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**Title**

*Clostridium difficile* colonizes alternative nutrient niches during infection across distinct 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. In this study, we orally challenged C57BL/6 mice with *C. difficile* and demonstrated that it was able to colonize the ceca in four separate mouse models of antibiotic-induced susceptibility after 18 hours of infection. However, 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. To more closely investigate the specific responses of *C. difficile* as it colonizes the ceca of mice, we performed *in vivo* *C. difficile*-focused RNA-Seq analysis from cecal content of infected mice. This approach revealed 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 used a reverse ecology approach and developed a novel substrate scoring algorithm within the genome-scale bipartite metabolic network of *C. difficile* str. 630 that 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. 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*. 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 SPF 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* adapted its nutrient utilization profile to colonize to high levels and express its virulence factors.

**Results**

**Levels of *C. difficile* sporulation and toxin activity vary between antibiotic-treated specific pathogen free and germ free 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 (*P* > 0.05; Fig. 2a). All antibiotic treated specific pathogen free (SPF) and germ free (GF) animals were colonized to ~1×108 C.f.u. per gram of content, while untreated SPF 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 (*P* < 0.001) 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 (*P* < 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.

***C. difficile* adapts the expression of genes for virulence and key sigma factors that are under the control of environmental nutrient concentrations.**

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 treatment models. We then narrowed our analysis to focus on genes that control or code for functions that have been linked to nutrient concentrations in the intestines during CDI. After observing differences in spore C.f.u. loads, we first examined transcription of known genes in the *C. difficile* sporulation pathway (Alvarez, 2007; Fimlaid, 2013; Saujet, 2014), limiting it to those with the highest overall gene expression (Fig. 3a). Across the four conditions where *C. difficile* colonized, we observed transcriptional profiles consistent with the spore density that we observed in the cecum (Fig. 2b). The mice treated with cefoperazone had the lowest spore density and had the highest level of expression for the anti-sigma factors *spoVG* and *spoVS.* These genes are involved in suppressing expression of genes found later in the sporulation pathway (Matsuno, 1999). The mice treated with streptomycin had the next highest density of spores and the highest expression of genes associated with sporulation activation (*spoIIAB*/*spoIIE*), but they also had relatively high levels of expression of *sspA* and *sspB*, which are genes that code for effectors that protect DNA from damage during dormancy. Next, mice treated with clindamycin *C. difficile* expressed genes associated with late stages of sporulation, including those for spore coat components (*cdeC*, *cotD*, and *cotJB2*), spore formation (*spoIVA*, *spoVB*, and *spoVFB*), and *sspA* and *sspB*. Finally, GF mice harbored the highest density of spores and those *C. difficile* primarily expressed the dormancy genes linked with the latest stages sporulation. Together these data demonstrate that *C. difficile* differentially expresses stages of sporulation between the gut environments it colonizes.

Expression of genes for quorum sensing and pathogenicity have been linked to changes in the nutrients that can be found in the environment of *C. difficil*e. Both the *agr* locus and *luxS* gene are thought to be associated with inducing the expression of *C. difficile* virulence (Lee, 2005; Martin, 2013). Considering the link between quorum sensing genes and toxin production, we expected the expression of genes for quorum sensing and toxin production and toxin titer to be concordant. Based on this model, we expected GF mice to have the highest levels of expression of genes for toxin production (Fig. 3b) and quorum sensing (Fig. 3c); however, these transcripts were not found in the germ-free mice. We also observed the highest level of expression for quorum sensing genes in cefoperazone-treated mice, but *tcdA* expression in these animals was not the highest among the different treatment groups. Interestingly, the levels of expression for genes associated with toxin production did not match the toxin titers observed in the animals. These results suggest that the relationship between toxin titer and the expression of genes for toxin production is even more complex than current models indicate.

