Determining the Role of Siderophores in *Clostridioides difficile* Pathogenesis

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Abstract

Clostridioides difficile (Cd) is the leading cause of antibiotic associated diarrhea. During colonization, *Cd* must obtain essential nutrients for growth, including iron, which is used both by host cells and bacteria for many cell processes. Very little free iron is available in a mammalian host due to many iron storage mechanisms. Bacterial pathogens have evolved numerous mechanisms for acquiring iron, including small, high-affinity molecules called siderophores. *Cd* grows poorly in iron depleted media (IDM). The siderophores ferrichrome, enterobactin, salmochelin, and yersiniabactin (YBT) restore Cd growth in this media comparable to supplemented iron. Pathogenic bacteria can gain a competitive advantage if they produce their own local pool of siderophore. The ability of *Cd* to produce and/or utilize siderophore from the environment may provide an advantage during infection. Analysis of sequenced *Cd* isolates revealed a small subset of isolates (74/1894 or 3.9%) that encode genes for siderophore biosynthesis, which are predicted to produce YBT. The genes predicted to be responsible for YBT synthesis (*irp2*) and uptake (*ybtPQ*) were knocked out using allelic exchange. These mutants were evaluated for siderophore production (Chrome Azurol S (CAS) assay), siderophore utilization (growth in IDM media) and pathogenesis (*Cd* mouse model). Compared to WT (VPI10463) and *ΔybtPQ*, the *Δirp2* strain reduces the rate that the CAS dye changes color, suggesting siderophore production is altered. In IDM supplemented with 2 μM YBT, the *ΔybtPQ* strain shows slightly impaired growth compared to the WT and *Δirp2* mutant. However, the *Δirp2* and *ΔybtPQ* mutants did not alter pathogenesis in the mouse model. *Cd* utilizes siderophores as an iron source and some *Cd* strains can produce a siderophore. The strain VPI10463 produces high levels of toxin, which may account for the lack of attenuation seen with the *Δirp2* and *ΔybtPQ* mutants due to how quickly the model progresses. Other isolates of *Cd* that exhibit lower virulence in mice are potentially better suited to examine the role of YBT in colonization. Additionally, *Cd* has three other transporters that may import YBT. Future work will focus on determining siderophore/transporter specificity to provide insight into how *Cd* competes with commensal bacteria for siderophore acquired iron.

Background

C. difficile

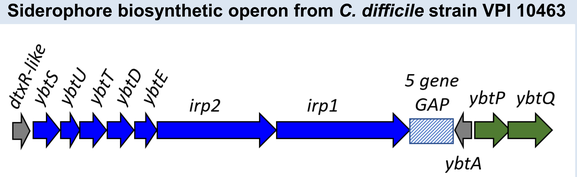
The main risk factor for *C. difficile* infection is taking antibiotics which disrupt the normal microbiota allowing *C. difficile* spores to germinate and colonize. ~223,900 people are infected with *C. difficile* every year and ~12,800 result in death (CDC, 2017).

Iron

Iron is an essential nutrient for most living organisms and most of the iron (~75%) in humans is found in heme associated with hemoglobin within erythrocytes. Surplus iron is quickly bound by host iron storage proteins transferrin and lactoferrin. One mechanism bacteria use to acquire iron are small molecules called siderophores. Some pathogenic bacteria can use siderophores to outcompete commensal bacteria and host cells by producing a local pool of unique “stealth” siderophore.

OBJECTIVE: Make clean deletions of putative siderophore biosynthetic and transporter genes to determine if *C. difficile* produced siderophore contributes to colonization.

Siderophore biosynthetic operon from *C. difficile* strain VPI 10463



grey = regulatory genes, blue = biosynthetic genes, green = tansport genes

Results

Can *C. difficile* utilize siderophore as a sole iron source?

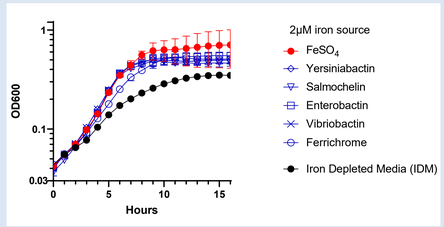


Figure 1. *C. difficile* strain VPI 10463 can utilize siderophores as sole iron source. An overnight culture was grown in BHIS and then sub-cultured 1:10 into BHIS + 2,2 bipyridyl (75 μM). Once the culture doubled, the cells were pelleted and washed with PBS. The pellet was resuspended in iron depleted media (IDM) and adjusted to an OD of 0.1 to inoculate IDM supplemented with $FeSO_4$ or siderophore.

Can *C. difficile* produce siderophore?

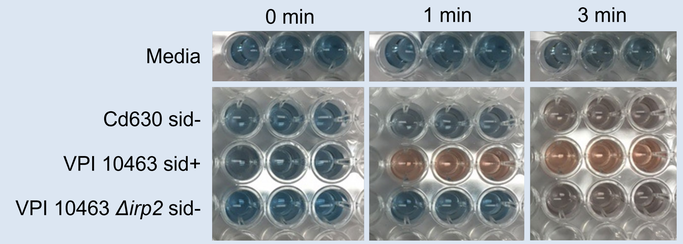


Figure 2. VPI 10463 produces a siderophore. Cultures were grown overnight in BHIS. The cells were pelleted and washed with PBS. The pellet was resuspended in low salt IDM, adjusted to an OD 0.5 and used to back-dilute 1:10 into low salt IDM. Subsequent cultures were grown at 37°C to an OD of 0.4 when 100 μl of supernatant was mixed 1:1 with Chrome Azurol S (CAS) dye and monitored for a color change. Cd630 does not encode the siderophore biosynthesis genes.

