colonies were counted to determine CFU/mL.

Subsequent assays were conducted using spores from the 630Δ*ermB* background with 5 µL of spores (5x10^9 spores/mL) suspended in 245 µL of either PBS or a solution of lysozyme in PBS (10mg/mL final concentration). After aerobic incubation at 37℃ spore suspensions were heat treated in a water bath at 65℃ for 30 minutes. Serial dilutions, spot plating, and colony counts were conducted following the same protocol as the previous assay.

2.5 EMS treatment, confirmation of mutagenesis via increase in rifampicin resistance, and assay to determine optimal EMS concentration to maximize mutagenesis

In order to mutagenize and confirm mutagenesis, vegetative cultures of *cdeC*::CT mutants from 630Δ*ermB* and R20291 background were streaked on BHIS agar plates with erythromycin or lincomycin respectively and incubated anaerobically overnight at 37℃. A single healthy colony was picked to inoculate a 10 mL BHIS broth tube and incubated anaerobically overnight at 37℃. The overnight cultures were diluted to an OD_{600} of 0.05 then grown anaerobically to OD_{600} of 0.5 (approximately 4 hours). 500 µL of the OD_{600} 0.5 cultures were aliquoted into 1.5 mL microcentrifuge tubes twice per culture. EMS (Sigma) was added at 1% v/v (5 µL) and the tubes were incubated anaerobically at 37℃ for 3 hours. The cells were then pelleted down and washed twice in 1mL BHIS broth then both pellets were resuspended together in 1mL BHIS broth. The 1mL of treated cell suspension was added to 7 mL of BHIS broth then incubated at 37℃ overnight to allow for cell recovery. Recovered cultures were plated on 70:30 media for sporulation. EMS treated and untreated *cdeC*::CT cultures were serially diluted in BHIS broth and 100 µL of each dilution was plated on BHIS agar plates and BHIS agar plates containing rifampicin (5 µg/mL) and grown anaerobically overnight at 37℃. EMS mutagenesis was considered to be successful if the frequency of rifampicin resistant CFU/mL was higher in mutagenized cultures than unmutagenized cultures.

To determine if increased EMS concentrations increased the frequency of mutagenesis, the same protocol was followed using varying concentrations of EMS. First, EMS concentrations of 1%, 2.5%, 5% and 10% (v/v) were tested. The EMS concentration range was then expanded to test 0%, 1%, 5%, and 10%. EMS treated and untreated *cdeC*::CT cultures were serially diluted in BHIS broth and 100 µL of each dilution was plated on BHIS agar plates to determine viability

and BHIS agar plates containing rifampicin (5 µg/mL) to determine the number of rifampicin resistant CFU/mL.

2.6 Lysozyme treatment, germination quantification, and enrichment for suppressor mutants

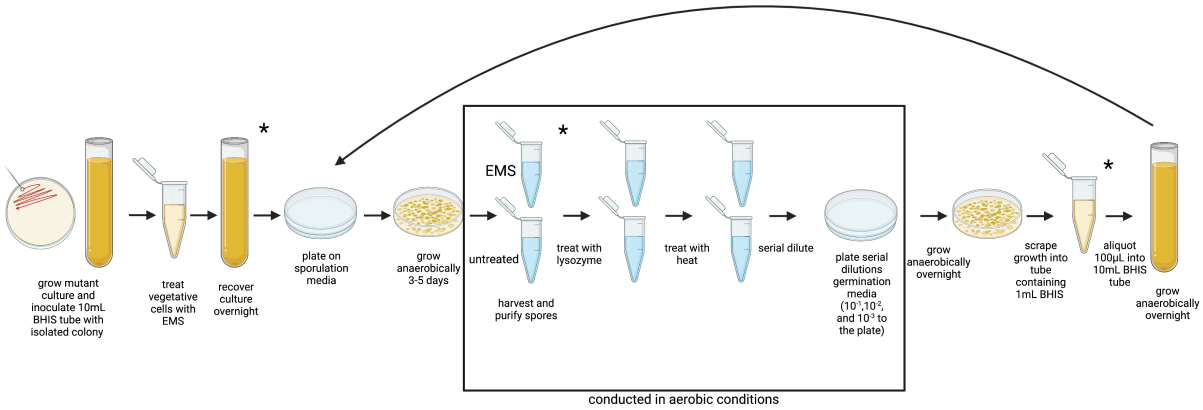

Figure 1. Schematic representation of the methods developed to enrich for suppressor mutants in EMS treated and untreated cdeC::CT spores. Vegetative cells are treated with EMS then recovered overnight and plated on sporulation media. Spores are grown anaerobically 3-5 days then harvested and purified. Phase contrast microscopy images are taken before and after treatment with lysozyme (10mg/mL) for 24 hours. Spore suspensions are heat treated then serial diluted and plated (10^{-1}, 10^{-2}, and 10^{-3} to the plate) and grown anaerobically overnight on germination media. Plates containing a lawn of growth are scraped into 1mL BHIS broth then 100µL is aliquoted into 10mL BHIS tube. The culture is grown anaerobically overnight and plated on sporulation media. The treatment process was repeated to 5th passage. Asterisks (*) indicate where stocks were taken.

Enrichment for suppressor mutations that confer resistance to lysozyme was performed through passages of treatment of *cdeC*::CT spores with lysozyme and heat to kill germinated spores (Figure 1). Treated spores were plated on BHIS agar plates containing ST to induce germination of surviving spores. Growth from these plates was harvested in order to produce new spores from the surviving cultures to be used in subsequent passages. Phase contrast microscopy images were taken before and after lysozyme treatment in order to quantify germination. First, 5 µL of 5 x 10^9 spores/mL stock spore suspensions in 630Δ*ermB* and R20291 backgrounds were resuspended in 245 µL of lysozyme (10 mg/mL final concentration) and

sonicated. Ten µL of the lysozyme spore suspension was pipetted on microscope slides with 1% agarose pads. Phase microscopy images were taken, and the lysozyme spore suspensions were incubated aerobically at 37℃ for 24 hours. After incubation the process of sonication and phase contrast microscopy was repeated to quantify germination. The lysozyme spore suspensions were incubated in a water bath at 75℃ for 15 minutes to kill germinated spores. The lysozyme spore suspensions were then serially diluted and plated on BHIS agar plates containing 0.1% ST to induce germination of surviving spores and erythromycin or lincomycin depending on the strain. The plates were incubated overnight at 37℃ anaerobically. To harvest survivors of the enrichment, plates were selected based on the most diluted sample that gave rise to a lawn of growth. Growth from the selected plates was scraped and resuspended in 1 mL BHIS broth. The resuspended growth was diluted by aliquoting 100 µL in BHIS broth and the culture was incubated overnight anaerobically at 37℃. 250 µL of the overnight culture was plated on 70:30 agar 4 times (total 1 mL of overnight culture plated) and incubated 3-5 days anaerobically at 37℃ to produce spores for the subsequent passage. The remaining resuspended growth was used to make a 1mL DMSO (80%) stock stored at -80℃ and a small sample of the resuspended scraped growth was stored at -80℃ for genomic DNA isolation and whole genome sequencing. Passaging of 630Δ*ermB* spores did not continue after passage 0 due to insufficient spore production.

