Clostridioides difficile utilizes the siderophore ferrichrome as an iron source through the ABC transporter FhuDBGC

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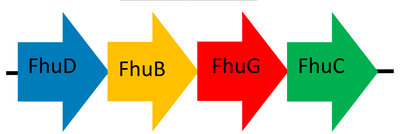
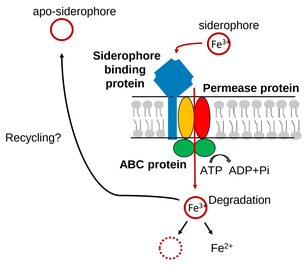
Abstract

Clostridioides difficile (*Cd*) is a Gram-positive, spore-forming bacterium. The primary risk factor for *Cd* Infection (CDI) is antibiotic use. Antibiotics disrupt the gut microbiota, allowing *Cd* to colonize the gastrointestinal tract. *Cd* infection starts when an individual ingests spores, which then germinate in the small intestine. The germinated vegetative cells then produce toxin and cause disease. *Cd* needs many nutrients to survive, including iron, an essential factor for both bacteria and host cells. Most iron in the body is sequestered by storage proteins. Bacteria use multiple mechanisms to acquire iron, including small molecules that bind iron called siderophores. ATP-binding cassette (ABC) transporters import the siderophore across the bacterial membrane. To determine if *Cd* strain 630 can use siderophores as a sole iron source, the bacteria were grown in iron-depleted media (IDM), with or without the addition of several siderophores. The siderophores ferrichrome, yersiniabactin, enterobactin, and salmochelin were able to restore growth and we hypothesize siderophore acquired iron contributes to colonization during CDI. The FhuDBGC transporter has been shown to uptake ferrichrome, a hydroxamate siderophore, in other organisms. Therefore, we took both genetic and biochemical approaches to determine if FhuD, the siderophore binding protein, is responsible for recognizing ferrichrome in *Cd*. Using CRISPRi targeting FhuD, growth with ferrichrome as the sole iron source was significantly reduced in IDM. Additionally, microscale thermophoresis studies demonstrated that purified FhuD binds ferrichrome with an affinity of ~39 nM. In both experiments' specificity was seen with ferrichrome compared to other siderophores. Based on these observations FhuD is responsible for recognizing ferrichrome. To examine the role of the FhuDBGC transporter during infection a strain lacking either the full operon (Δ*fhuDBGC*) or the binding protein (Δ*fhuD*) will be compared to wild-type in the mouse model of CDI. Creating clean deletions in *Cd* has historically been challenging, however, a recently published allelic exchange system that utilizes a toxin as counterselection has been successfully employed by our laboratory and will be used to create these deletion mutations. This work provides insight into how *Cd* competes for nutrients and the role of siderophores in iron acquisition during CDI.

Objectives

Fhu Operon





1) Is the FhuDBGC operon responsible for ferrichrome uptake in *C. difficile*?

2) Does the siderophore binding protein (FhuD) directly bind ferrichrome?

Introduction

- The primary risk factor for contracting *C. difficile* infections (CDI's) is antibiotic use. Antibiotics disrupt the gut microbiota allowing *C. difficile* to outcompete other organisms and colonize the gut. Vegetative cells produce a toxin that attacks the gut epithelium most commonly causing diarrhea.
- *C. difficile* needs nutrients to colonize and produce toxins in the gut. One essential nutrient is iron. Very little free iron is available in the body. Therefore, bacteria have multiple mechanisms aimed at acquiring iron.
- Siderophores are small molecules that scavenge iron. Bacteria use siderophores to acquire iron and ABC transporters to import the siderophores inside the cell.

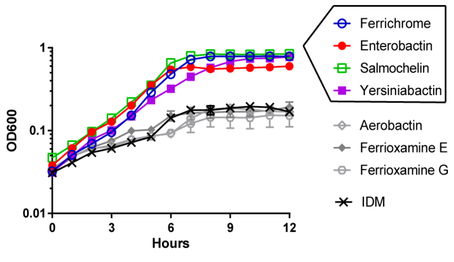


Figure 1. *C. difficile* utilizes siderophores as a sole iron source. An overnight culture of *C. difficile* 630 strain was sub-cultured 1:100 into BHIS + 2, 2-bipyridyl (75 μM) and grown for 4 hours. The cultures were pelleted and washed with PBS. The OD of the cultures were standardized before inoculation in iron depleted media (IDM) supplemented with 20 μM of siderophore pre-loaded with iron.

Hypothesis:

We hypothesize the transporter FhuDBGC is also responsible for ferrichrome uptake in *C. difficile*.

Results

GENETIC:

The addition of xylose induces the deactivated cas9 to bind to the signal guide RNA (sgRNA) and then acts as a roadblock for transcription.