Because the genes discussed thus far are connected to metabolism, we chose to next focus on the regulators of these processes. Sigma factors are master regulators of transcription and a subset have been shown to integrate signals from intra- and extracellular nutrient concentrations (Antunes, 2012; Fimlaid, 2013; Bouillaut, 2015; Donnelly, 2016). The transcription of the global repressor *codY* is responsive to intracellular concentrations of GTP and certain amino acids, which are *C. difficile* energy sources (Fig 3d). Highest transcription for this gene was found in cefoperazone-treated and GF mice. Expression of *ccpA* is dependent on relief of *codY* inhibition and its activity is linked to local concentration of rapidly metabolizable carbon sources. Cefoperazone-treated mice also exhibited increased transcription of *ccpA*, but the GF condition did not follow the same pattern. CcpA acts directly on *spoA0* (Fig. 3d), which positively regulates initiation of the sporulation pathway in *C. difficile*. Transcripts for *spoA0* were highly abundant in all conditions tested except for clindamycin-treated mice, where it was still moderately detectable. The *sig*-family of sigma factors is under the control of SpoA0 and regulate different stages of sporulation. The genes from this family with the highest total transcription (*sigA1*, *sigF*, *sigG*, *sigH*, and *sigK*) each demonstrate a unique pattern of expression between conditions. These results indicate that complete expression of sporulation likely integrates multiple levels of signaling and is more complex than a single metabolic switch. Both CcpA and Spo0A also regulate pathogenicity by acting on *tcdC*/*tcdR* (Fig. 3d). We found expression of the toxin negative regulator *tcdC* in all of the antibiotic-treated groups, but no detectable transcripts for the positive toxin A/B regulator *tcdR* were seen in any treatment. In addition to its effects on sporulation and virulence, CcpA also regulates the expression of other sigma factors that generally mediate distinct forms of *C. difficile* metabolism as needed. These targets include *rex* (general fermentation regulator) and *prdR* (Stickland fermentation regulator)(Fig. 3d). Although the expression of both has been shown to be linked to environmental proline concentrations, *rex* integrates additional signals from the intracellular NADH/NAD+ ratio to also control carbohydrate fermentation. Low-level transcription of *prdR* was found across all conditions, however *C. difficile* expression the *rex* gene highly in both cefoperazone-treated and GF mice. Combined, the variable expression of these sigma factors support the hypothesis that *C. difficile* adapts expression metabolism to fit its needs between colonized environments.

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

In the context of similar colonization between antibiotic-treated animals, differential expression of global metabolic control mechanisms that are under the control of specific nutrient concentrations suggests that *C. difficile* adapts to each environment when in competition with the resident microbiota. To test this further, we quantified the percentage of the total expression for all annotated genes on a per gene basis (Fig. 4). The distribution of points indicated that there were large scale differences in expression between each group (Fig. 4a), but lacking functional labels isn’t informative for uncovering differences in metabolic strategy. We then identified gene sets based on established *C. difficile* metabolism and displayed highest expression of those genes among the three antibiotic treatment groups. The highest overall expression was for those genes involved in amino acid catabolism (Table S2). These findings suggest that *C. difficile* utilizes amino acids for energy as a central part of its metabolismduring infection under any condition.

To assess more clear associations of each gene set with a given antibiotic group, we analyzed each set separately and how they relate to the others. We first chose to asses sugar transport systems since CodY has been associated with adaptive expression of phosphotransferase systems (PTS) and ABC transporters with many known differences in substrate specificities. Among the genes classified as PTS transporters (Fig. 4b) were overrepresented in both clindamycin and streptomycin-treated mice, while ABC sugar transporters (Fig. 4c) were overrepresented in the cefoparazone-treated mice. The most stark differences were seen in transcription for genes involved in sugar alcohol catabolism (Fig. 4d). Expression of these genes was entirely 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. Concordant patterns also emerged in genes associated with fermentation end steps (Fig. 4f) and polysaccharide degradation (Fig. 4g). Short chain fatty acids (SCFAs) and alcohols are the end products of both carbohydrate and amino acid fermentation in *C. difficile* through separate pathways with shared terminal steps. Transcripts for genes involved in *C. difficile* butyrate/butanol metabolism (*ptb*, *buk1*, *cat2*, and *adhE*) were more abundant in clindamycin-treated mice. Additionally, alpha/beta-galactosidase genes (*aglB* and *bglA*) were also overrepresented in clindamycin-treated mice (Fig. 4g). Together these patterns support polysaccharide fermentation in this condition. More subtle differences were seen in those gene associated with glycolysis (Fig. 4e). This category includes genes for not only the steps of glycolysis, but also several genes that mediate entry points of monosaccharides to this pathway. Transcripts for several genes in this group (*eno*, *gapA*, *gpmI*, *tpi*, and *pyk*) were overrepresented in cefoparazone-treated mice, however *fruK* is overrepresented in streptomycin-treated mice which catalyzes the committed step of glycolysis. Overall, these results support the hypothesis that *C. difficile* is able to adapt its metabolism to fit the nutrient availability across different susceptible environments.