2.7 Assay to determine optimal heat treatment conditions to select for lysozyme resistance

To determine if there is a more optimal heat treatment after incubation in lysozyme to kill a larger portion of germinated spores the characterization assay was conducted in 4 different heat treatment conditions. First, 5 µL of wild type and *cdeC*::CT spores ($5x10^9$ spores/mL) in the epidemically relevant R20291 background were suspended in 245 µL lysozyme (final

concentration 10 mg/mL) or PBS. After aerobic incubation at 37℃ for 24 hours spore suspensions were heat treated in a water bath at 65℃ for 30 minutes, 65℃ for 60 minutes, 75℃ for 15 minutes, or 85℃ for 15 minutes then serially diluted and spot plated on BHIS agar plates containing 0.1% ST then incubated anaerobically at 37℃ overnight. Colonies were counted to determine CFU/mL.

2.8 Construction of *cotL* mutant

pMTL-YN4 was linearized by restriction enzyme digestion with *Asc*I and *Sbf*I (New England Biolabs). Homology arms were amplified using High Fidelity Phusion DNA polymerase and the primers P1088, P1089, P1090 and P1091 (Table 1) resulting in a left homology arm of 1031 bp and a right homology arm of 1033 bp. All fragments were gel purified and a Gibson assembly reaction resulted in pMTL-YN4 *cotL* mutator plasmid carrying a deletion of *cotL* with 24 remaining amino acids. The mutator plasmid was isolated then transformed into CA434 cells. One mL of overnight cultures of CA434 cells containing the mutator plasmid and pR702 was centrifuged at 4000 rpm for 5 minutes and the supernatant discarded then the pellet was washed with 1mL of PBS without resuspending and centrifuged again at 4000 rpm for 3 minutes then the supernatant was discarded while anaerobically 200 µL of *C. difficile* Δ*pyrE* cells was pelleted and resuspended in 200 µL PBS. The *E. coli* pellets were entered into the anaerobic chamber and resuspended gently in the 200 µL of *C. difficile* PBS suspension then spot plated in 10 µL spots on BHIS agar plates and incubated overnight anaerobically at 37℃. Growth from spot plating was scraped and resuspended in 500 µL of PBS and 100 µL was plated on BHIS agar plates containing cefoxitin, D-cycloserine, and thiamphenicol (CCT) and incubated overnight anaerobically at 37℃. Transconjugants from the first BHIS CCT agar plate were picked and restreaked on BHIS CCT agar plates. A single healthy colony was picked and restreaked on a

new BHIS CCT agar plate. Passaging on BHIS CCT agar plates continued to the fourth passage then the same single healthy colony was used to patch plate on *C. difficile* minimal medium (CDMM), on CDMM containing 5-fluorotic acid (FOA) and uracil and used to inoculate a 10 mL BHIS tube containing thiamphenicol. The two plates and one tube were incubated anaerobically overnight at 37℃. Clones able to grow on minimal medium and not on minimal medium containing FOA and uracil contained the mutator plasmid. DMSO stocks were made from the BHIS overnight cultures of these first integrants. One hundred µL of the BHIS thiamphenicol overnight culture was pipetted into a new tube containing 10 mL BHIS broth and incubated overnight anaerobically at 37℃. Passaging of 100 µL into 10 mL BHIS broth was continued to the fourth passage then 2 µL of the fourth passage was resuspended in 1 mL PBS and 100 µL was plated on CDMM agar plates and incubated overnight anaerobically at 37℃. Colonies from the overnight CDMM plates were patch plated on BHIS and BHIS thiamphenicol agar plates and incubated overnight anaerobically at 37℃. Thiamphenicol sensitive clones were re-streaked on BHIS and BHIS thiamphenicol agar plates and incubated overnight anaerobically at 37℃. Isolated healthy thiamphenicol sensitive clones were picked to inoculate 10mL BHIS broth tubes and incubated overnight anaerobically at 37℃. DMSO stocks were made of the overnight cultures and tubes were stored at -80℃ for genomic DNA isolation and PCR screening for mutants. Construction of this mutant is ongoing.

2.9 Construction of transposon library as an alternative mutagenesis strategy

Attempts to conjugate the putative transposon plasmid pRPF215 into R20291 Δ*pyrE* Δ*cdeC* background with complemented *pyrE* were conducted. One mL of overnight cultures of CA434 cells containing the transposon plasmid and pR702 was centrifuged at 4000 rpm for 5 minutes and the supernatant discarded then the pellet was washed with 1mL of PBS without

resuspending and centrifuged again at 4000 rpm for 3 minutes. The supernatant was discarded while anaerobically 200 µL of *C. difficile* cells were pelleted and resuspended in 200 µL PBS. The *E. coli* pellets were entered into the anaerobic chamber and resuspended gently in the 200 µL *C. difficile* PBS solution then spot plated in 10 µL spots on BHIS agar plates and incubated overnight anaerobically at 37°C. Growth from spot plating was scraped and resuspended in 500 µL of PBS and 100 µL plated on BHIS agar plates containing cefoxitin, cycloserine, and thiamphenicol (CCT) and incubated overnight anaerobically at 37°C. Subsequently, there was no growth of transconjugants, so conjugation and construction of the transposon library did not continue.

3. RESULTS

3.1 Identifying concentrations of lysozyme that render *cdeC*::CT spores oxygen sensitive

Previous studies have reported that *cdeC* mutant spores have a permeable spore coat which leads to lysozyme diffusing into the peptidoglycan cortex and triggering germination, a phenotype not observed in wild type spores [2, 3]. Therefore, to use this phenotype in a genetic screen, we first tested whether lysozyme-germinated spores became inactivated in the presence of oxygen. For this, 5 µL of *cdeC*::CT mutant and wild type spores were incubated aerobically in 95 µL of 1, 2.5, 5, or 10 mg/mL lysozyme for 8 and 24 hours, while the control was incubated in PBS. After incubation spore suspensions were serially diluted and spot plated on BHIS agar plates containing ST then incubated overnight anaerobically at 37 ºC. This assay was conducted with only 1 biological replicate and 2 technical replicates. Our results showed that wildtype CFU/mL does not seem to be impacted by treatment with lysozyme for 8 hrs at any of the tested concentrations (Figure 2A). We expected a reduction of CFU/mL in lysozyme-triggered germinated *cdeC*::CT mutants due to exposure to oxygen. When *cdeC*::CT spores were treated for 8 hours we observed an increase in relative CFU/mL of around 100-fold in all lysozyme concentrations. Phase contrast microscopy indicates that lysozyme at all tested concentrations resulted in spores changing from phase bright to phase dark indicating germination (Figure 2C). When we increased incubation time in lysozyme to 24 hours the same trends were observed in wildtype and *cdeC*::CT (Figure 2B). Altogether, these results indicate that exposure to oxygen was not sufficient to kill lysozyme-germinated *C. difficile* spores. These results also demonstrate that lysozyme triggers germination of *cdeC*::CT spores that would otherwise remain dormant shown as an increase in CFU/mL of treated *cdeC*::CT spores.

