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Gut microbiota-produced succinate promotes *C. difficile* infection after antibiotic treatment or motility disturbance

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Summary

Clostridium difficile is a leading cause of antibiotic-associated diarrhea. The mechanisms underlying *C. difficile* expansion after microbiota disturbance are just emerging. We assessed the gene expression profile of *C. difficile* within the intestine of gnotobiotic mice to identify genes regulated in response to either dietary or microbiota compositional changes. In the presence of the gut symbiont *Bacteroides thetaiotaomicron*, *C. difficile* induces a pathway that metabolizes the microbiota fermentation end-product succinate to butyrate. The low concentration of succinate in the microbiota of conventional mice is transiently elevated upon antibiotic treatment or chemically-induced intestinal motility disturbance, and *C. difficile* exploits this succinate spike to expand in the perturbed intestine. A *C. difficile* mutant compromised in succinate utilization is at a competitive disadvantage during these perturbations. Understanding the metabolic mechanisms involved in microbiota-*C. difficile* interactions may help to identify approaches for the treatment and prevention of *C. difficile*-associated diseases.

Introduction

One of the most common causes of infectious antibiotic-associated diarrhea is the Gram-positive, spore-forming anaerobe *Clostridium difficile* (He *et al.* 2013). While *C. difficile* resides asymptomatically in the GI tract of 2–5% of the population, it can also cause pathology ranging from mild diarrhea to severe colitis, toxic megacolon, and other *C.*

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Author Contributions
J.A.F and J.L.S designed experiments, analyzed data and wrote the paper. J.A.F and K.J.W. performed experiments. D.M.B. conducted pathology scoring and imaging. B.C.W. contributed reagents.

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difficile-associated diseases (CDAD) (Rivera EV and Woods S 2003). Even after successful treatment with antibiotics such as vancomycin or metronidazole, roughly 20–30% of individuals experience disease recurrence (Pepin J *et al.* 2006, Johnson S 2009). Fecal transplants have been found to be effective in curing recurrent CDAD, consistent with the importance of a healthy gut ecosystem in outcompeting *C. difficile* (Khoruts *et al.* 2010; van Nood *et al.* 2013). The successful use of six bacterial species to cure persistent infection in mice, but failure of other cocktails of six or fewer species underscores the current poor understanding of ecological relationships that underlie *C. difficile* disease (Lawley *et al.* 2012). Additional mechanistic insight into how *C. difficile* exploits the perturbed gut ecosystem will facilitate prevention strategies to hold *C. difficile* at bay during the period of post-antibiotic vulnerability (Wilson and Perini, 1988; Ng *et al.* 2013).

The gut microbiota metabolizes dietary and host-derived nutrients and produces end products including heat, gases, organic acids (OAs) and short-chain fatty acids (SCFAs). Of all metabolites in the colon, dietary carbohydrates play the largest role in the formation of SCFAs and OAs (Macfarlane and Macfarlane 2003). The SCFAs acetate, propionate and butyrate are the most common end products of fermentation within the gut microbiota (Wong *et al.*, 2006). The OAs succinate and lactate are also common end-products of primary fermenters that are taken up and metabolized by other microbes. Several recent studies have highlighted the role of the microbiota-produced metabolites in pathogen colonization (Thiennimitr *et al.*, 2011; Ng. *et al.*, 2013; Maier *et al.* 2013), however the contribution of SCFAs and OAs on pathogenesis remains understudied. While antibiotics contribute to the majority of cases of hospital-acquired *C. difficile*, dietary and motility changes represent two unexplored perturbations that may contribute to cases of community-acquired *C. difficile* infection, which occur independently of antibiotic-exposure (Wilcox *et al.* 2008; Khanna *et al.* 2012). Shifts in diet have been shown to affect the composition and diversity of the microbiota (Turnbaugh *et al.*, 2009; Wu *et al.*, 2011; McNulty *et al.*, 2013), and the extent to which diet-induced transitions in microbiota membership share ecosystem disturbance-characteristics with antibiotic use is unknown. *C. difficile* and other opportunistic members of the microbiota have evolved mechanisms to capitalize upon such ecosystem alterations (Lozupone *et al.,* 2013; Ng *et al.* 2013). In our previous study, microbiota-liberation of the host mucosal monosaccharide sialic acid was identified as a nutrient source used by *C. difficile* and *Salmonella* during post-antibiotic expansion (Ng *et al.* 2013). Further characterization of how microbiota perturbations affect availability of metabolites that contribute to pathogen niches is needed.