CRISPRi Knockdown

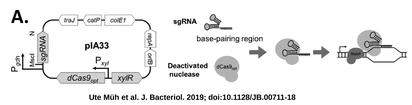
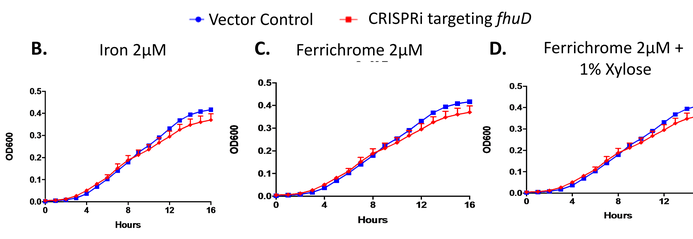
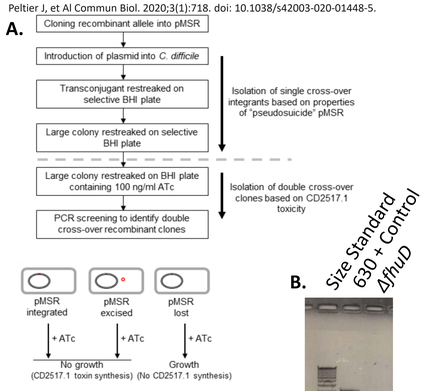


Figure 2. CRISPRi targeting *fhuD* shows a decreased ability to utilize ferrichrome. (A) Overview the CRISPRi knockdown system. Overnight cultures were grown in BHIS and then sub-cultured 1:1000 in BHIS + 2,2-bipyridyl (75μM). Once the cells had doubled, cultures were pelleted, washed with 1X PBS, washed with IDM, and then diluted to an OD of 0.1 to inoculate IDM. Cultures were supplemented with 2 μM iron (B), 2 μM ferrichrome (C), or 2 μM ferrichrome and 1% xylose (D).



In Frame Deletion

Peltier J, et Al Commun Biol. 2020;3(1):718. doi: 10.1038/s42003-020-01448-5.



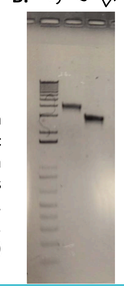


Figure 3. Allelic exchange system used to delete *fhuD*. (A) This allelic exchange system is based on using a toxin as counter selection that is induced by anhydrotetracycline (ATc). (B) Gel showing the deletion of *fhuD*. Wild type strain Cd630 (3591 bp) compared to Δ*fhuD* (2655 bp).

Growth Curve

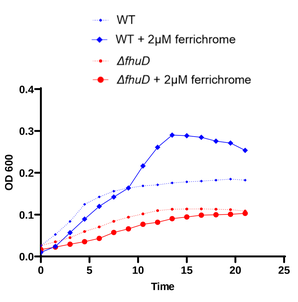


Figure 4. Preliminary data examining the growth of *ΔfhuD* in iron limiting conditions. Overnight cultures were grown in BHIS then back diluted 1:10 in BHIS + 2,2-bipyridyl (75μM). After the cells had doubled, the cultures were pelleted, and washed with twice with PBS or IDM. The OD of the cells were standardized to 0.1 before inoculating in IDM with (solid lines) or without (dashed lines) 2 μM ferrichrome .

BIOCHEMICAL:

MST is based on the detection of a temperature-induced change in fluorescence of a target (FhuD) as a function of the concentration of a non-fluorescent ligand (ferrichrome).

MicroScale Thermophoresis (MST)

A. Nanotemper technologies, 2014

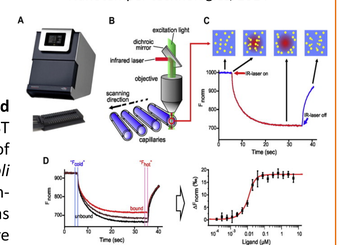
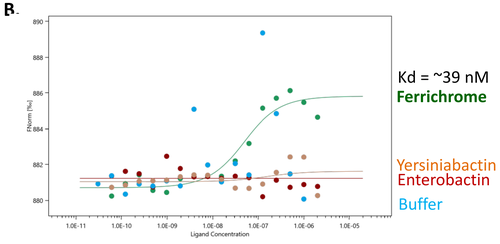


Figure 5. Ferrichrome directly binds to purified *fhuD* extracellular domain (A) Overview of MST technology. (B) The extracellular domain of FhuD was expressed and purified from *E. coli* BL21 cells using magic media and Strep-Tactin-tag column purification. Purified protein was labeled with Red-tris-NTA-2nd generation dye for use in MST.

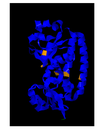
B.



Conclusions and Future Directions

1) *FhuD* directly binds ferrichrome and inhibition of *fhuD* expression impairs the ability of *C. difficile* to utilize ferricrhome as an iron source.

- Now that we have a clean deletion of *fhuD* we will examine the role of this mutation on colonization using the mouse model of CDI.
- Several studies have identified residues important for the *fhuD*/ferrichrome interaction. We plan to mutant these residues and determine if they are necessary for binding.



2) *FhuDGBC* specifically recognizes ferrichrome and is one of three potential ABC transporters responsible for siderophore upatake. We are interested in determining siderophore/transporter specificity with the other transporters.

- Use CRISPRi, in frame deletions and MST to examine the role of the Fpi and Ssu transporters and determine which siderophores they recognize.

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