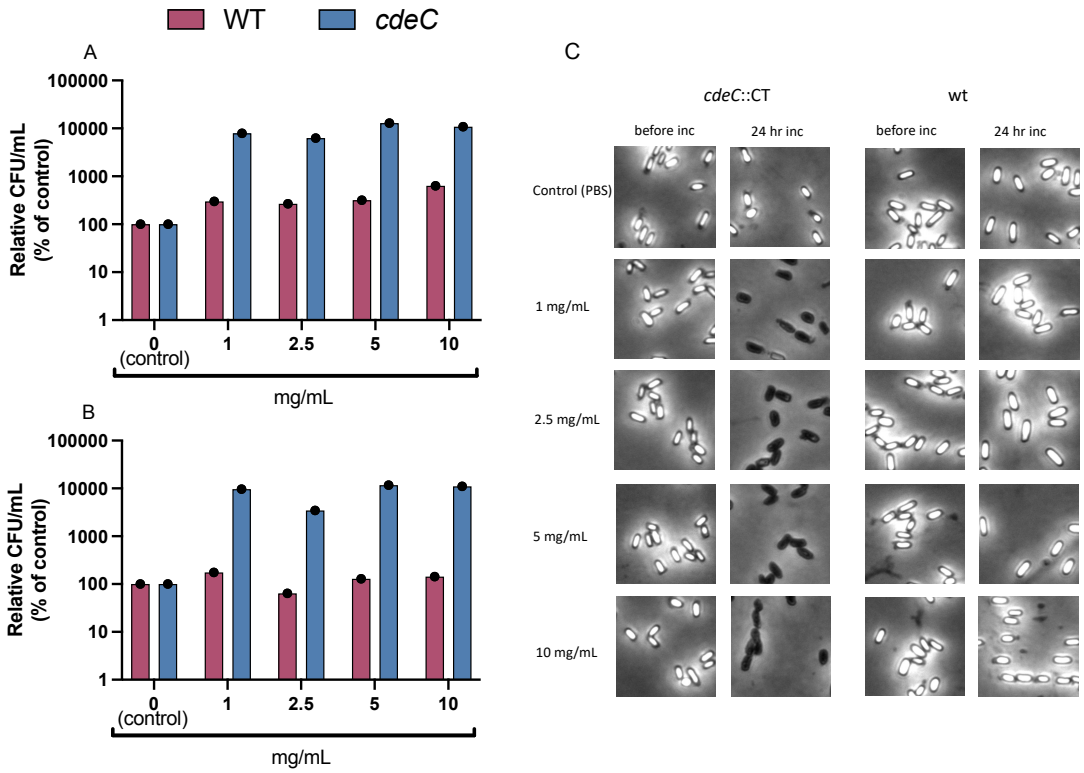

Figure 2. Effects of lysozyme concentration and incubation time on CFU/mL in *cdeC*::CT and WT spores in laboratory strain 630 background. WT and *cdeC*::CT spores were incubated aerobically in a lysozyme solution or PBS for 8 (A) or 24 hrs (B) at the indicated concentration and spot plated on BHIS agar with 0.1% sodium taurocholate and incubated anaerobically at 37°C overnight to determine CFU/mL. (C) Phase contrast images were taken before and after 24hr aerobic incubation. Results shown as percentage CFU/mL relative to untreated controls. Data points represent the average of 2 technical replicates.

3.2 Test of heat treatment to kill lysozyme germinated *cdeC*::CT spores

Since exposure to oxygen did not kill *cdeC*::CT lysozyme-germinated spores in the previous assay, we tested if the addition of heat would kill lysozyme-germinated spores. Wild type and *cdeC*::CT spores in the 630 laboratory strain were treated with lysozyme (final concentration 10 mg/mL) or PBS when indicated and heat treated at 65ºC for 30 mins when indicated in order to kill germinated spores. After heat treatment, spore suspensions were serially diluted and spot plated on BHIS agar plates with ST and incubate overnight anaerobically at 37ºC. We hypothesized that with the addition of heat treatment would kill *cdeC*::CT lysozyme-germinated spores. This assay was conducted with 1 biological replicate and 3 technical

replicates. Our results determined heat treatment of wild type spores decreased relative CFU/mL by approximately 1.5 logs both with and without treatment with lysozyme (Figure 3A). For *cdeC*::CT spores we observed a similar amount of killing when treated with heat alone, however, treating *cdeC*::CT spores with both lysozyme and heat resulted in a much more dramatic decrease in relative CFU/mL of over 3 logs (Figure 3B). Interestingly, in this assay treating *cdeC*::CT spores with lysozyme did not result in an increase in relative CFU/mL as seen in Figure 2. These differences may be due to both assays not being conducted with multiple biological replicates and variability of the data. These results are consistent with our hypothesis that treatment with lysozyme germinates *cdeC*::CT spores leaving cells susceptible to heat killing. In conclusion, in *cdeC*::CT mutant spores treatment with heat kills lysozyme-germinated spores.

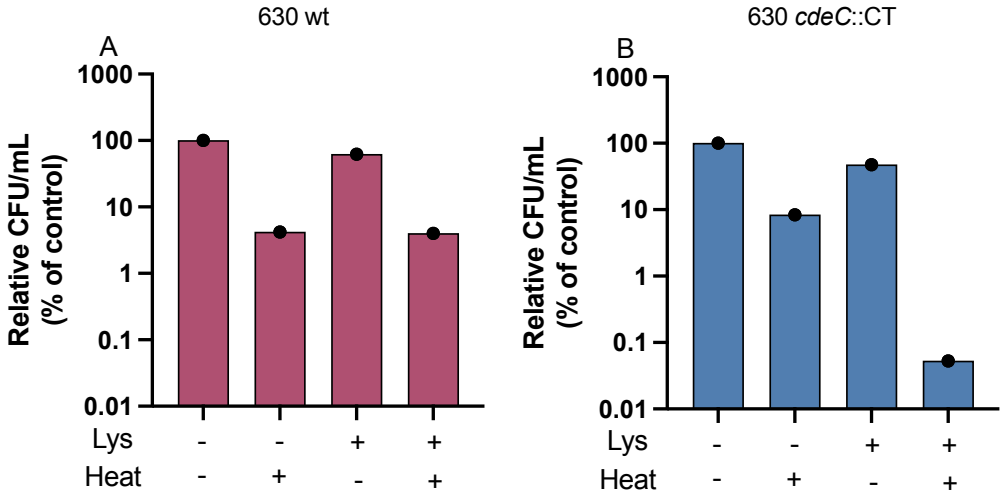

Figure 3. Effects of lysozyme incubation and heat treatment on CFU/mL in *cdeC*::CT and WT spores in 630 background. WT (A) and *cdeC*::CT (B) mutant spores were incubated in a lysozyme solution at a final concentration of 10 mg/mL or untreated in PBS for 24 hrs. Spores were either plated directly or heat treated in a water bath at 65°C for 30 mins then serially diluted and spot plated on BHIS agar with 0.1% sodium taurocholate to determine CFU/mL. Results are shown as percentage of CFU/mL relative to untreated controls. Data points represent the average of 4 technical replicates.