In this study, we aimed to understand the mechanisms underlying *C. difficile* expansion after microbiota disturbance, with a focus on microbiota-associated metabolites. We utilized germ-free mice colonized with a well-characterized representative of the prominent Bacteroides genus, *B. thetaiotaomicron* (*Bt*). We profiled the transcriptional response elicited by *C. difficile* in the presence and absence of *Bt in vivo* using standard, polysaccharide-rich dietary conditions versus a polysaccharide-deficient diet. We identified key metabolic strategies that *C. difficile* differentially regulates in these environmental conditions. *Bt* produces high levels of succinate during fermentation of dietary carbohydrates, and *C. difficile* couples reduction of succinate to butyrate with fermentation of remaining dietary carbohydrates. *C. difficile* relies on succinate in a conventional,

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antibiotic-treated setting as well as after gut motility disturbance to efficiently expand. This work sheds light on the important roles that microbiota-associated metabolites play in *C. difficile* expansion, which has implications for potential therapeutic strategies for enteric pathogens.

Results

Identification of key metabolic strategies for *C. difficile* colonization *in vivo*

We sought to identify colonization strategies employed by *C. difficile* within the gut. When *C. difficile* infects mice colonized with *Bt,* it attains higher population densities on day 1 post-infection when the mice were fed a polysaccharide-rich standard diet (2.1-fold). Previous work has shown that *C. difficile* obtains higher population densities in the presence of *Bt* in polysaccharide-deficient chow (Ng *et al.*, 2013) (Fig. 1a). We performed transcriptional profiling of *C. difficile* residing within the distal gut of gnotobiotic mice in these four conditions, to identify potential metabolic strategies employed by *C. difficile* in the presence of *Bt* that could explain its ability to attain higher densities (Fig. 1b). Five COG functional categories, including "carbohydrate transport and metabolism" are significantly differentially regulated ($p<0.01$; hypergeometric test) in response to the presence of *Bt* and three categories changed in response to polysaccharide availability in the diet (Supplemental Fig. S1a, S1b).

Several putative operons involved in nutrient acquisition and metabolism are responsive to changes in the gut environment. *C. difficile* expresses the sialic acid utilization pathway more highly in the *Bt*-bicolonized condition when mice are fed a polysaccharide-deficient diet (14.8–88-fold), consistent with our previous report (Ng *et al.* 2013) (Fig. 1b and Supplemental Table S1). In some cases, the changes are specific to a colonization state in a single dietary condition. For instance utilization pathways of sorbitol and fructose, both common dietary sugars found in many plants including fruit, are more highly expressed in the *Bt*-bicolonized condition when mice are fed standard diet, whereas the glucose utilization operon is more highly expressed in all other conditions (Supplemental Fig. S1c). Dietary change also affects *C. difficile* gene expression, and in standard diet, genes involved in sorbitol/glucitol utilization and cell-surface modification increase, whereas in polysaccharide-deficient diet, genes involved in acquiring cofactors such as cobalt and iron as well as cell-surface proteins and sugar ABC transporters increase (Supplemental Table S1). Because the genes involved in the conversion of succinate to butyrate were highly expressed in conditions where *C. difficile* achieves higher density (4.9–8.2-fold increase in expression in the *Bt*-biassociation relative to monoassociation) (Fig. 1b, Supplemental Table S1, Supplemental Fig. S1d, S1e), we pursued the importance of this pathway for gut colonization by *C. difficile*.

