**Title**

*Clostridium difficile* colonizes alternative nutrient niches during infection across susceptible murine gut environments

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**Abstract**

*Clostridium difficile* infection (CDI) as grown to be the greatest cause of hospital acquired infection in the United States. Susceptibility to CDI is induced by previous antibiotic exposure, which has been shown to alter the structure of the gut microbiome. These changes have been associated with changes in bacterial growth nutrient availability in the gut, often increasing concentrations of several useable by *C. difficile*. In this study, we orally challenged C57BL/6 mice with *C. difficile* str. 630 and demonstrated that it was able to colonize the ceca in three separate models of antibiotic induced susceptibility to the same high degree (~1×108 CFU/g content) within 18 hours of inoculation. However, despite equal vegetative cell load at this time point, the levels of both spore and toxin production vary between each antibiotic treatment group. The expression of both phenotypes have both been linked to environmental concentrations of certain substrates, and this indicated possible differences in the nutrient niche *C. difficile* inhabits between susceptible gut conditions. To more closely investigate the specific responses of *C. difficile* as it colonizes the GI tract of mice, we performed *in vivo* *C. difficile*-focused RNA-Seq analysis from cecal content of infected mice. This approach identified differences in expression for genes associated with life-cycle stages and pathogenicity between antibiotic pretreatments, in agreement with previous results. We then went on to observe numerous variations between condition in metabolic pathways associated with carbohydrate and amino acid catabolism, indicating that *C. difficile* likely colonizes alternative nutrient niches across the environments it colonizes. In order to assess which aspects of the gut environment *C. difficile* is exploiting during infection, we sought to identify the growth nutrients that are most likely being used the the pathogen in each colonized condition. To accomplish this we developed a novel substrate scoring algorithm within the genome-scale bipartite metabolic network of *C. difficile* str. 630. The calculation incorporates both network topology and transcript abundance for enzymes within the model to infer the likelihood that a given substrate in acquired from the environment as opposed to being produced by the organism itself. Using this technique, we first validated the approach by confirming the most central nodes reflect known components of *C. difficile* core metabolism. Applying the importance algorithm, we found that *C. difficile* indeed occupies alternative nutrient niches across each antibiotic class tested. Results from this analysis support specific sugar alcohols (D-sorbitol & mannitol) and host derived amino sugars (salicin & N-acetylneuraminate) as the most probable growth nutrients that *C. difficile* differentially utilizes across the susceptible gut environments it colonizes. We then confirmed the capacity of each implicated substrate to support substantial *C. difficile* growth *in vitro*. Furthermore, by employing liquid chromatography-mass spectrometry, we show that these substrates are significantly enriched in the conditions they were found to be most important by the network/transcript-based algorithm. This work has implications for elucidating specifics of the nutrient niche of *C. difficile* during infection, and may lead to the discovery of targeted measures for *C. difficile* colonization prevention.

**Introduction**

Infection by the Gram-positive, spore-forming bacterium Clostridium difficile has increased in both prevalence and severity across numerous countries over the last decade (Lessa 2012). In the United States in 2014, C. difficile was estimated to have caused >500,000 infections and resulted in ~$4.8 billion worth of acute care costs (Lessa, 2015). Clostridium difficile infection (CDI) causes an array of toxin-mediated symptoms ranging from abdominal pain and diarrhea, to the more life-threatening conditions pseudomembraneous colitis and toxin megacolon. Prior treatment with antibiotics is the most common risk factor associated with susceptibility to CDI (Leffler, 2015). It has been shown that antibiotic therapy alters the structure and function of the gut microbiota makes it susceptible to colonization by *C. difficile* (Young, 2004). This is referred to as colonization resistance, in which the gut microbiota inhibits the persistence or growth of a number of pathogenic bacteria. Colonization resistance can be achieved by multiple mechanisms including competition for physical space or growth nutrients (Lawley, 2013).

Mouse models have been an effective tool for studying the mechanisms of colonization resistance. Use of distinct antibiotic classes to vary the structure of the microbiota has been shown to result in varied levels of *C. difficile* colonization (Chen, 2008; Theriot, 2013; Schubert, 2015). Additionally, 18 hours after initially colonizing a cefoperazone treated mouse, *C. difficile* reaches its maximum vegetative cell density in the cecum before beginning to sporulate (Koenigsknecht, 2015). This provided a single timepoint in which to measure the largest population of metabolically active *C. difficile*. Building upon these results, others have shown that many of these antibiotic classes also alter the gut metabolome, increasing the concentrations of known *C. difficile* growth substrates (Antunes 2011; Theriot, 2014; Ferreyra, 2014; Jump, 2014). Taken together these results are a strong indication that the healthy gut microbiota inhibits the growth of *C. difficile* through limitation of substrates it needs to grow. The ability of a healthy gut community to exclude *C. difficile* colonization is suggestive of the nutrient-niche hypothesis (Freter, 1983; Wilson, 1988) in which an organism must be able to utilize a subset of available resources better than all competitors to colonize the intestine.

Based on its genome sequence and *in vitro* growth characteristics, *C. difficile* appears to be able to fill multiple nutrient niches. *C. difficile* has a relatively large and mosaic genome, it is amenable to a variety of growth substrates, and is able to colonize a diverse array of hosts suggesting that that it is a bacterial generalist (Sebaihia, 2006; Tracy, 2012; Songer, 2006). The ability to metabolize a variety of substrates is important since these substrates affect the regulation of genes involved in *C. difficile*’s pathogenesis. For example, *in vitro* transcriptomic analysis suggests that high concentrations of easily metabolized carbon sources, such as glucose or amino acids, inhibit toxin gene expression (Neumann-Schaal, 2015) and sporulation (Nawrocki, 2016). These genes are regulated by DNA-binding sigma factors, such as the pleiotropic regulator *ccpA,* which are under the control of environmental nutrient concentrations, especially carbohydrates (Bouillaut, 2015). Downstream effects of this regulation likely have enormous impact on the lifestyle and metabolic strategy of *C. difficile* when colonizing across sensitive hosts.