3.3 Survivor *cdeC*::CT spores are likely not spontaneous revertant or suppressor mutants

Since a portion of the *cdeC*::CT population was able to survive after treatment with heat and lysozyme we wanted to test if these survivors had mutated and gained resistance to lysozyme. Spores were made from three individual colonies selected from a plate of lysozyme and heat-treated *cdeC*::CT spores. Spores from these three clones along with spores from the original *cdeC*::CT stock and wild type spores were treated in the same conditions described in the previous assay. We observed relative CFU/mL for wild type spores varied slightly between treatment conditions (Figure 4A). There was no significant differences in relative CFU/mL between the control and the lysozyme and heat treated spores. Interestingly, heat treatment of wild type spores in PBS led to a slight increase in relative CFU/mL. Our results showed that relative CFU/mL for *cdeC*::CT mutant spores significantly decreased after lysozyme and heat treatment by over 1 log (Figure 4B). This same trend can be seen in two of the three survivor clones of *cdeC*::CT. We observed the first *cdeC*::CT survivor clone (C1) when treated with lysozyme and heat shows over 1 log decrease in relative CFU/mL (Figure 4C). These results are similar to those seen in Panel B in the original *cdeC*::CT mutant spores. Interestingly, treatment of the second clone (C2) with lysozyme and heat did not lead to any significant reduction in relative CFU/mL (Figure 4D). Similarly to the stock *cdeC*::CT spores, the third *cdeC*::CT survivor mutant clone (C3) treated with heat and lysozyme showed an over 1 log decrease in relative CFU/mL (Figure 4E). The phenotype of 2 of the 3 clones tested was similar to the phenotype of the original *cdeC*::CT stock and future assays assessing C2 may be of interest. From this data we conclude that it is likely that survival of *cdeC*::CT spores following lysozyme and heat treatment is stochastic. It is interesting to note that the increase in relative CFU/mL with lysozyme treatment alone observed in Figure 2 was not observed in any of the *cdeC*::CT spores

tested. Also, interestingly treatment with heat alone led to a slight increase in relative CFU/mL in all spores tested including in the wildtype spores.

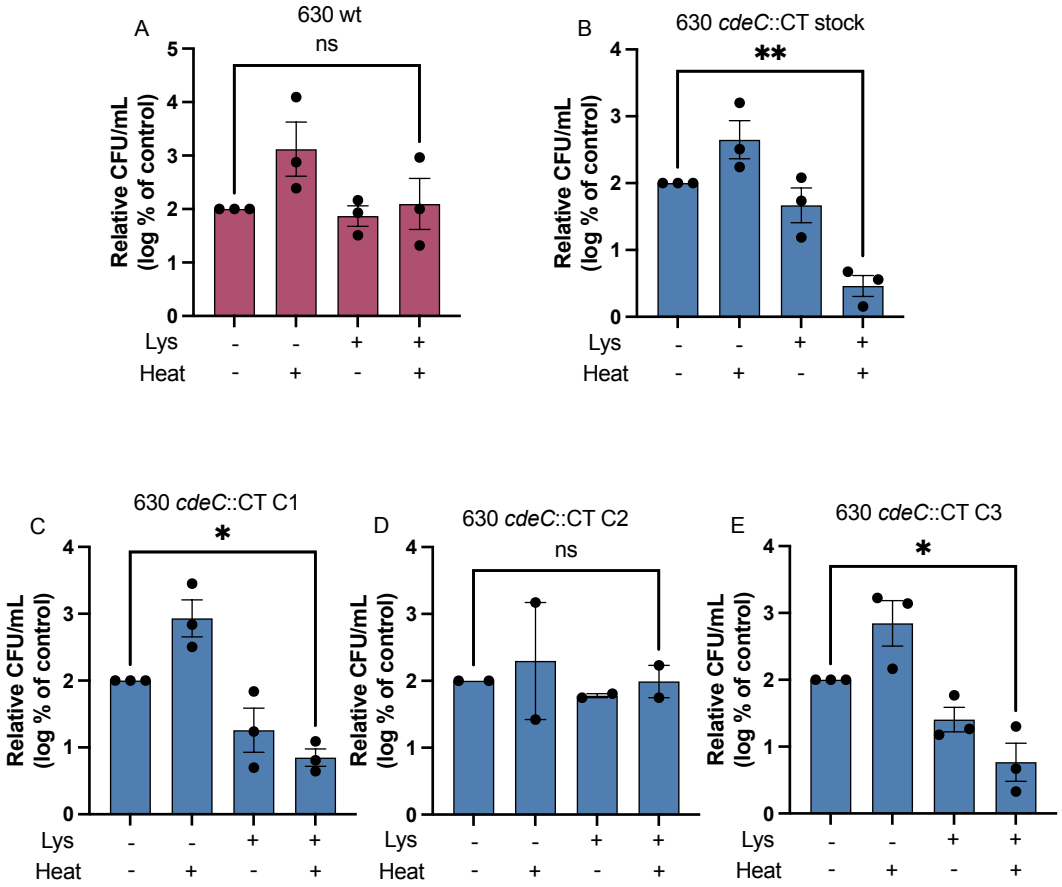

Figure 4. Effects of lysozyme and heat treatment on *cdeC*::CT mutant spore surviving clones. WT and *cdeC*::CT mutant spores were incubated in a lysozyme solution at a final concentration of 10 mg/mL or untreated in PBS for 24 hrs. Spores were either spot plated directly, or heat treated in a water bath at 65°C for 30 mins then spot plated on BHIS agar with 0.1% sodium taurocholate to determine CFU/mL. Three colonies of *cdeC*::CT mutant spores that survived initial lysozyme and heat treatment were isolated, spores of each clone were made and incubated in a lysozyme solution at a final concentration of 10 mg/mL or untreated in PBS for 24 hrs. Spores were either plated directly or heat treated in a water bath at 65°C for 30 mins then spot plated on BHIS agar with 0.1% sodium taurocholate to determine CFU/mL. Results shown as log 10 of the percentage of CFU/mL relative to untreated controls. Data was analyzed by one-way ANOVA and Asterisks (*) denote statistical difference at $P < 0.05$, (**) denotes statistical difference at $P < 0.01$.

