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. Pathogenic bacteria can gain a competitive advantage by producing local pool of siderophore. 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 yersiniabactin (YBT). We performed RNA-seq on one YBT positive strain (VPI10463) grown in rich media (BHIS) or iron restricted media (BHIS + deferoxamine). The genes for YBT biosynthesis and the predicted transporter YbtP/Q were highly induced. We hypothesize that YBT plays a role in *Cd* pathogenesis and may contribute to the increased disease severity observed for some of these isolates. We knocked out the genes predicted to be responsible for YBT synthesis (*irp2*) and uptake (*ybtPQ*). In iron limiting media supplemented with 2 µM YBT, the Δ*ybtPQ* strain shows impaired growth compared to the WT and Δ*irp2* mutant. However, the Δ*irp2* and Δ*ybtPQ* mutants did not alter pathogenesis in the mouse model of *Cd* infection. VPI10463 produces high levels of toxin contributing to the rapid progression of disease in mice. Siderophore utilization is likely to be important while *Cd* is competing for nutrients. Therefore, siderophore uptake may be more important during prolonged colonization, which is not modeled well using strain VPI10464 due to how quickly the mice succumb to disease. We are currently working to make these mutants in other isolates of *Cd* that exhibit lower virulence in mice to examine the role of YBT in colonization. Based on these observations, *Cd* utilizes YBT as an iron source and YbtPQ is necessary for YBT uptake. Greater knowledge about the role of siderophores in *Cd* iron acquisition and the differences in the pathogenesis of *Cd* isolates could lead to improved treatment options.

Introduction

C. difficile

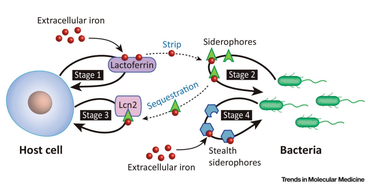
The main risk factor for *C. difficile* infection is taking antibiotics which disrupt the normal microbiota allowing *C. difficile* spores to germinate and colonize. ~223,900 people are infected with *C. difficile* every year and ~12,800 result in death (CDC, 2017).

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.

Does *C. difficile* produce its own siderophore?

Do siderophores contribute to iron acquisition during colonization?



(Wilson *et al.*, 2016)

Background

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

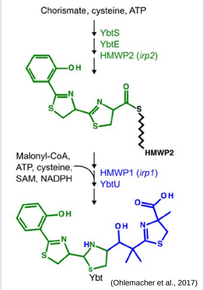
Siderophore biosynthetic operon from *C. difficile* strain VPI 10463

- The biosynthesis locus is ~30% homologous to yersiniabactin from Yersinia. Grey = regulatory genes, blue = biosynthetic genes, green = transport.
- 16.8% (28/166) strains in our collection are positive for yersiniabactin by PCR for *irp2* (Clinical isolates from Michigan)
- 74/1893 (3.9%) genomes in NCBI call siderophore synthesis genes using FeGenie

Table 1. FeGenie finds siderophore genes in strains VPI 10463 and Cd86, but not Cd630. FeGenie is a program that uses a database of hidden Markov models (HMMs) based on genes related to iron acquisition, storage, and reduction/oxidation. (Garber et al., 2020 Front. Microbiol.)

	VPI	Cd630	Cd86
Iron aquisition-iron transport	20	17	18
Iron aquisition-heme transport	0	0	0
Iron aquisition-heme oxygenase	0	0	0
Iron aquisition-siderophore synthesis	6	0	7
Iron aquisition-siderophore transport potential	11	11	11
Iron gene regulation	17	18	17
Iron oxidation	0	0	0
Possible iron oxidation and possible iron reduction	0	0	0
Probable iron reduction	0	0	0
Iron reduction	0	0	0
Iron storage	1	1	1
Magnetosome formation	0	0	0

Biosynthesis of the polyketide-nonribosomal peptide compound yersiniabactin



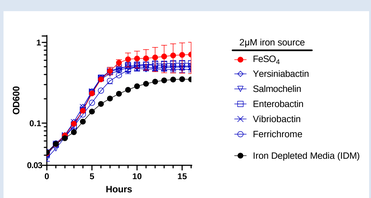
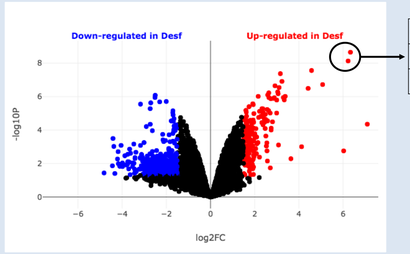


