ALLELIC EXCHANGE OF THE FLAGELLIN GENE *FLIC* OF *CLOSTRIDIUM DIFFICILE*: GENERATION OF A KNOCKOUT MUTANT

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ABSTRACT

Allelic Exchange of the Flagellin Gene *fliC* of *Clostridium difficile:*
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Clostridium difficile is an antibiotic-resistant Gram-positive bacterium. It has become a major concern for hospitals and acute care medical wards where antibiotics are used more readily. Therapies and preventative measures are being developed to control the disease, and therefore it is crucial to understand the basis of its colonization of susceptible hosts (*e.g.* antibiotic-treated patients). Genetic manipulation of the *C. difficile* genome can be achieved through allelic exchange technologies. Precise alterations are made in the PCR-ribotype 027 strain, R20291 to generate a deletion mutant of the flagellin gene *fliC.* By following mutagenesis protocols, this mutant can be obtained to serve as a tool and for studies on *C. difficile* motility and chemotaxis.

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CHAPTER I

INTRODUCTION

Clostridium difficile poses a great threat to antibiotic-treated patients and is a huge burden to hospitals and healthcare facilities worldwide. *C. difficile* causes severe diarrhea and morbid colitis. Broad-spectrum antibiotic therapy perturbed the normally protective colonic microbiome and allows the antibiotic-resistant pathogen to grow in the created niche. Because antibiotics are used more readily in hospitals and acute care medical wards, the incidence of infection is increasing [1]. Moreover, growing numbers of patients are having adverse effects. The disease alone costs the United States healthcare system more than $1.1 billion per year [1]. Thus, it is important to understand the pathogenesis and virulence of *C. difficile* at the molecular level in order to develop new treatments and preventative measures to control the disease. One potential target is motility.

The *C. difficile* flagella (and thus motility) has been linked to pathogenesis in prior studies. The idea that other virulence components besides its two virulence factors, clostridial toxins A and B, contribute to the disease is accepted [2]. Baban et al. (2013) tested the hypothesis by comparing a flagellated wildtype to strains which flagella genes were inactivated. Their results concluded that the structural components of the flagellum are what is needed for adherence and colonization of the intestinal epithelium during infection [2]. The *fliC* gene codes for flagellin – the structural component of the flagella. *fliC* negative mutants do not have the necessary means to propel itself forward. Herein, I will introduce a deletion of the *fli*C gene into *C. difficile* using a newly-developed allelic exchange technology. This system requires the prior creation of a *pyrE* deletion mutant (already pre-existing in the Sorg laboratory). In wild-type

cells, the *pyrE* gene product provides sensitivity to 5-fluoorortic acid (FOA), which is converted into a toxic product during growth [5-fluorouracil (5-FU)]. 5-FU is misincorporated into DNA or RNA, which will impede replication and transcription, leading to cell death. [3] Though a *pyrE* mutant is a uracil auxotroph, it is resistant to FOA, which enables for modification of the target genes by allelic exchange and counterselection of single integrants of the *fliC* mutagenesis plasmids in the *C. difficile* genome. The *pyrE* mutation is restored back to wild-type after mutagenesis of the target gene (*i.e.*, the *fliC* gene). Mutations in *fliC* should not be detrimental or lethal to *C. difficile*. A prior study dissected the bacterium and identified a set of 404 essential genes that are required for growth of the bacteria; *fliC* was nonessential for growth [4]. The *fliC* mutant will be used as a control in the motility assays that I developed for the laboratory in a previous year's research. By modifying essential biological processes at the molecular level, we can elucidate the molecular pathogenesis of *C. difficile* [5].