Conversely, the expression of genes associated with amino acid catabolism were consistently expressed at relatively equal levels across the conditions (Fig. 4h). This gene category includes those enzymes involved in Stickland fermentation (*arg*, *fdh*, *grd*, and *prd* loci) as well as several general peptidases (*pep* family). Additionally, genes for the metabolism of the host-derived amino sugars N-acetylglucosamine and N-acetylmannosamine were also expressed at consistent levels across each treatment group (*glm*, *nan*, *mur*, and *acd* loci) (Fig. 4i). Along similar lines with closely related molecules, a number of genes for other monosaccharide catabolism were expressed relatively evenly between each condition. This includes glycolysis-associated genes (*fba*, *fbp*, *gap*, and *pfk)* as well as several genes (*gal*, *man*, *pmi*, and *tag* loci) for bringing different monosaccharides into glycolysis (including galactose, mannose, and tagatose). Combined, these findings suggest that catabolism of amino acids and specific monosaccharides are core components of the *C. difficile* nutritional strategy during infection.

**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 (BC) and overall closeness centralization index (OCCI) for all enzyme and metabolite nodes in the bipartite metabolic network *C. difficile* str. 630 generated for this study (Table S3). In biological terms BC reflects the amount of influence a given hub has on the overall flow of metabolism through the network (Potapov, 2005), and OCCI indicates those enzymes and substrates that are the most central components of the organism’s metabolism (Ma, 2003). For both enzymes and substrates, the 18 of top 20 nodes with the highest BC values are involved in glycolysis, fermentation, and amino acid synthesis. In agreement almost all nodes with the largest OCCI values were involved in glycolysis and amino acid synthesis as well. Enzymes that score highly in both metrics included pyruvate kinase, aspartate aminotransferase, and formate C-acetyltransferase while substrates consistently scoring most highly were pyruvate, acetyl-CoA, D-glyceraldehyde 3-phosphate. This indicated to us that the topology of the network reflects established bacterial physiology.

Moving beyond a strictly topological analysis of the *C. difficile* metabolic network, we sought to utilize transcriptomic data to infer which metabolites *C. difficile* is most likely obtaining from its environment in each condition. To accomplish this we mapped normalized transcript abundances to the enzyme nodes in the network, and due to the coupling of transcription and translation in bacteria, we were able to use this information as a proxy for enzyme levels. The importance of each metabolite was measured as the log-transformed difference between the average transcript levels of enzymes that use the metabolite as a substrate and those that generate it as a product. A metabolite with a high importance score is most likely obtained from the environment because the expression of genes for enzymes that produce the metabolite are low. We first ranked the importance scores to identify the most important metabolites for each treatment group (Table S5), however a more informative effort is to identify the core metabolites that are essential to *C. difficile* in any condition. To achieve this goal, we compared the top 50 scoring metabolites from each treatment group to find those metabolites that were important across all conditions (Fig. 5b). Each of the metabolites conserved between all groups scored more highly than the levels simulated from random transcript distributions (vertical black lines). 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 it has been shown that *C. difficile* produces acetate during fermentation and metabolizes it effectively to utilize in glycolysis (Karlsson, 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. S7) suggesting that *C. difficile* is utilizing acetate. These findings provided validation for our metabolite importance algorithm as well as supporting known elements of *C. difficile* metabolism.

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 between treatment groups to uncover the most important patterns of nutrient utilization by *C. difficile* in each (Fig. 5c). Only metabolites that were most important in the antibiotic-treated conditions all score more highly than their simulated median scores. Each group of metabolites contained at least one known carbohydrate growth substrate of *C. difficile* (Ng, 2013; Theriot, 2014). This included close analogs of D-fructose, mannitol, N-acetylneuraminic acid, salicin, and D-sorbitol. Furthermore, in GF mice where no other competitors are present, our model indicates that *C. difficile* is more likely to acquires several amino acids (lysine, leucine, and isoleucine) from the environment instead of expending energy to produce them itself. These data support the hypothesis that *C. difficile* may exploit alternative nutrient sources between the susceptible environments it colonizes.