3.4 Enrichment for suppressor mutants in EMS mutagenized *cdeC*::CT spores

Since the phenotype of lysozyme-triggered germination and heat killing of lysozyme germinated spores was established in *cdeC*::CT mutant spores in previous assays we used it to conduct a genetic screen for suppressor mutants. We hypothesized that treatment of *cdeC*::CT

spores with EMS would induce point mutations in genes involved in outer spore layer assembly that would allow mutant spores to recover wild type resistance to lysozyme. To enrich for these suppressor mutants, we passaged EMS treated *cdeC*::CT in lysozyme and heat treatment. To quantify changes in germination, phase contrast images were taken before and after every lysozyme treatment. Vegetative cells in the epidemically relevant R20291 background containing the *cdeC*::CT mutation were grown and treated with 1% (v/v) EMS to induce random mutagenesis and a control untreated culture was also grown. Spores were made from each vegetative cell culture and were suspended in a lysozyme solution (final concentration of 10 mg/mL) and incubated 24 hours aerobically at 37°C. Phase contrast microscopy images were taken before and after each lysozyme treatment and cells were counted as one of the following: phase bright, phase gray, phase dark, or vegetative cells. After lysozyme treatment and phase contrast imaging spores were heat treated at 75°C for 15 minutes to kill germinated spores. The heat treatment was increased from previous assays to try to increase the number of germinated spores killed. Heat treated spore suspensions were then serially diluted and dilutions plated on BHIS agar plates with ST anaerobically overnight at 37°C. Growth from plates of the 10^{-2} and 10^{-3} dilutions was collected and resuspended in 1mL BHIS broth. BHIS tubes with 100 μL aliquots of the resuspended scraped growth were incubated anaerobically overnight at 37°C, plated on 70:30 sporulation media, and grown anaerobically 3-5 days at 37°C. Spores were then harvested and purified for the next passage. This procedure was repeated for five additional passages. Figure 5 shows the expected phase contrast microscopy results in the event of successful enrichment for suppressor mutants.

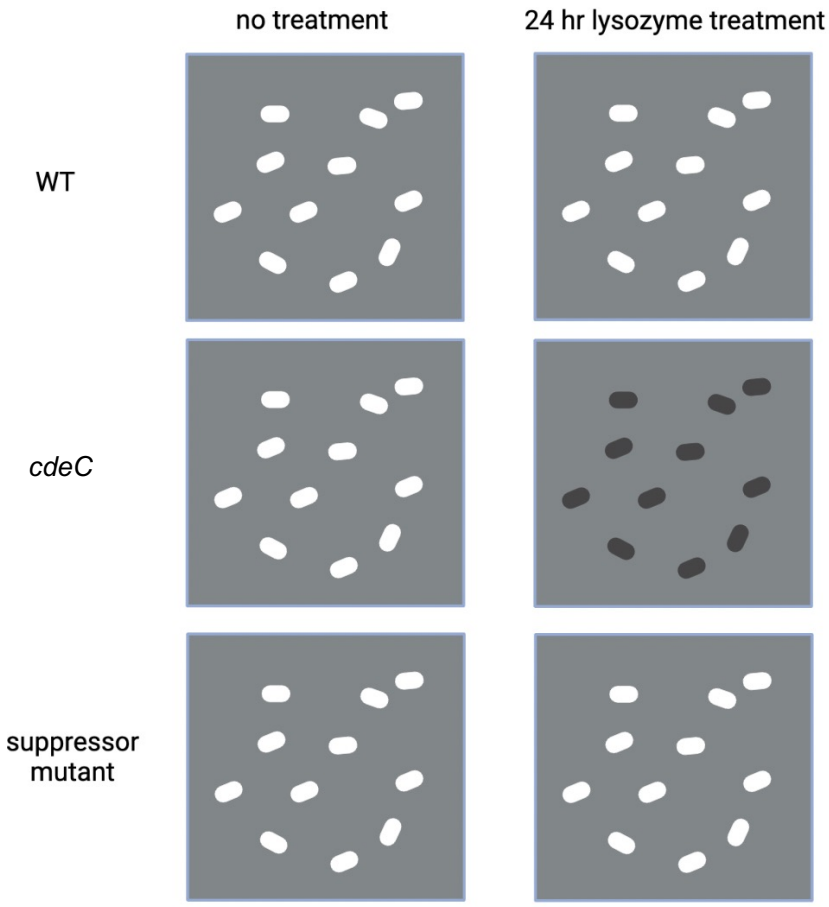

Figure 5. Expectation for phase contrast microscopy with enrichment for suppressor mutations.

We quantified the percentage of phase bright and phase dark spores immediately following lysozyme treatment for passage 0 and for each of the five subsequent passages for both mutagenized and unmutagenized *cdeC*::CT spores (Figure 6). Phase contrast images from passage 0 are also shown. was no detectable increase in the percentage of phase bright spores after lysozyme treatment. In conclusion, the current genetic screen was insufficient to enrich for suppressor mutants.

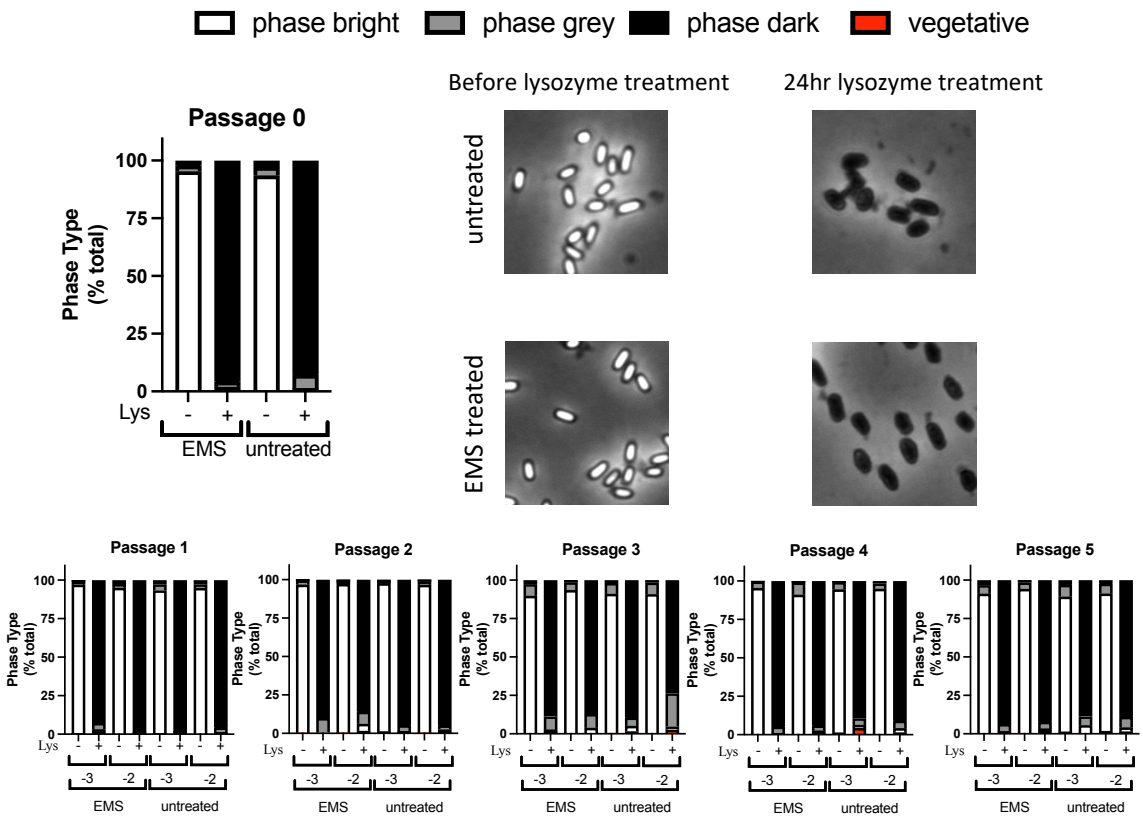

Figure 6. Quantification of percentage of germinated spores in EMS mutagenized and not mutagenized *cdeC*::CT spores of R20291 background. Phase contrast microscopy of both untreated and EMS treated spores of *cdeC*::CT were taken before and after 24hr aerobic lysozyme treatment (final concentration 10mg/mL). Spore suspensions were serially diluted, plated, and grown aerobically overnight at 37°C. Growth from 10^{-2} and 10^{-3} dilutions was scraped and diluted then plated on 70:30 sporulation media to grow spores for the next passage.