Previous transcriptomic studies of *C. difficile* have mainly focused on transcription of virulence factors, *in vitro* (Matamouros, 2007; Antunes, 2011), with some work characterizing transcription during colonization of germ free mice (Janoir, 2013; Kansau, 2016). Relevant to our hypothesis, *C. difficile* up-regulated several phosphotransferase systems (PTS) and ABC transporters in germ free mice, alluding to metabolic adaptation to nutrient availability *in vivo* (Kansau, 2016). Although these analyses are informative, they are either primarily directed toward the expression of virulence factors or lack the context of the gut microbiota which *C. difficile* must compete against for substrates. Metabolomic analyses have also been used to more directly assay changes in bacterial metabolism as they relate to CDI (Theriot, 2013; Jump, 2014); however, these methods cannot focus on *C. difficile* specifically and more closely exhibit echoes of metabolism, not currently active processes. In contrast to these methods, *in vivo* *C. difficile* transcriptomic analysis from conventionally-raised animals may provide unique insight into its active metabolic pathways in a more realistic model of infection. Integrating transcriptomic data with genome-scale metabolic modeling has previously aided in identifying the most active aspects of an organism’s metabolism and which substrates are preferred by the organism (Patil, 2005; Borenstein, 2008; Suthers, 2009). Apply these methods to study *C. difficile* colonization would allow us to directly test our hypothesis.

Based on the ability of *C. difficile* to grow on a diverse array of carbon sources and its ability to colonize a variety of communities, we hypothesized that it focuses its metabolism to fit the context of the community it is attempting colonize. To test this hypothesis, we employed a mouse model of infection to compare the response of *C. difficile* to the gut environment caused by different classes of antibiotics (Fig. 1A). The antibiotics used in this study included cefoperazone, clindamycin, and streptomycin (Table 1). These antibiotics differentially affect the structure of the gut microbiota (Schubert, 2015). Each has also been shown to alter the gut metabolome relative to untreated animals (Antunes, 2011; Theriot, 2014; Jump, 2014). As such, we predicted that *C. difficile* would encounter a unique subset of nutrients and competitors in each environment, which would necessitate distinct adaptive responses. To determine whether *C. difficile* is a generalist and differentially responds to each condition, we assayed for differences in the amount of sporulation and toxin activity phenotypes and used metabolic models built using *C. difficile* expression data. In each of the three antibiotic conditions we challenged with *C. difficile*, as well as in germ free mice, we observed that *C. difficile* altered its metabolic gene expression to colonize to high levels and produce varied amounts of toxin.

**Results**

**Levels of *C. difficile* sporulation and toxin activity vary between germ free and conventionally-raised mice.**

Due to the connection between metabolism and sporulation and toxin production in *C. difficile*, we measured both phenotypes at 18 hours post infection. This time period was selected based on previous work indicating this was a time that would yield the highest number of *C. difficile* vegetative cells in the cecum relative to the number of spores (Koenigsknecht, 2015). There was not a significant difference in the number of vegetative cells between any susceptible condition tested (all *p*-values < 0.05; Fig. 2A). All antibiotic treated and gnotobiotic animals were colonized to ~1×108 CFU/g content, while untreated conventionally-raised mice maintained colonization resistance to *C. difficile*. Despite having the same number of vegetative *C. difficile* cells, large differences were detected in the density of *C. difficile* spores. Significantly more spores (all *p*-values < 0.01) were detected in gnotobiotic mice than in the antibiotic treated mice (Fig. 2B). The spore densities in both streptomycin and clindamycin-treated mice were significantly higher than that in cefoperazone-treated mice. There was significantly more toxin titer in gnotobiotic animals than any other colonized group (all *p*-values < 0.01), but toxin titer also varied between antibiotic treatment groups (Fig. 2C). Although similar toxin titers were found in both the cefoperazone and clindamycin-treated groups, toxin titer was not detectable in the streptomycin-treated animals. These results indicate that *C. difficile* was able to colonize different communities to a consistently high level, but that the density of spores and toxin titer varied by treatment. High levels of both spores and toxin titer in gnotobiotic mice is most likely due to temporal changes in infection dynamics when no other bacteria are present. Because of this, we chose to focus our analysis on the antibiotic-treated conventionally-raised mice.

***C. difficile* adapts expression of genes for key sigma factors controlling metabolism and pathogenesis across antibiotic pretreatments.**

To more closely investigate the responses of *C. difficile* to colonizing distinct susceptible gut environments, we performed whole transcriptome analysis of *C. difficile* during infection of the antibiotic pretreatment models. To narrow our analysis of the transcriptomic data, we chose to focus on genes that code for sigma factors, sporulation, pathogenesis, and quorum sensing.

*C. difficile* sigma factors are the master regulators of transcription and a subset have been shown to respond to environmental nutrient concentrations (Antunes, 2012; Fimlaid, 2013; Bouillaut, 2015; Donnelly, 2016). The global nutritional regulator *codY* transcript was highly expressed in each condition, despite apparent differences in toxin and spore production (Fig. 3A). Canonically, *codY* is a strong negative regulator of toxin production and sporulation and could demonstrate a more complex regulatory network as *C. difficile* integrates multiple environment nutrient signals. Save for a few instances, it appeared that expression the sigma factors of interest were generally higher in cefoperazone-treated animals. The largest contrasts were seen in expression of *sigE*, *sigG*, and *sigK, which are involved in sporulation and* were approximately 10-fold less expressed in cefoperazone than the others. This agrees with the measured differences in CFU due to spores (Fig. 2B) where cefoperazone pretreatment resulted in delayed sporulation.