**Important carbon sources according to network-based approach support *C. difficile* growth *in vitro* and reveal a hierarchy of nutrient preference.**

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. As such, the most effective negative control is growth in media lacking carbohydrates but containing amino acids.

At least one carbohydrate highlighted as distinctly important in each of the antibiotic treatment groups provided high levels of *C. difficile* growth relative to no carbohydrate control (*P* << 0.001) (Fig. 6a). This included D-fructose (streptomycin; OD600 = 0.671), mannitol (cefoperazone; OD600 = 0.464), salicin (clindamycin; OD600 = 0.888), N-acetylneuramininic acid (GF; OD600 = 0.556). Interestingly, the growth curve for D-sorbitol showed no difference over the negative controls. Furthermore, when assessing the consistently high scoring metabolites shown in Fig. 5c N-acetylglucosamine (OD600 = 0.671) (*P* << 0.001) (Fig. 6a). These results further support the importance scores and the hypothesis that *C. difficile* is able to adapt its physiology to carbohydrate availability.

To further investigate if preference and not just availability could play a role in which carbon source *C. difficile* metabolizes at a given time, we also grew *C. difficile* str. 630 in combinations of carbon sources from Fig. 5d. (Fig. 6b)

Shared metabolites tested for growth…need a new color for shared (purple?)

**Discussion**

Collectively, our results support our hypothesis that *C. difficile* occupies separate nutrient niches across susceptible gut environments. This behavior could be due to a variety of factors including variations in concentration of certain nutrients or competition with other microorganisms for growth resources. Several previous mass spectrometry-based efforts have been made to study intestinal content obtained from either SPF mice pretreated with the antibiotics used in this study or raised in GF conditions (Antunes, 2011; Theriot, 2013; Matsumoto, 2012; Jump, 2014). These investigation reveal that many of the substrates predicted to be used by *C. difficile* in a given condition through metabolic modeling (Fig. 4c&d), are increased in the gastrointestinal tract of mice in the corresponding treatment group. For example, cefoperazone treatment resulted in a 553-fold increase in mannitol concentration in the cecum of mice prior to *C. difficile* colonization. This trend is also true in streptomycin and clindamycin pretreatments, as well as in GF mice with different *C. difficile* growth substrates enriched in each. Together, these results provide evidence that our network-based approach accurately predicts which metabolites *C. difficile* chooses to metabolize in a given environments and that these changes are most likely due to nutrient availability.

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).

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.

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 GF 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.

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.

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).

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). C. difficile as an autotroph could explain the appearance of CO2 in the shared metabolite list.

http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0062157

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 overrepresented in cefoperazone-treated SPF mice with no concordant shifts in either sporulation or toxin activity. 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.

Discrepancies with spore c.f.u. and sporulation transcripts may reflect the fact that RNA extraction is primarily only effective on vegetative cells and that distinct subpopulations could be undergoing sporulation at different rates. This largely does not impact results from metabolic modeling because metabolite importance scores reflect average demand at the level of the entire population of *C. difficile*. Because of this, these scores aren’t subject to the effect of subtle differences in metabolism across small groups within the entire collection of activity within the species.

germ free distinct metabolites appear to be mostly auxotrohpies, suggests C. diff can skip a lot of biosynthesis in the absence of competition

Along with highlighting known growth substrates being differentially utilized between conditions, this network-based approach also provides a hypothesis-generating platform to identify new edges of competition of auxotrophies from the organism of interest.

**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 GF 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 distilled drinking water *ad libitum* for 5 days with 2 days recovery with untreated distilled 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 C.f.u. 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 C.f.u. 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 C.f.u. 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, C.f.u.s from spores were quantified. Significant differences were determined by Wilcoxon test and Holm-Bonferroni correction.

***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, library preparation, and sequencing.** 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. Prior to library construction, quality and integrity as measured again using the Agilent RNA 6000 Pico Kit.