3.5 Assay to determine optimal heat treatment conditions to select for lysozyme resistance

Since there was no enrichment for suppressor mutants with wild type like resistance to lysozyme-triggered germination we wondered if the heat treatment used was too severe and had killed a portion of dormant wild-type and/or cdeC::CT spores. To test this hypothesis and possibly identify a more optimal heat treatment, R20291 wild type and *cdeC*::CT mutant spores were incubated for 24 h in either PBS or PBS with lysozyme (10 mg/mL), heated in a water bath at either 75°C for 15 min, 85°C for 15 min, or 65°C for 30 min, then serially diluted and plated on BHIS agar with ST. It is important to note that enrichment for suppressors was conducted

using a heat treatment of 75°C for 15 minutes. These assays were conducted with 1 biological replicate and 4 technical replicates. We observed an over 2 log reduction in relative CFU/mL for wildtype spores treated with 75°C for 15 minutes heat without treatment with lysozyme (Figure 7A). The heat treatment at 85°C for 15 minutes resulted in death of nearly all heat-treated spores and so would not be useful in a genetic screen for suppressor mutants resistant to lysozyme-triggered germination (Figure 7B). After heating at 65°C for 30 minutes there was no observable reduction in relative CFU/mL of *cdeC*::CT spores treated with heat alone, and there was a reduction of over 1 log in the double treatment (Figure 7C). There was a reduction in relative CFU/mL between the control and heat-treated wild type spores of over 1 log that may have reduced the chances of enriching for suppressors, however, it was less dramatic than in the 75°C for 15 minutes treatment over 2 log reduction in relative CFU/mL. The reduction in relative CFU/mL for wildtype spores treated with 75°C for 15 minutes, along with other errors in experimental design, could potentially explain the lack of enrichment for suppressor mutants after EMS mutagenesis seen in Figure 6. It is possible suppressor mutants that were resistant to lysozyme-triggered germination and remained dormant were killed by this heat treatment. Altogether, we conclude that if future enrichment assays are conducted, heat treatment should be reduced and 65°C for 30 minutes is a better heat treatment option since it did not kill as much of the dormant spore population as the current heat treatment.

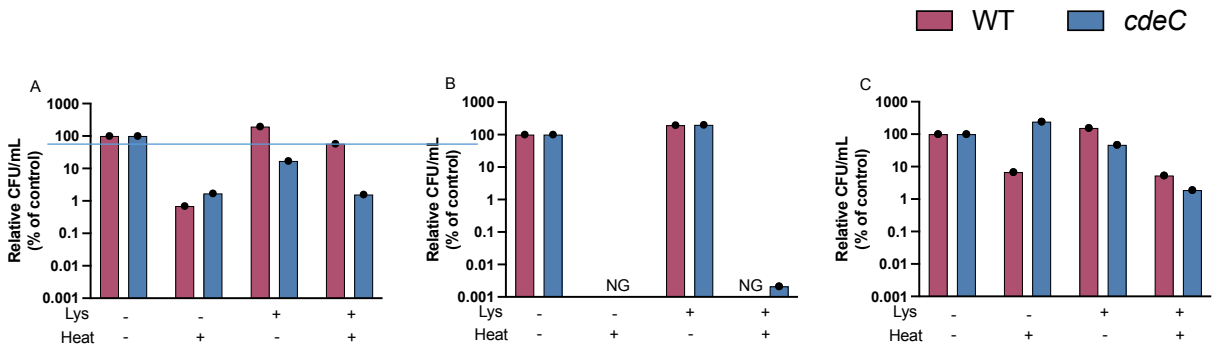

Figure 7. Effects of varying heat treatment on relative percentage of CFU/mL in *cdeC*::CT and WT spores in R20291 background. WT and *cdeC*::CT mutant spores were incubated in a lysozyme solution at a final concentration of 10 mg/mL or untreated in PBS for 24 hrs. Spores were either plated directly or heat treated in a water bath at 75℃ for 15 mins (panel A), 85 ℃ for 15 mins (B), or 65 ℃ for 30 mins (C) then spot plated on BHIS agar with 0.1% sodium taurocholate to determine CFU/mL. Individual replicates of CFU/mL divided by the mean CFU/mL of the unheated and untreated controls. Results shown as percentage CFU/mL relative to untreated controls. Data points represent the average of 4 technical replicates. "NG" indicates no growth above the detection limit of 200 CFU/mL.

3.6 Assay to determine optimal EMS concentration to maximize mutagenesis

Since there was not successful enrichment for suppressor mutants, we tested varying concentrations of EMS to see if the frequency of mutagenesis was too low for suppressors to be detected in the genetic screen. In order to determine if the concentration of EMS used to mutagenize spores was optimal, an assay comparing the frequency of rifampicin resistant CFU/mL to viable CFU/mL after treatment with different concentrations of EMS was conducted. Vegetative cells of R20291 *cdeC*::CT mutants were grown in BHIS broth tubes overnight, diluted to OD_{600} = 0.05 and grown to OD_{600} = 0.5. Cultures were treated by pipetting EMS to final concentrations (v/v) of 0%, 0.5%, 1%, and 2% and incubating the cultures anaerobically at 37°C for 3 hours. The concentration of EMS used to induce mutagenesis before enrichment passages was 1% (v/v). Cells were then washed in BHIS broth and incubated overnight anaerobically at 37°C to allow for recovery. Recovered cultures were serially diluted and spot plated on BHIS agar plates to determine viable CFU/mL and on BHIS agar plates containing rifampicin to determine the frequency of mutations that confer rifampicin resistance (Figure 8).

We show that all concentrations tested did not significantly impact viability. The titer of viable cells remains close to 10^7 CFU/mL. When the cultures were plated on medium with rifampicin, we saw that the control not treated with EMS showed <10^2/mL Rif resistant CFU/mL. Cultures treated with 0.5% EMS contained 10^2 Rif resistant CFU/mL indicating a mutant frequency of 10^{-5}. Cultures treated with 1 and 2% EMS contained around 10^3 Rif resistant CFU/mL indicating a frequency of rifampicin resistant colonies of 10^{-4}. C. difficile spontaneous resistance to rifaximin, a derivative of rifampicin, is 10^{-8} [11].