Figure 1. ***C. difficile* strain VPI 10463 can utilize siderophores as sole iron source**. An overnight culture was grown in BHIS and then sub-cultured 1:10 into BHIS + 2,2 bipyridyl (75 µM). Once the culture doubled, the cells were pelleted and washed with PBS. The pellet was resuspended in iron depleted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

Results

Figure 2. ***ybtPQ* is highly upregulated in iron depleted conditions.** *C. difficile* strain VPI10463 was grown 6 hours in either BHIS or BHIS + desferroxamine (Desf) before RNA analysis. Data are mean of 3 replicates. Rockhopper RNAseq pipeline was used.



Gene	logFC
ybtP	6.3
ybtQ	6.2

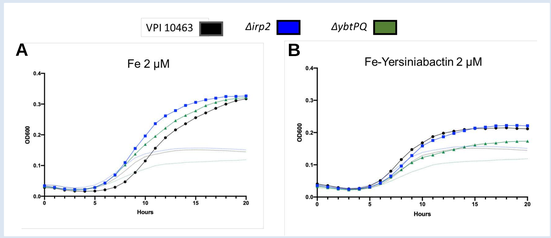
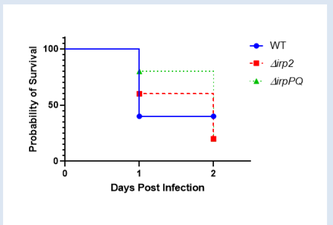


Figure 3. *ΔybtPQ* is deficient in yersiniabacitn uptake. An overnight culture was grown in BHIS and then sub-cultured 1:10 into BHIS + 2,2 bipyridyl (75 µM). Once the culture doubled, the cells were pelleted and washed with PBS. The pellet was resuspended in IDM and adjusted to an OD of 0.1 to inoculate IDM alone (dashed lines) or supplemented with A. $FeSO_4$ or B. yersiniabactin.



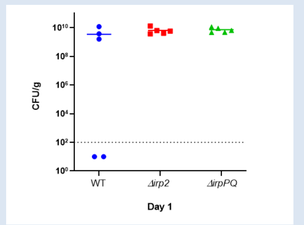


Figure 4. Knockouts of *irp2* and *ybtPQ* do not alter the pathogenesis of VPI 10462 in a mouse model of CDI. C57Bl6J mice (5-8 weeks) were treated with cefoperozone (0.5 mg/mL) *ad libitum* in sterile drinking water for five days and then switched to normal water for 2 days before administering *C. difficile* WT and Δ*irp2* (~ 900 spores) or Δ*ybtPQ* (~400 spores) by oral gavage. A. Survival in days post infection. B. CFU/g of *C. difficile* on day 1.

Conclusions

- Δ*ybtPQ* may be important for yersiniabactin uptake. Strains that do not encode the siderophore locus, are still able to utilize yersiniabactin suggesting a secondary mechanism of uptake.
- There was no difference between WT and mutants Δ*irp2* and Δ*ybtPQ* 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.

Strain	Toxin ng/mL
VPI	7139
Cd088	895
Cd111	1412
Cd086	869

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 organism contributes to pathogenesis.
- **What is the advantage of producing a local pool of siderophore vs “stealing” xenosiderophores from the commensal microbiota during infection?** Although the Δ*irp2* or Δ*ybtPQ* mutations did not have a clear phenotype in VPI10463, we still hypothesize yersiniabactin may provide a growth advantage during colonization in strains that produce less toxin.
- ***C. difficile* has three ABC transporters, do they have specificity for a siderophore or are promiscuous?** These transporters are highly conserved and even those strain lacking the siderophore biosynthetic genes utilize siderophore.

Disclaimer:
The opinions expressed in this presentation are the author's own and do not reflect the view of the Food and Drug Administration, the Department of Health and Human Services, or the United States government. Funding: FDA/CBER