To further investigate the discrepancies in sporulation, we chose to quantify specific changes in expression within the *C. difficile* sporulation pathway (Fig. 3B). The gene list was derived from Saujet et. al., 2014 where the authors reviewed the complex cascade of regulation that occurs as *C. difficile* completes the steps of sporulation. The resulting profiles further support regulation differences across environments in which the pathogen has colonized. A striking difference was that in Cefoperzone treated animals, *C. difficile* highly expresses a number of putative anti-sigma factors including *spoIIAA,* *spoIIAB,* and *spoVG* involved in suppressing other sporulation-related gene expression (Matsuno, 1999). This pattern was not seen in either Streptomycin or Clindamycin pretreatments, possibly explaining the observed differences in spore CFU. Instead, these conditions induced expression of genes for spore coat components, coat assembly, and spore morphogenesis (including *cotD*, *cotJB2*, *dpaA*, *gpr*, *sodA*, *spoIVA*, *sspB*), all associated with later sporulation stages (Permpoonpattan, 2013). These results further reinforce the connections previously made to the differences seen in spore-specific CFU quantification. Along with sporulation, expression of PaLoc genes has been linked to changes in the nutrients that can be found in the environment of *C. difficil*e (Fig. 3C). We found approximately 3-fold higher expression of the toxin A gene, *tcdA*, than the toxin B gene, *tcdB*, across all pretreatments. This relationship has long been known to exist at least *in vitro* with another strain of *C. difficile* (Hundsberger, 1997). This could have possible implications in the relative importance of toxin A for causing virulence, or temporal differences that toxin B may be more important later during infection. Additionally, we found large differences in the regulation factors most associated with toxin production, *tcdC* (negatively regulating anti-sigma factor) and *tcdR* (positive regulating sigma factor) (Carter, 2012). Between both genes, only *tcdC* transcript was detectable at 10-fold greater abundance than that of *tcdR* across all groups. This was interesting as toxin activity was detectable in each condition, but only the negative regulator of toxin expression was seen implying a more complex regulation of toxin production than this mechanism alone. Finally *tcdE*, the gene for a holin-like protein associated with *C. difficile* toxin secretion, was only detectable in cefoperazone pretreatment.

**Genes sets from multiple *C. difficile* metabolic pathways are differentially expressed between colonized environments.**

In the context of similar colonization, spore, and toxin levels, differential expression of genes for sigma factors and sporulation suggest that *C. difficile* is adapting to environmental nutrient concentrations. To test this further, we identified gene sets based on established *C. difficile* metabolism and quantified the differential expression of genes in each gene set (Fig. 2). Although we observed overlap in the levels of expression of metabolic gene-sets, the largest differences were observed in carbohydrate metabolism. Among the genes classified as sugar transporters, those that are PTS transporters (Fig. 4H) were overrepresented in the clinda and strep-treated mice and ABC sugar transporters (Fig. 4I) were overrepresented in the cef-treated mice. Further differences in carbohydrate metabolism were visible when comparing the polysaccharide catabolism expression set (Fig. 4D) with genes associated with beta-glucoside utilization locus (*bgl* operon), canonically linked with salicin or arbutin metabolism being overrepresented in the clindamycin-treated mice. Sugar alcohol catabolism genes were absent from clindamycin-treated mice and expression of genes for mannitol utilization (*mtl* operon) were overrepresented in cefoparazone-treated mice and expression of genes for sorbitol utilization (*srl* operon) were overrepresented in streptomycin-treated mice. Conversely, the expression of genes associated with amino acid catabolism were consistently expressed at relatively equal levels across the conditions (Fig. 4F). Short chain fatty acids (SCFAs) are the product of both amino acid and carbohydrate fermentation in *C. difficile*. Gene for XXXX were overrepresented in clindamycin-treated mice, while a gene for XXXX was not expressed in clindamycin-treated mice. Overall, these results support the hypothesis that C. difficile is able to adapt its metabolism to fit the nutrients present in different susceptible environments.

cdf:CD630\_03340 adhE; bifunctional acetaldehyde-CoA/alcohol dehydrogenase

cdf:CD630\_24250 ptb; phosphate butyryltransferase

cdf:CD630\_24260 buk1; butyrate kinase

cdf:CD630\_01550 4-hydroxyphenylacetate decarboxylase, activating subunit HpdA

These points all trend toward the center of the plotting area and show no apparent differences between any treatment group. This gene category includes those enzymes involved in Stickland fermentation (*arg*, *fdh*, *grd*, *had*,and *prd* operons) as well as several general peptidases (*pep* family). The known Stickland fermentation pathway in *C. difficile* couples the oxidation and reduction of proline an glycine to generate ATP (Neumann-Schaal, 2015). Transcription of specifically the *grd* and *prd* operons indicates that both proline and glycine are present and the associated metabolic pathways have most likely been activated (Bouillaut, 2013). This suggests that the catabolism of environmental amino acids may be a central part of the metabolism of *C. difficile* str. 630 during infection.

Both systems are known carbohydrate importers, however activation of some PTS transporters in response to environmental concentrations of certain carbohydrates has been shown to negatively regulate the expression and function of many ABC transporters in some bacterial species (Deutscher, 2006).

**Bipartite metabolic models underscore known bacterial metabolism and reveal adaptive nutritional strategies of *C. difficile* during infection.**

We represented the metabolic network of *C. difficile* str. 630 as a directed bipartite graph using the genome annotation. Enzymes and metabolites were represented by nodes and their interaction by the edge between the nodes (Fig. 5A). To validate our metabolic network, we calculated Betweenness centrality for all enzyme and metabolite nodes in the bipartite metabolic network *C. difficile* str. 630 generated for this study (Table S3). In biological terms, this metric reflects the metabolites and functionalities that are most critical to the overall metabolism of the organism. Enzymes scoring highly included pyruvate kinase, 1-deoxy-D-xylulose-5-phosphate synthase, and transketolase. The metabolites that scored highly included pyruvate, acetyl-CoA, and D-glyceraldehyde 3-phosphate. These highly scoring enzymes and metabolites are known to be involved in numerous central pathways including glycolysis, the citric acid cycle, and amino acid biosynthesis, which are all critical to life for most free-living bacteria. This indicated to us that the topology of the network reflects established bacterial physiology.