Stranded RNA-Seq libraries were made constructed with the TruSeq Total RNA Library Preparation Kit v2, both using the manufacturer's protocol. The Agilent DNA High Sensitivity Kit was used to measure concentration and fragment size distribution before sequencing. High-throughput sequencing was performed by the University of Michigan Sequencing Core in Ann Arbor, MI. For all groups, sequencing was repeated across 4 lanes of an Illumina HiSeq 2500 using the 2x50 bp setting, to normalize for stochasticity in oligo binding to flow cell. 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 to give a per base report of gene coverage. Unless indicated otherwise, each collection of reads was then 1000-fold iteratively subsampled to 97,930 reads to generate bootstrapped medians for each gene. A detailed protocol for red curation can be found in the Github repository associated with this project: <https://github.com/SchlossLab/Jenior_Transcriptomics_2015/blob/master/doc/drylab_protocol.txt>

**Reaction Annotation & Bipartite Network Construction.** The metabolism of *C. difficile* str. 630 was represented as a directed bipartite graph with both enzymes and metabolites as nodes. Briefly, models were constructed using KEGG ortholog gene annotations to which transcripts had been mapped.

KO to reactions first

genes are lost

reactions are lost

need to address huge dependence on annotation...

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, with enzymes that catalyze multiple reactions represented as single nodes. 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 and overall closeness centralization indices were calculated using the igraph R package found at <http://igraph.org/r/>.

**Metabolite Importance Calculation.** 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 (Fig. 5a). 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. If a compound is more likely to be produced, the more negative the resulting score will be. To calculate the importance of a given metabolite (**m**), we used rarefied transcript abundances mapped to respective enzyme nodes. This is represented by **to** and **ti** to designate if an enzyme creates or utilizes **m**. The first step is to calculate the average expression of enzymes for reactions that either create a given metabolite (i) and consume that metabolite (ii). For each direction, the sum of transcripts for enzymes connecting to a metabolite are divided by the number of contributing edges (**eo** or **ei**) to normalize for highly connected metabolite nodes:

(i) *µ*i = Σti ÷ *n*(eo) (ii) *µ*o = Σto ÷ *n*(ei)

Next the raw metabolite importance score is calculated by subtracting the creation value from the consumption value to weight for metabolites that are likely acquired exogenously:

(iii) mi = 2√(*µ*i – *µ*o)

The difference is Log2 transformed for comparability between scores of individual metabolites. This results in a final value that reflects the likelihood a metabolite is acquired from the environment. Untransformed scores that already equal to 0 are ignored and negative values are accounted for by transformation of the absolute value then multiplication by -1. These methods have been written into a single python workflow, 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>.

**Transcriptome Randomization and Probability Distribution Comparison.** As sequencing replicates of *in vivo* transcriptomes were not feasible, we applied a Monte-Carlo style simulation to distinguish calculated metabolite importances due to distinct transcriptional patterns for the environment measured from those metabolites that are constitutively important. With this in mind we took an iterative approach of randomly reassigning transcript abundance for enzyme nodes and recalculating metabolite importances, and ten times the amount of enzymes remaining in the *C. difficile* str. 630 metabolic network after biochemical reaction annotation (= 11830 iterations) was used as the number of iterations for each treatment group. This approach was chosen over fitting a simulated transcriptome to a negative binomial distribution because it created a more relevant standard of comparison for lower coverage sequencing efforts. Using this method, each substrate node accumulated a random probability distribution of importance scores which were then used to calculate the median and 99% confidence interval. By comparing the calculated scores to their associated random probability distribution, we were able to distinguish important metabolites from those that would have consistent scoring across all conditions. 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 would yield biologically uninformative comparisons. This strategy is also included within the standard 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 5 mg/mL, 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. 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% O2). *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 1× 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 2-way ANOVA with Holm-Bonferroni correction.

**Figure Legends**

**Table 1 | Antibiotics used during *C. difficile* infection models.**

**Figure 1 | Experimental time lines for mouse model pretreatments and *C. difficile* infection.** Nine wild-type C57BL/6 mice were included in each pretreatment group. **(a)** Streptomycin or **(b)** cefoperazone administered *ad libitum* in drinking water for 5 days, with 2 days recovery with untreated drinking water before infection, **(c)** a single clindamycin intraperitoneal injection on Day -1 prior to infection, or **(d)** no antibiotic pretreatment (for both SPF control and GF mice). If no antibiotics were administered in the drinking water, mice were given untreated drinking water for the duration of the experiment beginning 7 days prior to infection. At the time of infection, mice were challenged with 1×103 *C. difficile* str. 630 spores at the time of infection. Sacrifice and necropsy was done 18 hours post-challenge and cecal content was then collected.