C. difficile relies upon microbiota-produced succinate to expand in the intestine

A similar succinate-to-butyrate pathway has been described for *Clostridium kluyveri* (Kenealy and Waselefsky, 1985; Wolff *et al.* 1993), which enables regeneration of NAD+ from NADH. Succinate metabolism does not directly yield ATP, however it acts as an electron sink allowing for the oxidation of electron carriers (Wolff *et al.* 1993). Notably, *C.*

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difficile's induction of this NAD+ regenerating pathway is coincidently expressed with genes that enable consumption of the sugar alcohol, sorbitol, as well as other dietary sugars, including fructose, which require NAD+ for catabolism (Supplemental Fig. S2). We quantified the levels of SCFAs and OAs in the four *in vivo* conditions (Supplemental figure S3a, S3b). In monoassociation, *Bt* produces high levels of succinate (Supplemental Fig. S3a). Succinate is a common by-product of primary fermenters such as *Bt*, and is typically consumed by various secondary fermenters in the gut (Fischbach and Sonnenburg, 2011). In *Bt*-biassociation, *C. difficile* produced higher levels of butyrate compared to its monoassociation (3.8-fold, $p<0.01$) (Fig. 1c), consistent with its use of *Bt*-generated succinate to produce butyrate.

We next tested the metabolic role of the putative *C. difficile* succinate-to-butyrate pathway. Using ClosTron targeted mutagenesis (Heap *et al.* 2010) we mutated CD2344, a putative succinate transporter, of *C. difficile,* creating a new strain *Cd-CD2344*$^{-}$. Although both wild-type and the mutant strain grow equally well in minimal medium (MM) containing glucose or water (Fig. 2a), *Cd-CD2344*$^{-}$ is unable to use succinate for growth (Fig. 2a). GC-MS measurement of SCFAs within the medium from the cultures at the maximum optical density revealed *C. difficile* wild-type and *Cd-CD2344*$^{-}$ show no differences in SCFA production when grown in MM-glucose (Figs. 2b, Supplemental Fig. S3c). The mutant *Cd-CD2344*$^{-}$ produces significantly less butyrate than wild type *C. difficile* grown in minimal medium containing succinate (11.4-fold, $p<0.0001$) (Fig. 2b) and does not deplete succinate from the medium (5-fold, $p<0.05$) (Fig. 2c). When *C. difficile* and *Cd-CD2344*$^{-}$ are grown in minimal medium containing ^{13}C-succinate, only wild-type *C. difficile* is able to produce ^{13}C-butyrate ($p<0.001$ and Fig. 2e, $p<0.05$)(Fig. 2d), and no ^{13}C-propionate or ^{13}C-acetate are found as byproducts, demonstrating that this pathway is specific for the conversion of succinate to butyrate and is not functional in the mutant strain.

We conducted several experiments to assess whether the succinate-to-butyrate pathway is necessary for *C. difficile* to expand in the presence of *Bt. C. difficile*-monoassociated mice were fed a polysaccharide-deficient diet, which unlike the standard diet, is known to not to include sorbitol as a component. *C. difficile* is able to achieve marginally increased densities with succinate addition in a monoassociation, however a more dramatic benefit is observed when succinate is administered in the presence of the fermentable sugar alcohol, sorbitol, a common carbohydrate present in many fruits (Fig. 3a). In contrast, *Cd-CD2344*$^{-}$ does not reach high densities when colonizing GF mice supplemented with succinate and sorbitol-containing water (7.1-fold, $p<0.05$) (Fig. 3b), consistent with the inability of the mutant to consume succinate effectively. In mice colonized with succinate-producing *Bt*, *Cd-CD2344*$^{-}$ is unable to reach the same densities as wild type (8.8-fold, $p<0.0001$) (Fig. 3c and Supplemental Fig. S3d) and produces lower levels of butyrate compared to wild-type *C. difficile* (1.9-fold, $p<0.001$) (Fig. 3d), demonstrating that the succinate-to-butyrate pathway is important for *C. difficile*'s expansion in the presence of *Bt*. There are no significant differences in any other SCFAs or OAs quantified from the cecal contents of bi-associated mice (Supplemental Fig. S3e). *Cd-CD2344*$^{-}$ also induces less severe inflammation compared to wild-type *C. difficile* in *Bt*-associated germ-free mice (Supplemental Fig. S4).