To move beyond a static representation of the *C. difficile* metabolic network we mapped transcript data to the enzyme nodes. This mapping allowed us to quantify the importance of each metabolite. The importance of a metabolite was measured as the log-transformed difference between the average enzyme transcript levels that used the metabolite as a substrate or generate it as a product. A substrate with a high importance score is mostly likely obtained from the environment because the expression of genes for enzymes that produce the substrate are low. We ranked the importance scores to identify the most important metabolites for each treatment group (Table S5). In both cefoperazone and streptomycin treatment group, several Stickland fermentation intermediates, including propanoate and phosphonoacetate, were scored as highly important relative to scores from randomly assigned transcript abundances. In the clindamycin and gnotobiotic conditions, the mucus derived amino sugars N-acetylglucosamine and N-acetylneurminic acid and glycolysis intermediates scored highly. Well-studied enzyme cofactors, such as Fe2+, were consistently important. Several amino acids were scored highly including proline, threonine, cysteine, leucine, isoleucine, and valine. Many of which are know or suspected auxotrophies of *C. difficile* (Ho, 2015; Neumann-Schaal, 2015). *C. difficile* is known to acquire these substrates from its environment to support its growth. The confirmation of previous results demonstrates that our importance-defining algorithm was robust and detects biologically relevant patterns of expression.

We sought to identify the core metabolites that are essential to *C. difficile* str. 630 in any condition. To accomplish this goal, we compared the top 100 scoring metabolites from each treatnebt group to find those metabolites that were important across all conditions (Table S4). The host derived amino sugar N-acetyl-D-glucosamine was found to be consistently important, indicating that it may be an integral component of the nutrient niche for *C. difficile* under any condition. Other conserved metabolites included fructose derivatives, which are inputs and lactate, which is a product of the anaerobic glycolytic pathway. Additionally, acetate was found to be important in all conditions and is a common byproduct of Stickland fermentation and has been shown to be further metabolized to butyrate by other closely-related species of Clostridia (Li, 2008). We directly tested the relative concentration of acetate in cefoperazone-treated *C. difficile*-infected mice versus mock-infected mice. We found that *C. difficile* colonization led to a significant decrease in the levels of acetate (Fig. S7A) suggesting that *C. difficile* is utilizing acetate.

Returning to our hypothesis that C. difficile adapts its metabolism to fit the surrounding community, we identified those metabolites that were uniquely important to each condition in which *C. difficile* colonized. We cross-referenced the top 25 positively scoring substrates from each treatment group to uncover patterns of unique nutrient utilization by *C. difficile* (Fig. 5B). All of these metabolites were more important than scores for the metabolites where transcript abundances were randomly assigned (black vertical lines). Each group of metabolites contained at least one known carbohydrate growth substrate of *C. difficile*. These included D-fructose, mannitol, N-acetylneuraminic acid, salicin, and D-sorbitol (Ng, 2013; Theriot, 2014). These data support the hypothesis that *C. difficile* may exploit alternative carbon sources between the susceptible environments it colonizes.

**Important carbon sources according to metabolic networks support *C. difficile* growth *in vitro*.**

To validate the biological relevance of substrates identified as uniquely important to C. difficile metabolism through our network-based analysis, we tested whether *C. difficile* was able to utilize each substrate for *in vitro* growth. This was performed using the modified defined *C. difficile* minimal media, supplemented individually with the selected carbohydrates (Theriot, 2013) implicated by high importance scores. Because *C. difficile* can use amino acids for growth through Stickland fermentation, but is auxotrophic for several amino acids, the negative control is growth on media lacking glucose but containing amino acids. At least one carbohydrate highlighted as important in each of the antibiotic treatment groups provided high levels of *C. difficile* growth relative to the negative control (corrected *p*-values << 0.001) (Fig. 5C). This included D-fructose (Streptomycin; OD600 = 0.671), mannitol (Cefoperazone; OD600 = 0.464), salicin (Clindamycin; OD600 = 0.888), N-acetylneuraminate (Germ-free; OD600 = 0.556). Interestingly, the growth curve for D-sorbitol (Streptomycin) showed no difference over the negative controls. These results further support the importance scores and the hypothesis that *C. difficile* is able to adapt its physiology to carbohydrate availability.

**Discussion**

Our results indicate that *C. difficile* occupies separate nutrient niches across susceptible gut environments based on which substrates are available. Both *in vivo* and *in vitro* data support that *C. difficile* shifts its metabolic focus to alternative sources of carbon across susceptible gut environments. This phenotype could be due to a variety of elements including increased concentration of a given set of nutrients, or competition with other microorganisms for those substrates. Sporulation and toxin production have been linked to environmental concentrations of growth substrates for *C. difficile* (Antunes, 2012). In these experiments, the level of both phenotypes varied between antibiotic pretreatment groups and initially suggested differences in metabolic cues received by *C. difficile* in each of condition. By directly examining the transcription of specific metabolic pathways, we were able to gain a greater understanding of the plasticity that *C. difficile* possesses for nutrient utilization.

Initial transcriptional analysis revealed that there was a large amount of variability in the expression of sporulation genes between pretreatments. Aside from the regulating sigma factor *spo0A*, which was consistently highly expressed, *spo* family genes were only expressed highly in cefoperazone treated mice. These genes are integral to Stage V sporulation and contribute to the assembly of the spore coat (Pettit, 2014) whose expression illustrates that this population of *C. difficile* has begun its final phase before transmission either other antibiotic group. In addition to lifestyle associated genes, *C. difficile* str. 630 also demonstrated clear trends of expression for genes associated with separate aspects of its carbon metabolism between pretreatment groups. In both cefoperazone and streptomycin pretreatments, *C. difficile* displayed increased relative abundance in gene expression associated with catabolizing monosaccharides and simple sugar alcohols. *C. difficile* expression in the clindamycin treated animals seemed to favor fermentation of polysaccharides. These differences could signify a focus in nutrient catabolism for *C. difficile* in some treatments and virulence/transmission in others. It has also been previously shown that this bacterium is capable of cleaving host associated glycans and liberating short amino sugars, which is a functionality *C. difficile* lacks (Sebaihia, 2006).