CHAPTER II

METHODOLOGY

I will be following a basic mutagenesis protocol involving the use of *pyrE* alleles to make modifications to the *C. difficile* genome. The procedures I will be using are familiarized with the laboratory. *C. difficile* CRG2359 (R20291 strain, *pyrE* mutant) was constructed previously by another laboratory. The vector I will use to generate my mutation is pJS166. In this plasmid, 1 kb upstream and 1 kb downstream of the *fliC* gene were cloned and inserted into the *E. coli – C. difficile* mutagenesis / shuttle vector pMTL-YN4. The resulting plasmid was introduced into *E. coli* HB101pRK24 in order to conjugate the DNA into *C. difficile*. The mutagenesis plasmid will be introduced into the *C. difficile pyrE* mutant strain. The pMTL-YN4 mutagenesis plasmid (pJS166, encoding a region to create *fliC* deletion) is segregationally unstable in *C. difficile.* Thus, this plasmid has difficulty replicating and colonies normally appear small. However, the plasmid can integrate into the genome through the cloned homology region and single-integrants can be detected by screening for large, fast growing colonies on the agar medium. The colonies will be isolated on a *C. difficile* defined minimal medium (CDMM) supplemented with FOA. FOA selects for strains that have recombined the integrated plasmid out of the genome by counterselecting the *pryE* allele on the integrated plasmid. Depending on how the plasmid recombines from the locus, I will have either restored the wildtype allele of the gene or created a mutation, which will be screened for by PCR amplification of the *fliC* region using *fliC*-locus-specific primers. The wild-type *pyrE* allele then is restored in the *fliC* knockout mutant by use of a different plasmid (pMTL-YN2). Most of the resources I need to complete this project will be

available for me to utilize in the laboratory. Research articles and references can be found in the library database.

CHAPTER III

RESULTS

In order to generate a deletion of the *fliC* coding sequence, plasmid DNA containing upstream and downstream regions, with the *fliC* coding sequence deleted, was transferred into *C. difficile* using conjugation from an *E. coli* donor. The *E. coli* HB101pRK24 donor was transformed with the KO plasmid and transformants were selected on LB medium supplemented with chloramphenicol and ampicillin. The resulting strain was conjugated with *C. difficile* Δ*pyrE* recipient, CRG2359, and plated on rich, BHI medium supplemented with uracil (BHI-uracil). The mating was allowed to incubate for 8 – 24 hours. *E. coli* only and *C. difficile* only controls were included to ensure that any observed transconjugants were due to the conjugal transfer of the plasmid and not background growth by the parental strains. Growth from the BHI-uracil agar medium was collected and plated on medium supplemented with thiamphenicol, uracil, D-cycloserine, and kanamycin (BHIS-TUCK) to select for single integrants. Growth streaked onto BHIS-TUCK plates grew after 3 to 4 days of incubation, and yielded very few colonies.

The *catP* gene carried on the pMTL-YN4 plasmid encodes thiamphenicol resistance. Antibiotic-resistant colonies indicate conjugal transfer of the plasmid into *C. difficile*, because daughter cells will carry a copy of *catP* in their genome. Because *E. coli* has no resistance to D-cycloserine, this antibiotic provides further counterselection against the *E. coli* donor. To isolate strains that contain a single integration of the plasmid in the *C. difficile* chromosome, large, fast growing colonies were screened. These colonies were streaked onto another BHIS-TUCK plate, and DNA was extracted. A PCR was ran using two primer pairs designed by the Sorg Lab to verify its purity and identity. One primer pair amplifies a region of the *tcdB* gene that codes for a

virulence factor of *C. difficile*, Toxin B. The other pair amplifies a region of the *catP* gene, a component of the pMTL-YN4 shuttle vector. A colony positive for both genes indicates successful conjugation of the plasmid containing a region for deletion of *fliC* into the *C. difficile* genome.

Unfortunately, several attempts at conjugation led to no transfer of the plasmid into *C. difficile*. Surprisingly, though antibiotic-resistant, the results indicated that the growth was *E. coli* and not *C. difficile.* Incubation times and mating mixes were adjusted accordingly. The summary of these mating reactions can be found in Tables 1 and 2.