**Figure 2 | *C. difficile* sporulation and toxin activity quantification after 18 hours of infection. (a)** Vegetative *C. difficile* c.f.u. per gram of cecal content (n=9 mice per group). No significant differences were observed in between any group colonized by *C. difficile*. **(b)** *C. difficile* spore c.f.u. per gram of cecal content. Significantly more spores were detectable in GF mice compared to any of the antibiotic-treated SPF groups (\**P* < 0.05). **(c)** Toxin titer from cecal content measured by activity in Vero cell rounding assay (n=9 mice per group). Reported values are the log10 transformed reciprocal dilution factor of the most dilute well in which cell rounding was observed. GF mice also displayed significantly more toxin activity than all other groups (\**P* < 0.05). UD\* in **a**,**b**,**c** indicate that all points in this groups were undetectable and are significantly different from all other groups in each assay (\*\*\**P* << 0.01). Median values are shown for each group with significant differences calculated using Wilcoxon rank-sum test with the Holm-Bonferroni correction. Dotted lines denote the limit of detection for both assays, and differences were calculated using the this value. Undetectable points are shown just below the limit of detection for clarity.

**Figure 3 | Select *C. difficile* gene set expression compared between treatment group.** Relative abundances of rarefied transcript data for each gene from pooled transcriptomic sequencing of *C. difficile* in each condition. **(a)** Select genes from the *C. difficile* sporulation pathway with the greatest variation in expression between the conditions tested. **(b)** Genes for effector proteins included in the *C. difficile* pathogenicity locus. **(c)** Genes that have been associated with quorum sensing in *C. difficile*. **(d)** Select sigma factors which expression/activity is under the control of environment nutrient concentrations. Relative abundances were calculated from the total expression within each treatment group for all genes shown in **a**-**d**. UD indicates genes from which transcript was undetectable.

**Figure 4 | *C. difficile* expression of gene sets for alternative carbon metabolism across antibiotic pretreatments.** Ternary plot of all genes with with any transcripts mapping to them are included. Mapped transcript counts again were iteratively rarefied and the median expression of each gene was used. 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. 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 associated group. Colored points within each gene set are sized relative to the greatest transcript abundance among the three conditions. **(a)** Point sizes were determined based on the expression from the condition with the highest value for each gene. Highest overall expression across all experimental groups was seen in genes associated with Stickland (amino acid) fermentation. **(b** – **i)** Groups from **a** are also plotted separately in without size differences for calrity. Genes included in each group and transcript abundances can be found in Table S2. Colored asterisks refer to groups that display significantly different distributions by factorial MANOVA (*P* < 0.05).

**Figure 5 | 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 metabolites), with 2000 edges connecting them. The size of enzyme nodes is relative to the number of normalized reads mapping to genes 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)** Metabolites shared among the 50 highest scoring between all conditions. **(c)** Most highly scoring distinctly important metabolites from each treatment group. The top 25 scoring metabolites from each group was cross-referenced against all others to arrive at metabolites differentially important between environments. Points in **b** & **c** are the calculated metabolite importance scores based on the given sequenced transcriptome. Gray vertical lines in **b** & **c** display the median and 99% confidence interval from 11830-fold iterative transcript abundance randomization. Measured scores of the most important metabolites are shown to be greater than the ranges associated with random noise, supporting a valid biological signal. Red points are distinct from the random probability distribution to an equivalent degree as *P* < 0.05.

**Figure 6 | C. difficile growth curves using metabolites predicted from network approach. (a)**  *In vitro* growth curves validating identified growth nutrients from shared analysis. In black are CDMM controls variations with the indicated constituents. At least one experimental metabolite indicated as uniquely important from each group supports *C. difficile* growth significantly more than – Glucose + Amino Acids controls (*P* << 0.001, F-values > ). **(b)** distinct metabolites from each group. **(c)** combinations

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

**Supplementary Figure 2 | *C. difficile* str. 630 gene 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 | *cdtR expression*** High expression of *cdtR*, the positive regulator of binary toxin production, was detected in cefoperazone and to a smaller degree in streptomycin-treated mice. This is in spite of *C. difficile* str. 630 possessing neither *cdtA* nor *cdtB* genes.

**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 7 | Acetate data supporting network output.** Description.

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**Conflict of Interest**

The authors declare no conflict of interest.

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