Metabolic modeling results from both cefoperazone and streptomycin-treated mice indicated carbohydrates, known to be *C. difficile* growth nutrients, are preferred in these conditions and are also enriched *in vivo*. These carbon sources are both commonly provided by the host’s diet, but are easily metabolized by a number of resident bacterial species and are therefore highly competed for by the healthy gut microbiota. In the antibiotic-treated state, the normal competitors for these nutrients may be missing and allows for *C. difficile* to get the substrates it needs to grow. Interestingly, findings from clindamycin-treated animals using the same approach suggest that salicin, and breakdown analog 4-hydroxybutanoic acid, are also likely to be used by *C. difficile*. Prior research has shown that salicin is readily fermented by *C. difficile* (Nakamura, 1982). Both are members of the nonsteroidal anti-inflammatory drug (or NSAID) class of molecules. Although the exact source is unknown salicin availability in the gut could be derived from the mice’s plant-based diet, possibly following release during degradation of cell wall molecules by other gut bacterial species (Pierpoint, 1994). What makes this result the most interesting is that molecules in the same class have been shown to actually *increase* severity of CDI symptoms in mice (Trindade, 2016). Network analysis also supports that in germ free mice, *C. difficile* utilizes N-acetylneuraminic acid as an important carbon source. The capacity to catabolize this amino sugar, and others like it, is highly distributed among bacterial species that inhabit the mammalian gut (Vimr, 2004). This means that N-acetylneuraminic acid is strongly competed for by the healthy microbiota. Since the largest increase in availability of the molecule is seen when there are no other microbes present, this could demonstrate a preference of *C. difficile* for N-acetylneuraminic acid as a growth substrate above most others. From these data it seems that *C. difficile* alters it’s nutrient preference to fit what is most easily obtained.

Other conserved features include several central fructose derivatives as well as a likely byproduct, lactate, from the anaerobic glycolytic pathway. This is not surprising as this is known to be a key component of *C. difficile* metabolism, and is likely active to some capacity across all conditions. The lactate produced in this manner is also possibly further fermented to short-chain fatty acids, primarily butyrate (Janoir, 2013).

Many of the top scoring compounds appear to be associated with both fermentation and glycolysis, suggesting that these forms of *C. difficile* metabolism are always active to some degree across conditions and are central to the organism’s nutritional strategy.

Several previous gas & liquid chromatography-mass spectrometry studies of intestinal content obtained from mice have been done using either conventionally-raised then pretreated separately with the antibiotics used in this study or raised in germ free conditions. These studies reveal that many of the substrates predicted to be used by *C. difficile* in a given condition through metabolic modeling (Fig. 4C), are increased in each respective group. For example, cefoperazone treatment resulted in a 553-fold increase in mannitol concentration in the cecum of mice prior to *C. difficile* colonization (Theriot, 2013). It was also shown that fructose is significantly increased in streptomycin-treated mice and would be available to *C. difficile* (Antunes, 2011). Although salicin was not directly assayed for in clindamycin-treated mice, there was a significant increase in 2-hydroxybutryate (a byproduct of salicin metabolism) 24 hours following *C. difficile* infection and indicated that salicin is likely being utilized in this condition (Jump, 2014). Finally, it was demonstrated that N-acetylneuraminic acid is significantly increased in the colons of germ free versus those ex-germ free mice recolonized with microbiota from colon content of conventionally-raised mice (Matsumoto, 2012). Together, these results provide evidence that our network approach accurately predicts which substrates *C. difficile* chooses to metabolize when colonizing different environments.

In light of the results presented here, our method strongly supports that *C. difficile* metabolizes alternative carbon sources across susceptible gut environments for growth. Together, they demonstrate that the novel metabolic modeling approach presented here highlights which substrates an organism is likely using at the time of transcriptomic sequencing. Ultimately, these genome-scale metabolic models provide a framework to predict phenotype changes of an organism across environments. Our results may also indicate that further considerations are needed when attempting to design more targeted probiotics for the prevention or elimination of *C. difficile* from the human gut. Different classes of antibiotics may each result in a unique gut environment which *C. difficile* can exploit. Therefore, the best approach may be to consider each of these as a separate problem which different collections of bacteria can restore to a resistant state. Supporting this hypothesis is that the current, most effective microbiome-focused treatment for *C. difficile* infection is fecal microbiota transplant. By even transiently implanting the entire metabolic functionality of a resistant gut community, one could imagine a rapid out-competition for resources by a healthy community of specialists and leading to *C. difficile* clearance. This could be supported by combining the metabolic modeling technique described here using metatranscriptomic analysis of susceptible versus resistance gut bacterial communities.

In conclusion, our findings indicate that *C. difficile* is able to cope with unique nutrient availabilities across vulnerable gut environments which it colonizes. These data support that separate classes of antibiotics cause susceptibility to CDI through slightly different means by opening unique nutrient niches to *C. difficile*. Context-dependent limitation of certain *C. difficile* growth nutrients by specific members of the gut microbiota may be the key to colonization resistance. In the future, further considerations may be needed to discover targeted probiotics that prevent colonization or eliminate *C. difficile* from the human gut. By understanding vacant nutrient niches in CDI susceptible individuals, preventative microbiome-focused therapies could be personalized to each patient.

Given that many of the differences in expression dynamics seen may also be due to variations in bacterial quorum sensing, we went on to measure transcription of known quorum sensing genes in *C. difficile* (Fig. 3D). These systems have been linked to the lifestyle switch from growth/division to virulence/transmission of *C. difficile* (Darkoh, 2015; Carter, 2005). Transcripts for *agrB*, *agrD*, and *luxS* were found at nearly equivalent levels across all antibiotic pretreatments. This illustrated that the observed changes in phenotype and expression may not be directly due to changes in the levels of *C. difficile* quorum sensing occurring.

**Materials and Methods**

**Animal care and antibiotic administration.** Six-to-eight week-old specific pathogen free (SPF) C57BL/6 mice were obtained from a single breeding colony maintained at the University of Michigan for all experiments. Six-to-eight week-old germfree C57BL/6 mice were obtained from a single breeding colony maintained at the University of Michigan for all experiments. All animal protocols were approved by the University Committee on Use and Care of Animals at the University of Michigan and carried out in accordance with the approved guidelines. Specified SPF animals were administered one of three antibiotics; cefoperazone, streptomycin, or clindamycin (Table 1). Cefoperazone (0.5 mg/ml) and streptomycin (5.0 mg/ml) were administered in the drinking water for 5 days with 2 days recovery with untreated drinking water prior to infection. Clindamycin (10 mg/kg) was given via intraperitoneal injection 24 hours before time of infection.