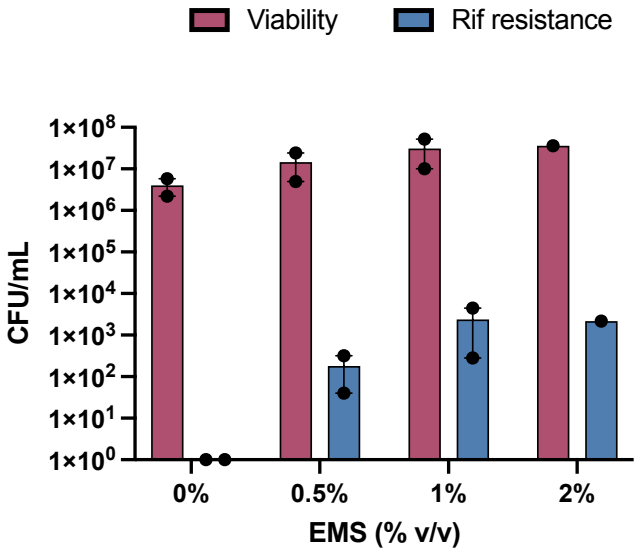

Figure 8. Effects of varying concentrations of EMS on CFU/mL in *cdeC*::CT spores in R20291 background. BHIS liquid cultures were grown overnight anaerobically at 37℃ then back diluted to OD_{600} = 0.05 and grown in the same conditions to OD_{600} = 0.5 then treated with EMS to the indicated final concentration (v/v). Cultures were grown 3 hrs in the same conditions then washed twice. Cultures recovered overnight in the same conditions then serially diluted and spot plated on BHIS agar plates without antibiotics (Viability) or with rifampicin to final concentration 5µg/mL (Rif resistance). Two biological replicates were plotted. Error bars indicate standard error of the means.

To determine if higher concentrations of EMS would increase the frequency of mutagenesis, we repeated the assay with EMS concentrations of 0%, 1%, 5% and 10% (Figure 9). In this assay the viability of the control and 1% treatments remained at 10^7 CFU/mL like the previous assay, however, the viability of cultures treated with 5% and 10% EMS was reduced to

undetectable levels meaning that these concentrations of EMS render the cultures inviable. Interestingly, in this assay cultures treated with 1% EMS showed growth on plates containing rifampicin of around 10^4 CFU/mL indicating a mutant frequency of 10^{-3}, which is an increase of 1 log compared to the 1% treatment in the previous assay. This difference may be due to biological variability. Overall, our results show that increasing the concentration of EMS above 1% results in inviable cultures and does not increase the frequency of mutagenesis. The current frequency of mutations that confer rifampicin resistance is approximately 10^{-4} and at each passage of the screen 10^7 spores are treated. After treatment of *cdeC*::CT spores with lysozyme and heat there was approximately 1 log reduction in CFU/mL meaning selection is occurring at around 10^6 CFU/mL. It is likely that the amount of suppressor mutants induced at this frequency of mutagenesis is too low to be detected at this level of selection. In conclusion, the frequency of mutagenesis in the current screen does not produce suppressor mutants at a detectable level. Screening for suppressor mutants may be more successful in a mutant background with a more dramatic phenotype of lysozyme triggered germination and heat killing of germinated spores, possibly the *cotL* mutant background. Alternatively, other mutagens or mutagenesis strategies could produce higher frequencies of mutagenesis that may be detectable in the current screen.

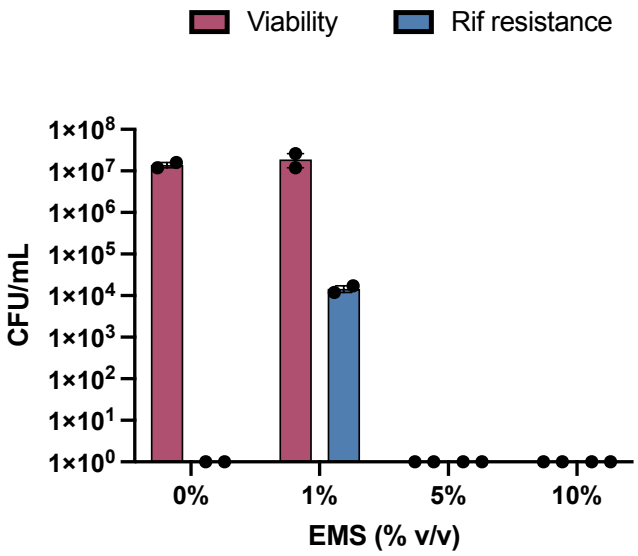

Figure 9. Effects of increased concentrations of EMS on CFU/mL in *cdeC*::CT spores in R20291 background. BHIS liquid cultures were grown overnight anaerobically at 37°C then back diluted to OD_{600} = 0.05 and grown in the same conditions to OD_{600} = 0.5 then treated with EMS to the indicated final concentration (v/v). Cultures were grown 3 hrs in the same conditions then washed twice. Cultures recovered overnight in the same conditions then serially diluted and spot plated on BHIS agar plates without antibiotics (Viability) or with rifampicin to final concentration 5µg/mL (Rif resistance). Two biological replicates were plotted. Error bars indicate standard error of the means.

4. DISCUSSION

The role of dormant spores in the pathogenesis of *Clostridiodes difficile* is dependent on outer spore layers resistant properties. While much remains unknown of the construction of outer spore layers, mutants of the outer spore layer genes *cdeC* and *cotL* are susceptible to lysozyme permeation of the spore coat resulting in germination [1,2,3]. We hypothesized that a phenotype of lysozyme-triggered germination in the *cdeC*::CT mutant background could be used in a genetic screen for suppressor mutants to identify determinants related to outer spore layer assembly.

4.1 Lysozyme combined with heat treatment renders a screenable phenotype

A first conclusion of this work is that lysozyme germinated spores were viable in an aerobic environment. This was unexpected, given the oxygen sensitivity of *C. difficile* vegetative cells. . However, aeration, temperature, pressure, and salt concentrations all impact the saturation of oxygen in a solution [22]. It is possible that the conditions used in the current screen of spore incubation in lysozyme and PBS solutions in microcentrifuge tubes at 37°C was suboptimal and conditions may be altered to implement oxygen killing of germinated spores by increasing oxygen concentrations. Increasing the levels of oxygen in the solution might more efficiently kill germinated spores without impacting dormant spores. This work subsequently showed that the inclusion of a high temperature incubation after lysozyme treatment killed approximately 99% of *cdeC*::CT mutant spores, which made it possible to do an enrichment for suppressor mutants. This finding that lysozyme-germinated *cdeC*::CT spores are heat sensitive is similar to the results of a previous study that showed that incubation of wild-type spores in a solution of germinants in room air resulted in sensitivity to heat [14].