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Post-antibiotic succinate accumulation is a key nutrient for *C. difficile* expansion

We next used antibiotic-treated conventional mice to determine the importance of succinate for *C. difficile* expansion in the context of a complex microbiota. Antibiotic treatment of conventional mice results in a significant reduction in the levels of the SCFAs acetate and butyrate and a concomitant significant increase in succinate levels (87-fold, $p<0.001$) (Fig. 4a). In a competition experiment, wild-type *C. difficile* outcompetes *Cd-CD2344*$^{-}$ (7.3-fold, $p<0.05$) (Fig. 4b). These data demonstrate that succinate is one of several key nutrients promoting *C. difficile* expansion after antibiotic treatment.

Microbiota-derived succinate is utilized by *C. difficile* upon non-antibiotic perturbations

We tested whether perturbations to the gut microbiota other than antibiotics could enable *C. difficile* to gain access to succinate and expand to cause disease. A significant proportion (22–48%) of community-acquired *C. difficile* infections are not associated with recent antibiotic use (Wilcox *et al.* 2008; Khanna *et al.* 2012). Our previous work established that changes in gut motility induced by oral polyethylene glycol (PEG) treatment decreases diversity of the gut microbiota and significantly increases the presence of members of the family Peptostreptococcaceae, the same family that includes *C. difficile* (Kashyap *et al.* 2013). Given that members of the same family often share general metabolic characteristics, we wondered whether PEG-induced motility disturbance could share molecular features with antibiotic disturbance and result in *C. difficile* infection. Mice were administered a 10% PEG solution in drinking water, resulting in mild diarrhea and reduced gastrointestinal transit time (Kashyap *et al.* 2013). After five days of PEG treatment, mice were infected with *C. difficile. C. difficile* efficiently expands within the gut of mice with PEG-induced diarrhea (Fig. 4c) with the pathogen reaching densities comparable to post-antibiotic treatment (Ng *et al.* 2013). Diarrhea reduces the levels of butyrate and acetate and increases succinate (80-fold, $p<0.01$) (Fig. 4d), mirroring the changes that occur after antibiotic treatment. In competition, wild-type *C. difficile* outcompetes *Cd-CD2344*$^{-}$ in the presence of PEG (16.4-fold, $p<0.05$) (Fig. 4b), although in single infections of antibiotic-treated mice, *Cd-CD2344*$^{-}$ reached similar density levels as wild-type *C. difficile* (Supplemental Fig. S4c). These results demonstrate that succinate and other nutrients promote the expansion of *C. difficile* in the presence of PEG-induced diarrhea.

Discussion

Our results demonstrate that *C. difficile* relies upon the microbiota-produced metabolite succinate after antibiotic treatment as well as after the induction of diarrhea to efficiently expand and cause disease. The ability of *C. difficile* to reach high densities in the gut via pools of microbiota-produced metabolites is consistent with our previous work showing that both *C. difficile* and *Salmonella typhimurium* rely on microbiota-liberation of the host mucosal monosaccharide, sialic acid, as a nutrient source during post-antibiotic expansion (Ng *et al.* 2013). The succinate utilization pathway that *C. difficile* upregulates regenerates NAD+. In other studies, genes from both the nan operon (*CD2240* and *CD2241*) and the succinate-to-butyrate pathway (*CD2338*, *CD2339*) were found to be present in the *C. difficile* spore proteome (Lawley *et al.* 2009), consistent with the utility of these pathways soon after germination. Our transcriptional profiling screen also identified *C. difficile* genes

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specifying an ethanolamine utilization pathway. The previous report of ethanolamine metabolism by Salmonella within the gut suggests that metabolic strategies common to the antibiotic-associated pathogens likely extend beyond the ones identified (Thiennimitr *et al.* 2011). Building an understanding of the hierarchy and relative importance of nutritional substrates used by antibiotic-associated pathogens is an important goal of future work.