***C. difficile* infection and necropsy.** *C. difficile* strain 630 spores were prepared from a single large batch whose concentration was determined a week prior to challenge for all experiments. On the day of challenge, 1×103 *C. difficile* spores were administered to mice via oral gavage in ×1 phosphate-buffered saline (PBS) vehicle. Subsequent quantitative plating for CFU was performed to ensure correct dosage. Infection negative control animals were given an oral garage of 100 μl ×1 PBS at the same time as those mice administered *C. difficile* spores. 18 hours following infection, mice were euthanized by carbon dioxide asphyxiation. Necropsy was then performed and cecal content was split into three small aliquots (~100 μl). Two were flash frozen immediately for later DNA extraction and toxin titer analysis respectively. The third aliquot was quickly moved to an anaerobic chamber for CFU quantification. The remaining content in the ceca (~1 ml) was emptied into a stainless steel mortar in a dry ice/ethanol bath using 1 ml of sterile PBS. This process was repeated for each mouse within a treatment group to pool content into a single large sample (9 mice across 3 cages) to compensate for cage effects as much as possible while maximizing sequencing depth. The content was then finely ground and stored at -80° C for subsequent RNA extraction.

***C. difficile* cultivation and quantification.** Differential plating was performed to quantify both C. difficile spores and vegetative cells respectively. Cecal samples were weighed and serially diluted under anaerobic conditions (6% H, 20% CO2, 74% N2) with anaerobic PBS. Samples were plated on CCFAE plates (fructose agar plus cycloserine, cefoxitin, and erythromycin) and counted for CFU after 24 hours of anaerobic growth at 37° C (Buggy, 1997). It is important to note that the germination agent taurocholate was omitted from these plates in order to only quantify vegetative cells. Undiluted samples with then heated at 60° C for 30 minutes in order to eliminate vegetative cells and leave only spores (Sorg, 2001). These samples were then serially diluted under anaerobic conditions in anaerobic PBS and plated on CCFAE + taurocholate. Plating was simultaneously done for heated samples on CCFAE to ensure all vegetative cells had been eliminated. After 24 hours of anaerobic incubation at 37° C, CFUs from spores were quantified. Significant differences were determined by Holm-Bonferroni corrected Wilcoxon tests.

**Measurement of residual antibiotics in cecal content.** Disk diffusion assays were performed using separate, newly plated lawns of *Clostridium difficile* str. 630 and *Bifidobacterium longum* subspec. *longum* on BHIS agar. 500 μl of pooled cecal content within each treatment group were anaerobically centrifuged at 1,000 rpm for 5 minutes. Sterile filter papers disks were then soaked with 20 μl supernatant from spun cecal content and added to plated bacteria in triplicate. Plates were anaerobically incubated overnight at 37° C and zones of inhibition were measured the following day (Fig. S3).

***C. difficile* toxin titer assay.** Vero cell rounding assay was performed on mouse cecal content as previously described (Leslie, 2014). Cells were grown to a confluent monolayer in DMEM, supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin . The cells then were transferred to a conical tube and centrifuged at 1,000 rpm for 5 minutes to pellet the cells. 1×105 cells were seeded in each well of a 96-well plate and incubated for 4 hours. Filtered cecal content was serially diluted 1:10 in ×1 PBS. Control wells were given a volume of either a 1:25 dilution of anti-toxin serum. Following the incubation, samples were added to the Vero cells and the plate was incubated overnight at 37° C. Plates were viewed after 24 hours at ×10 magnification for cell rounding. The cytotoxic titer was defined as the reciprocal of the highest dilution that produced rounding in 80% of the cells. Significant differences were determined by Holm-Bonferroni corrected Wilcoxon tests. A detailed protocol with product information can be found at: <https://github.com/jlleslie/Intraspecific_Competition/blob/master/methods/Verocell_ToxinActivity_Assay.Rmd>

**RNA extraction and library preparation.** This procedure was adapted from the RNA isolation protocol defined by Lopez-Medina et. al., 2011. In order to generate enough mRNA biomass contributed by *C. difficile*, we pooled cecal content from all mouse replicates into a single large isolation for each treatment group. This was beneficial because it not only normalized subtle variation in expression between samples, but also simplified the technical process of isolating high-quality RNA from multiple treatment groups at once. 3 ml of lysis buffer (2% SDS, 16 mM EDTA and 200 mM NaCl) contained in a 50 ml polypropylene conical tube was first heated for 5 minutes in a boiling water bath. The hot lysis buffer was added to the frozen and ground cecal content. The mixture was boiled with periodic vortexing for another 5 minutes. After boiling, an equal volume of 37° C acid phenol/chloroform was added to the cecal content lysate and incubated at 37° C for 10 minutes with periodic vortexing. The mixture was the centrifuged at 2,500 g at 4° C for 15 minutes. The aqueous phase was then transferred to a sterile tube and an equal volume of acid phenol/chloroform was added. This mixture was vortexed and centrifuged at 2,500 g at 4° for 5 minutes. The process was repeated until aqueous phase was clear. The last extraction was performed with chloroform/isoamyl alcohol to remove acid phenol. An equal volume of isopropanol was added and the extracted nucleic acid was incubated overnight at -20° C. The following day the sample was centrifuged at 12000 g at 4° C for 45 minutes. The pellet was washed with 0° C 100% ethanol and resuspended in 200 μl of RNase-free water.