	Mating 1	**Mating 2**	**Mating 3**	**Mating 4**
C. difficile Δ*pyrE*	R20291 CRG1496*	R20291 CRG2359	R20291 CRG2359	R20291 CRG2359
E. coli donor strain	HB101pRK24_ pJS166	HB101pRK24_ pJS166	HB101pRK24_ pJS166	HB101pRK24_ pJS166
Heat treatment of *C. difficile* overnight culture	Heatshock at 48°C for 15 minutes	Heatshock at 48°C for 15 minutes	Heat shock at 52°C for 5 minutes	Heat shock at 52°C for 5 minutes
Mating Mix	• 500 µL HB101 pellet resuspended in 500 µL of CRG1496	• 500 µL HB101 pellet resuspended in 1 mL of CRG2359 • 250 µL HB101 pellet resuspended in 1 mL of CRG2359**	• 500 µL HB101 pellet resuspended in 1 mL of CRG2359 • 250 µL HB101 pellet resuspended in 1 mL of CRG2359	• 500 µL HB101 pellet resuspended in 1 mL of CRG2359 • 250 µL HB101 pellet resuspended in 1 mL of CRG2359
Conjugation medium	BHI agar	BHI agar	BHI-uracil agar	BHI-uracil agar
Incubation time	8 hours	8 hours	24 hours	24 hours
Selection medium	BHIS-TKU	BHIS-TKU and BHIS- TKC	BHIS-TKU and BHIS-TKC	BHIS-TUCK
PCR Results	Conjugation unsuccessful	Conjugation unsuccessful	Conjugation unsuccessful	Conjugation unsuccessful

Table 1. *Conjugation summary and details*

*The incorrect strain was used in this mating experiment

**Varying amounts of HB101 was used for mating to determine the smallest amount of *E. coli* required to reduce *E. coli* background

	Mating 5	Mating 6	Mating 7	Mating 8
C. difficile Δ*pyrE*	R20291 CRG2359	R20291 CRG2359	R20291 CRG2359	R20291 CRG2359
E. coli donor strain	HB101pRK24_ pJS166	HB101pRK24_ pJS166	HB101pRK24_ pJS166	HB101pRK24_ pJS166
Heat treatment of *C. difficile* overnight culture	Heatshock at 52°C for 5 minutes	Heatshock at 52°C for 5 minutes	Heat shock at 52°C for 5 minutes	Heat shock at 52°C for 5 minutes
Mating Mix	1 mL HB101 pellet resuspended in 200 µL of CRG1496	1 mL HB101 pellet resuspended in 200 µL of CRG2359	500 µL HB101 pellet resuspended in 1 mL of CRG2359	500 µL HB101 pellet resuspended in 1 mL of CRG2359
Conjugation medium	BHI-uracil agar	BHI-uracil agar	BHI-uracil agar	BHI-uracil agar
Incubation time	24 hours	24 hours	26 hours	26 hours
Selection medium	BHIS-TUCK	BHIS-TUCK	BHIS-TUCK	BHIS-TUCK
PCR Results	Conjugation unsuccessful	Conjugation successful	Conjugation unsuccessful	Conjugation unsuccessful

Table 2. *Subsequent conjugation summary and details*
After several failed attempts at conjugation between *E. coli and C. difficile*, the mating mix was altered again.

The only conjugation that led to conjugal transfer of the plasmid encoding a *fliC* deletion was in Mating 6. However, when transconjugants were restreaked onto a fresh BHIS-TUCK plate, no growth was observed after a week of incubation.

Due to such a low transfer rate of the plasmid into the *C. difficile* genome, mating is a very inefficient process. So far, attempts at conjugation have led to no transfer of the KO plasmid from *E. coli* HB101pRK24 into *C. difficile* CRG2359. Conjugation has been unsuccessful.

CHAPTER IV

CONCLUSION

Though methods have been recently published to increase the mating efficiencies between *C. difficile* and *E. coli*, they remain poor (an observation other laboratory members have observed). So far, there has been no transfer of the pMTL-YN4 plasmid into *C. difficile.* However, once the plasmid has been successfully integrated into the bacterium's genome, single integrants will be detected by growing them on defined media with FOA. FOA selects for strains that have recombined the integrated plasmid out of the genome. Mutations will be screened for by PCR using *fliC*-specific primers. The wild-type *pryE* allele will be restored in the *fliC* knockout mutant by the use of a different plasmid. A non-motile R20291 mutant-*fli*C can be used as a negative control in further chemotaxis and motility assays. In future experiments, this *flic* mutant will be used to discover the chemoattractants and repellants that *C. difficile* uses during motility. This information can be used to formulate alternative treatment options for *C. difficile*-associated diseases, aside from antibiotics.

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