Metabolomics studies on antibiotic-treated communities have shown that there are dramatic differences in host responses, bile acid composition, SCFA levels, and carbohydrate availability after antibiotic-treatment (Antunes *et al.* 2011; Theriot *et al.* 2014). Our work reveals that *C. difficile* responds to these changes by adjusting its metabolic pathways. The well-described role of diet in dictating fermentation products of the gut microbes, such as SCFAs and OAs, implies a potential additional link between our food and pathogen susceptibility. The OA succinate is not typically detected in the large intestine of healthy humans. However, succinate has been shown to accumulate in the large intestine of pigs with antibiotic-associated diarrhea (Tsukahara and Ushida, 2002). Combined with the elevated succinate we observe after PEG- or antibiotic-treatment, these data suggest that metabolic cross-feeding between gut resident microbes typically depletes succinate, and such interactions are disrupted by microbiota perturbations. It is well known that diet can have a profound impact on gut motility changes, and we have previously shown in a humanized mouse model that diet-induced motility changes are accompanied by large changes in SCFA profiles within the microbiota (Kashyap *et al.* 2013). How dietary interventions may promote or ameliorate CDAD is currently unknown, though dietary strategies that alter the composition and metabolic capabilities of the microbiota have proven successful against other enteric pathogens (Cummings *et al.* 1996; Ramakrishna *et al.* 2000; Muir *et al.* 2004; Alam *et al.* 2005; Alvarez-Acosta *et al.* 2009; Rabbani *et al.* 2009). Our data demonstrate that dietary change can rewire crossfeeding relationships relevant to pathogen expansion. Upon ecosystem disturbance the identity of available nutrients depends upon diet: *C. difficile* is able to capitalize upon available mucus-derived sialic acids in polysaccharide-deficient dietary conditions and high levels of succinate in the presence of a polysaccharide-rich diet.

This work points to common molecular features of gut microbiota disturbances, which create vulnerability to *C. difficile* expansion. Important questions include which alterations within the dynamic environment of the gut qualify as a “disturbance” and what are the common and specific molecular correlates of different disturbances (Antunes *et al.* 2011; Theriot *et al.* 2014). Microbiota-encoded metabolic pathways and the small molecules they produce represent therapeutic targets to prevent or combat *C. difficile* diseases.

Experimental Procedures

Animal diets and treatments

Germ-free (GF) Swiss-Webster mice of 6–8 weeks of age were utilized for experiments. All experiments were in accordance with A-PLAC, the Stanford IACUC. Mice were fed either an autoclaved standard diet (Purina LabDiet 5K67) or custom polysaccharide-deficient diet (BioServ) (Sonnenburg ED *et al.* 2010). All breeder and experimental isolators were periodically assessed for germ-free status by both culture-based methods (aerobic and

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anaerobic) as well as 16S-based PCR screens using universal primers. Sterility of multiple irradiated custom diets from this vendor (Bio-Serv) has been monitored in the lab over a five-year span with no samples or gnotobiotic mice fed the diets testing positive for contamination. For water supplementation experiments, succinic acid (Acros Organics) and sorbitol (Alfa Aesar) were administered at 1% (w/v) concentration in water after filtration through 0.22 μm Millipore filters. All mouse experiments presented in the manuscript are representative of at least two independent replicates of the experiment.