Following the manufacterer's protocol, samples were then treated with 2 μl of Turbo DNase for 30 minutes at 37° C. RNA samples were retrieved using the Zymo Quick-RNA MiniPrep according the manufacterer's protocol. Completion of the reaction was assessed using PCR for the V6 region of the 16S rRNA gene (Kozich, 2013). Quality and integrity of RNA was assessed using the Agilent RNA 6000 Nano kit for Total Prokaryotic RNA. The Ribo-Zero Gold rRNA Removal Kit Epidemiology was then used to deplete Prokaryotic and Eukaryotic rRNA from the samples according the manufacterer's protocol. Also, prior to library construction, quality and integrity as measured again using the Agilent RNA 6000 Pico Kit and stranded RNA-Seq libraries were made constructed with the TruSeq Total RNA Library Preparation Kit v2, both using the manufacturer's protocol. 2x50 bp sequencing was finally performed using an Illumina HiSeq 2500. A detailed protocol for this entire procedure containing product numbers and quality-control primer sequences can be found in the Github repository associated with this project: <https://github.com/SchlossLab/Jenior_Transcriptomics_2015/blob/master/doc/wetlab_protocol.txt>

**Sequence curation, read mapping, and normalization.** Raw transcript sequencing read curation was performed in a two step process. Residual 5’ and 3’ Illumina adapter sequences were trimmed using CutAdapt (Martin, 2011) on a per library basis. Reads were quality trimmed using Sickle (Joshi, 2011) on the default settings. An average of ~300,000,000 total reads (both paired and orphaned) remained after quality trimming. Mapping was accomplished using Bowtie2 (Langmead, 2009) and the default stringent settings. ~1,600,000 reads in sample each mapped to the annotated nucleotide gene sequences of *Peptoclostridium difficile* str. 630 from the Kyoto Encyclopedia of Genes and Genomes (KEGG). Optical and PCR duplicates were then removed using Picard MarkDuplicates (<http://broadinstitute.github.io/picard/>), leaving ~150,000 reads per sample for final analysis. The remaining mappings were converted to idxstats format using Samtools (Li, 2009) and the read counts per gene were tabulated. Discordant pair mappings were discarded and counts were then normalized to read length and gene length. Each collection of reads was then 1000-fold iteratively subsampled to 97,930 reads to generate bootstrapped medians for each gene.

**Bipartite Metabolic Modeling.** The metabolism of *C. difficile* str. 630 was represented as a directed bipartite graph with both enzymes and substrates as nodes. Briefly, models were constructed using KEGG ortholog gene annotations to which transcripts had been mapped. Using the reaction\_mapformula.lst file located in /kegg/ligand/reaction/, KEGG orthologs were converted to lists of input and output compounds associated with each enzymatic reaction respectively. As compounds are associated with the enzyme, it is labeled as either an input or output of the reaction that enzyme mediates. This process is repeated for all enzymes in the given bacterial genome. The resulting data structure is an associative array of enzymes associated with lists of both categories of substrates (input and output), which serves as the architecture of the network. The final metabolic network of *C. difficile* str. 630 contains a total of 1205 individual nodes (447 enzymes and 758 substrates) with 2135 edges. Transcriptomic mapping data is then re-associated with the respective enzyme nodes prior to substrate importance calculations. Betweenness-centrality was calculated using the igraph package in R.

The substrate importance algorithm favors metabolites that are more likely acquired from the environment (not produced within the network), and will award them a higher score. This is accomplished by first calculating the relative importance of compounds as both inputs and outputs of reactions in the network and then subtracting the output score from the input score (Fig. 4A). If a compound is more likely to be produced, the more negative the resulting score will be. These methods have been written into a single python script, along with supporting reference files, and is presented as BiGSMAll (BacterIal Genome-Scale Metabolic models for AppLied reverse ecoLogy), available in a public Github repository at <https://github.com/mjenior/bigsmall>.

To calculate substrate importance, we used rarefied transcriptomic abundances mapped to their respective enzymes (represented by **to** and **ti**), representing the reactions they mediate that either create or utilize the substrate (**s**). The sum is then taken of transcript abundances for enzyme nodes that connect with a given substrate node with the same direction edge (**eo** or **ei**). These values are then divided by the total number of edges that contributed to the previous calculation, creating an average transcription value as either input or output of reactions for the substrate node. The intent is to normalize for highly central substrate nodes that have many connecting enzymes and would score highly regardless of differential expression. The normalized transcription of output reactions is then subtracted from that of input reactions to yield a value that reflects whether synthesis or utilization of the substrate might be favored at the time of measurement. This value is then base-2 log transformed for ease of comparability between high and low transcript abundance associated with a substrate. Zeroes were left unchanged and negative values are accounted for prior to transformation using absolute value with the sign reapplied afterward following transformation. The presumption of our approach is that enzymes that are more highly transcribed are more likely to utilize the substrates they act on due to coupled bacterial transcription and translation.

By comparing the calculated scores to their simulated mean and standard deviation from iterative randomization, we were able to detect which substrate scores were most likely not from consistent high scoring due to a large degree of centrality.

**Mathematical validation of metabolite importance scores.** As sequencing replicates of *in vivo* transcriptomes were not feasible, we sought to apply a modified Monte-Carlo simulation to distinguish calculated metabolite importances due to transcriptional patterns, from consistently important compounds or those scores derived from random chance. With this in mind we took an iterative approach of randomizing assignment of transcript abundance to enzyme nodes and recalculating metabolite importances. Each substrate node accumulated a distribution of importance scores that were then used to calculate a confidence interval. Ultimately, scores from measured values were then compared against simulated medians and confidence intervals to assess for those outside the expectation by random chance for a network with the same topology. This is a superior approach to topology randomization as the landscape of the network itself was created through natural selection and any large-scale alterations to that would yield biologically uninformative comparators. These simulations and comparisons are available within the BigSmall workflow presented above.

**Anaerobic *in vitro C. difficile* growth curves.** The carbon-free variation of *C. difficile* Basal Defined Medium (NCMM) was prepared as previously described in Theriot et. al., 2014. Respective carbohydrate sources were added at a final concentration of 5000 mg/L, also as outlined in the aforementioned study. A solution of the required amino acids was made separately and added when noted at identical concentrations to the same study. 245 μl of final media mixes were added to a 96-well sterile plate arranged as shown in Table S4. A rich media growth control was also included, consisting of liquid Brain-Heart Infusion + 0.5% cysteine. Edge wells were skipped in order to avoid false changes to optical density due to dessication.