Is siderophore production involved in pathogenesis?

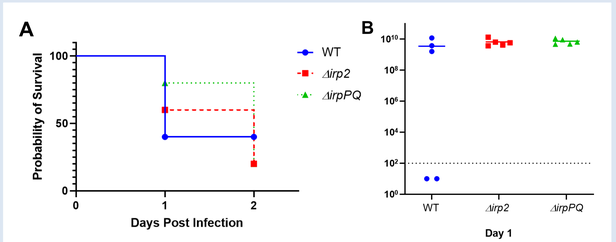


Figure 3. Siderophore production is not required for full pathogenesis of *C. difficile* strain VPI 10463 in a mouse model of CDI. C57Bl6J mice (5-8 weeks) were treated with cefoperazone (0.5 mg/mL) *ad libitum* in sterile drinking water for five days and then switched to normal water for 2 days before administering *C. difficile* WT and *Δirp2* (~ 900 spores) or *ΔybtPQ* (~400 spores) by oral gavage. A. Survival in days post infection. B. CFU/g of *C. difficile* on day 1.

What genes are involved in siderophore uptake?

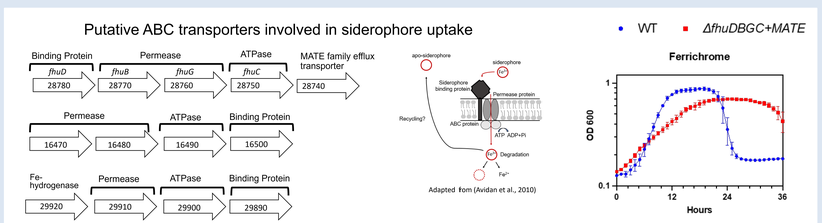


Figure 4. Uptake of ferrichrome by ABC transporter *FhuDBGC*. An overnight culture of Cd630 (blue) or *ΔfhuDBGC+MATE* (red) was grown in BHIS and then sub-cultured 1:10 into BHIS + 2,2 bipyridyl (75 μM). Once the OD doubled, the cells were pelleted and washed with PBS. The pellet was resuspended in IDM and adjusted to an OD of 0.1 to inoculate IDM supplemented ferrichrome (2 μM). Data are representative of three independent replicates.

Condition	Low iron (IDM vs Fe)	Enterobactin vs Fe	Salmochelin vs Fe	Yersiniabactin vs Fe	Ferrichrome vs Fe	Vibrobactin vs Fe
UP	13	0	0	1	2	0
DOWN	9	0	35	0	0	0

Table 1. Few genes are differentially expressed in the presence of siderophore. RNAseq was performed on RNA extracted from VPI10463 cells grown for 6 hours in IDM supplemented with either $FeSO_4$ or siderophore (2 μM). The resulting reads were aligned to VPI 10463 using Kallisto. Normalization and differential expression were performed using Limma with a $\log_2$ fold change cut off of 2 and P-value cut off of 0.01. Genes involved in iron uptake are up-regulated under low iron conditions (IDM vs Fe). Many of the same genes involved in iron uptake are down-regulated in the presence of salmochelin when compared to iron supplementation.

Conclusions and Future Directions

1. VPI 10463 produces siderophore as measured by the CAS assay. However, upon further incubation, *Δirp2* and Cd630 supernatants chelate CAS media at the same rate. Likely, an interfering chelator is produced.
2. There was no difference between WT and mutants *Δirp2* and *ΔybtPQ* in the mouse model of CDI. The two WT mice that survived were not colonized with *C. difficile*. Colonization with *C. difficile* strain VPI 10463 in mice causes rapid disease progression due to the high amounts of toxin this strain produces. We have identified several other strains that contain the siderophore locus, but produce less toxin based on data from Carlson *et al.*, 2014 Anaerobe. We are in the process of making deletions in these strains.
3. In strain Cd630 and VPI 10463 the transporter FhuDBGC shows specificity for ferrichrome. We are currently examining the ability of the other two transporters (16470-16500 and 29920-29890) to utilize different siderophores.
4. In the presence of salmochelin several iron regulated genes are down-regulated. These include the three putative siderophore transporters that are upregulated in low iron conditions. We are investigating why salmochelin turns down iron acquisition mechanisms compared to iron or the other siderophores tested.

Big picture questions for the future:

- **Only some strains of *C. difficile* have the genes to make a siderophore, is siderophore production important for colonization?** Similar to predictions in *C. difficile,* yersiniabactin is only produced by some Yersinia and *E. coli* strains, which in those organisms contribute to pathogenesis. Although the *Δirp2* or *ΔybtPQ* mutations did not have a clear phenotype in VPI 10463, yersiniabactin may provide a growth advantage during colonization in other strains.
- **What is the advantage of producing a local pool of siderophore vs “stealing” xenosiderophores from the commensal microbiota during infection?** Those strains lacking the siderophore biosynthetic genes are still able to utilize siderophore. In the future, we plan to compete yersiniabactin producing vs yersiniabactin non-producing strains in the mouse model of *C. difficile* infection