4.2 Survivor of lysozyme and heat treatment is potential spontaneous suppressor

Another observation from this work is that one survivor clone of lysozyme-heat treatment may be a spontaneous suppressor mutant. Spores made from isolated clones C1 and C3 of surviving *cdeC*::CT spores followed the same trend as stock *cdeC*::CT spores. C1 and C3 show significant reduction in relative CFU/mL when treated with lysozyme and heat, however, spores of the C2 clone show no significant reduction after the same treatment. It is possible that isolation of spontaneous suppressors may be of interest in future studies.

4.3 The current screen is insufficient to detect and enrich for suppressor mutants

We concluded that the current genetic screen is insufficient to enrich for and detect outer spore layer suppressor mutants. Since we determined a phenotype of lysozyme-triggered germination and heat killing of lysozyme germinated spores in *cdeC*::CT mutant spores we conducted a genetic screen to detect and enrich for suppressor mutants resistant to lysozyme-triggered germination. We screened for suppressor mutants by first lysozyme germinating then heat killing *cdeC*::CT mutant spores that were EMS mutagenized and quantified the germination phenotype using phase contrast microscopy. After 5 passages of the enrichment protocol, we observed no evidence of suppressor mutants with increased resistance to spore coat permeation with lysozyme. One possible explanation for the unsuccessful enrichment may come from the experimental design of the enrichment process. In each passage approximately 2.5 x 10^7 spores were treated with lysozyme (total volume 250 μL). After lysozyme and heat treatment we serially diluted the spore suspensions then plated 100 μL of the serial dilutions on BHIS agar plates with ST. The growth on the plates scraped into 1mL of BHIS broth and used in subsequent rounds of enrichment represented only 10^5 and 10^4 spores decreasing our chances of carrying suppressor mutants over into the next passage. We then further diluted the growth by aliquoting

100 μL of the resuspended scraped growth in 10 mL BHIS broth and plating 1 mL of the overnight growth of that culture on 70:30 media to make spores for the next passage. In future enrichment designs it would increase chances of detecting and enriching for suppressor mutants to plate the total volume of the spore suspension without dilutions and not to dilute the resuspended growth before plating on 70:30 media to make spores for subsequent passages.

It may also be the case that the phenotype of lysozyme germination and heat killing is not strong enough to select for suppressor mutants in the *cdeC*::CT mutant background. TEM analysis of *cdeC* mutants show evidence of defective outer spore layer assembly including a reduction in exosporium and spore coat size, however, TEM analysis of *cotL* mutants show a lack of exosporium layer and large reduction in the spore coat layer [1,2,3]. Future experiments characterizing lysozyme-triggered germination in *cotL* mutant backgrounds may show a more dramatic phenotype of lysozyme permeability compared to *cdeC* mutants that may be more easily applied to screen for suppressor mutants.

4.4 Heat treatment kills a portion of dormant spores

We also concluded that this heat treatment, which was used during the enrichment process, is too severe to use in the current genetic screen because it kills a portion of dormant spores. Future experiments would benefit from reducing the temperature of heat treatment. From our assay the optimal heat treatment of the three tested is 65°C for 30 minutes as it does not significantly reduce relative CFU/mL alone but does decrease relative CFU/mL of *cdeC*::CT spores after lysozyme and heat treatment compared to heat treatment alone. Another consideration is using an alternative to heat to kill germinated spores. As previously discussed, it

is possible that conditions could be altered in order to expose lysozyme treated spores to a concentration of oxygen sufficient to render germinated spores inviable.

4.5 The current mutagenesis treatment is insufficient to produce detectable levels of suppressor mutants in the current screen

We concluded that the mutagenesis frequency currently achievable using EMS is not sufficient to screen for suppressor mutants in the *cdeC*::CT background using the current screen and experimental design. EMS concentrations above 2% (v/v) killed treated cultures. This indicates that the maximum mutagenesis frequency achievable by EMS is the level of the 2% treatment, approximately 10^{-3}. The current screen uses the phenotype of lysozyme germination and heat killing in *cdeC*::CT spores, which is not sensitive enough, and leads to a reduction in CFU/mL of approximately 1 log (detection limit of 10%). The current mutagenesis frequency is 2 logs below the detection limit (0.1%). Consequently, we have considered the use of other mutant backgrounds with more sensitive phenotypes for future experiments. An example of a more selective phenotype can be seen in a *spo0A* mutant background. Spo0A is a master transcriptional regulator and mutants of *spo0A* are incapable of forming spores [4]. The *spo0A* mutant background has a detection limit of appearance for suppressor mutants 10^{-7}[4]. Mutations in *cotL*, the gene coding for a lysine rich spore coat protein, have been shown to produce germination null spores with defective spore coats that result in the degradation of the cortex and germination when treated with lysozyme [1]. The *cotL* mutant background has a detection limit of 10^{-6}[1]. We predict that, based on this comparative decrease in detection limit, using the *cotL* mutant background in the current genetic screen will result in the successful enrichment for and detection of suppressor mutants involved in outer spore layer assembly.

5. SUMMARY AND CONCLUSIONS

5.1 Summary

The aim of this series of experiments was to develop a genetic screen that would identify suppressor mutants in an outer spore layer mutant background that confer resistance to lysozyme-triggered germination in order to identify genes related to outer spore layer assembly. The outer spore layers are integral in *C. difficile* spores' interaction with their environment as well as their resistance to decontaminants. Identification of genes involved in assembly would create viable avenues for future studies and targets for future therapies. First, we conducted a series of assays and concluded that there was a phenotype of lysozyme-triggered germination in *cdeC*::CT mutant spores that could be used in a genetic screen for suppressor mutants. Then, a method of chemical mutagenesis, enrichment, and screening for suppressor mutations was designed using the characterization of *cdeC*::CT mutants sensitivity to lysozyme-triggered germination. Since enrichment for suppressor mutants was unsuccessful we tested if the heat treatment conditions were too severe and killing spores that were not lysozyme germinated. We also tested the frequency of rifampicin resistance at different concentrations of EMS treatment to see if a higher concentration could increase the frequency of mutagenesis. We predict that the following changes to experimental design may lead to a successful genetic screen of suppressor mutants of genes related to outer spore layer assembly. First, future experiments may test the replacement of heat treatment with longer exposure to oxygen or exposure to higher concentrations of oxygen. Second, if heat treatment is used, the heat treatment conditions need to be altered in order to kill only germinated spores without significantly impacting dormant spores. Third, in order to increase the frequency of mutagenesis other avenues of mutagenesis should be considered. Finally, we believe that implementing the genetic screen in a different mutant

background with a more dramatic difference in phenotype from wild type spores such as *cotL* mutants would greatly increase the chance of identifying suppressor mutants.

5.2 Conclusions

The first major conclusion of this study is that there is a phenotype of lysozyme germination and heat killing in the *cdeC*::CT background. However, we concluded that the current genetic screen is not capable of detection and enrichment for suppressor mutants. After testing a range of heat and mutagenesis treatments we also concluded that the heat treatment used was killing a portion of dormant spores lowering the likelihood of enriching for suppressors and that the mutagenesis frequency is low and not generating enough suppressor mutants to be detectable in the current screen.

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