All culturing and growth measurement were performed anaerobically in a Coy Type B Vinyl Anaerobic Chamber (3.0% H, 5.0% CO2, 92.0% N, 0.0% O). *C. difficile* str. 630 was grown for 14 hours at 37° C in 3 ml BHI + 0.5% cysteine. Cultures were then centrifuged at 2000 rpm for 5 minutes and resulting pellets were then washed twice with sterile, anaerobic 1x phosphate-buffered saline (PBS). Washed pellets were resuspended in 3 ml more PBS and 5 μl of prepped culture was added the each growth well of the plate containing aliquoted media. The plate was then placed in a Tecan Sunrise plate reader, heated to 37° C. Plates were incubated for 24 hours with automatic optical density readings at 600 nm taken every 30 minutes. OD600 values were normalized to readings from wells containing sterile media of the same type at equal time of incubation. Growth rates and other curve metrics were determined by differentiation analysis of the measured OD600 over time in R to obtain the slope at each time point. Significant differences were determined by Holm-Bonferroni corrected 2-way ANOVA.

**Figure Legends**

**Figure 1 | Experimental timelines and *C. difficile* phenotype quantification 18 hours after infection.** **A)** Experimental timelines for the antibiotic treatments used to sensitize animals to *C. difficile* colonization and infection (n = 9 per treatment group). **B)** Toxin titer measured by activity in Vero cell rounding assay. *C. difficile* monoassociated with germfree mice displayed significantly more toxin activity than any antibiotic treatment (corrected *p*-values < 0.05). **C)** Vegetative *C. difficile* CFU assessed through differential plating (plated without germinant) and **D)** *C. difficile* spore CFU measured with an alternative plating strategy (heating to 65° C for 30 minutes, plated with germinant). No statistical differences were observed in vegetative cell quantification, however germfree mice had significantly more spores than antibiotic treatment groups (corrected *p*-values < 0.05). Differences in spore quantification were noted between antibiotic pretreatment groups, however they were not significant. Conventionally raised mice that received no antibiotics remained resistant to *C. difficile* colonization.

**Figure 2 | Select *C. difficile* gene set expression compared between treatment group.** The median of subsampled gene abundances, shown with 95% confidence interval. **A)** Select sigma factors which their expression has been linked to environment cues. **B)** Majority of known genes from the *C. difficile* sporulation pathway. Many key genes for sporulation display differential expression between treatment groups. **C)** Transcript recruitment to pathogenicity loci, including both the standard PaLoc genes as well as *cdtR* but not the genes for binary toxin as they are absent in str. 630. **D)** Genes associated with quorum sensing in *C. difficile* are detected at approximately even levels in each antibiotic pretreatment.

**Figure 3 | *C. difficile* expression of gene sets for alternative carbon metabolism across antibiotic pretreatments. A)** Ternary plot of all genes with with any transcripts mapping to them are included. Coordinates for each gene are determined based on their relative abundance across each of the antibiotic treatment. The more over-represented transcription of a gene is in a single condition, the more it is placed near the corner corresponding to that treatment. Points placed near the center have equal expression in each of the conditions measured, conversely those genes toward the sides and corners are overrepresented in the groups associated with those edges. Colored points within each gene set are sized relative to the greatest transcript abundance among the three conditions. Groups from (A) are also plotted separately in **B)** through **I)** without size differences so all points are visible.

Mapped transcript read counts again were rarefied iteratively and the means of each gene were plotted as relative abundances along the three axes for the antibiotic pretreatment groups (Fig. 3). Each point represents a unique gene from the annotated genome of *C. difficile* str. 630 with position reflecting the ratio of transcription for that gene in all three antibiotic pretreatments. Points near a corner indicates approximately 100% of the total transcription for that gene across all pretreatments is contributed by *C. difficile* that has colonized mice given the antibiotic associated with given corner. By extension, points that are closer to the center of the plot area demonstrate nearly equal expression across each condition and imply that these functions may be constitutively expressed. Furthermore, a point directly along an axis conveys that 0% of its measured expression was found in the condition associated with the opposite corner. Genes included in each group and subsampled abundances can be found in Table S2.

**Figure 4 | Genome-scale bipartite metabolic modeling results using the transcriptome of *C. difficile* str. 630 in each colonized environment. A)** Largest component from the bipartite genome-scale metabolic model of *C. difficile* str. 630. This portion of the graph includes 1070 nodes (404 enymes and 666 substrates), with 2000 edges connecting them. The size of enzyme nodes is relative to the number of normalized reads mapping to nucleotide gene sequence.Transcript abundances are from *C. difficile* str. 630 during infection of cefoperazone treated mice. The expanded window displays metabolite importance algorithm and an example of a single importance calculation based on local gene transcription. **B)** Top scoring unique important substrates in each antibiotic treatment, top 25 from all were cross-referenced to each other. Monte Carlo simulated means are indicated by the lines, all substrates are calculated as more important than if transcripts abundances were randomly assigned throughout the entire metabolic network. **C)**  *In vitro* growth curves validating identified growth nutrients from (B). In black are CDMM controls variations with the indicated constituents. At least one experimental growth substrate indicated as uniquely important from each group supports *C. difficile* growth significantly more than – Glucose + Amino Acids control wells (corrected *p*-values << 0.001).

**Figure 5 | *in vivo* concentrations from targeted LC-MS analysis of predicted *C. difficile* nutrient sources from metabolic models. A)** Heatmap, group by treatment group on x-axis and hierarchical clustering on y-axis. Comparing No Antibiotics to each treatment group. **B)** Bar charts of the actual concentrations for the substrates identified through metabolic modeling, shown in Figure 4.

**Supplementary Figure 1 | Spore % of total CFU and toxin to spore correlations.** Continued analysis of wetlab phenotypic data. **A)** Spore %. **B)** Spore toxin correlation w/o germfree. **C)** Spore toxin correlation w/ germfree.

**Supplementary Figure 2 | *C. difficile* str. 630 exome coverage for each condition.** Normalized read abundances mapped to the full length genome of *C. difficile* str. 630 for each of the antibiotic pretreatments.

**Supplementary Figure 3 | Expression of specific KEGG gene families.** Description. A). B). C).

**Supplementary Figure 4 | Substrate scores outside of at least two standard deviations from simulated means.** Description. A). B). C).

**Supplementary Figure 5 | Additional growth curves.** Description.

**Supplementary Figure 6 | Acetate data supporting network output.** Description.

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**Conflict of Interest**

The authors declare no conflict of interest.

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