For antibiotic treatment, Streptomycin (Sigma) was administered at a concentration of 20 mg in 200 μl of PBS by gavage per mouse at 24 hours pre-infection, and mice were starved at 18 hours pre-infection with *C. difficile*. For PEG treatments, PEG 3350 (Miralax™) was administered at a 10% concentration in the drinking water continuously for 5 days prior to infection and removed at the time of infection.

Bacterial strains and culture conditions

*B. thetaiotaomicron (*VPI-5482; ATCC 29148), was grown anaerobically (6% H_2, 20% CO_2, 74% N_2) overnight in TYG medium as previously described (Sonnenburg JL *et al.* 2005).

C. difficile strain 630, JIR8094 and *Cd-CD2344* were cultured in Brain Heart Infusion (Becton Dickinson, MD) supplemented with 5 mg/ml of Yeast Extract (Remel) (BHIS) anaerobically (6% H_2, 20% CO_2, 74% N_2). *C. difficile* growth curves were generated using minimal medium (MM) composed of Farmtech Tryptone (peptone) (EMD), beef extract (Becton Dickinson), yeast extract (Remel), monosodium phosphate (EMD), and disodium phosphate (EMD). OD_{600} was monitored using a BioTek PowerWave 340 plate reader (BioTek, Winooski, VT) every 30 minutes, at 37°C anaerobically (6% H_2, 20% CO_2, 74% N_2). Fecal densities (CFU) of wild-type *C. difficile* after colonization of germ-free or *Bt*-monoassociated mice were quantified by duplicate sampling with 1 μl loops and subsequent dilution and spot plating on brain heart infusion agar (Becton Dickinson) with 10% v/v of defibrinated horse blood (Lampire Biological Laboratories) supplemented with 25 mg/L of erythromycin. To selectively quantify *C. difficile* fecal densities in antibiotic-treated conventional mice and in *Bt*-biassociation experiments involving *C. difficile* mutants, CDMN plates were utilized as previously described (Ng *et al.* 2013). Because Erm is not preset in CDMN plates, there is no difference in the recovery of *Cd-CD2344*$^-$ and wild-type *C. difficile* on CDMN plates.

ClosTron generation of *Cd-CD2344*$^-$ followed detailed protocols for targeted gene disruption in *C. difficile* (Heap JT *et al.* 2010). SOEing PCRs with primers IBS, EBS1d, EBS2 and EBS (Supplemental Table S2) were used to assemble and amplify the product for intron targeting to the 365/366 nucleotide region of the *CD2344* gene, as outlined in the TargeTron users' manual (Sigma Aldrich). The retargeting sequence was digested with BsrGI/HindIII and cloned into pMTL007C-E2. The resulting plasmid was transformed into HB101/pRK24 for conjugation into JIR8094 (O'Connor JR *et al.* 2006) (a generous gift from Aimee Shen) to generate *Cd-CD2344*$^-$. Integration was confirmed by PCR. For all experiments involving *Cd-CD2344*$^-$, the wild-type isogenic background strain, JIR8094, was used as the wild-type control.

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Expression analysis

Genome-wide transcriptional profiling of *C. difficile* was conducted using custom-made Affymetrix GeneChips, which contain probes for all annotated coding sequences for *C. difficile* strain 630 as well as intergenic regions. RNA was purified from cecal contents and *in vitro* culture and cDNA was prepared, fragmented and labeled as described (Sonnenburg JL *et al.* 2005). GeneChip data were RMA-MS normalized as described and log2 transformed (Ng *et al.* 2013). Statistical significance for differential gene expression was determined using Significance Analysis of Microarrays (SAM) (Ng *et al.* 2013). The delta parameter was adjusted to achieve a FDR less than 1%, and this delta value was used to select significantly-regulated genes.

Quantification of fermentation metabolites

Frozen cecal contents (100–300mg) or frozen supernatants from *in vitro* growth curves were resuspended in 0.1% formic acid in water (Honeywell) and blended with a pellet pestle (Kimble chase). Acetate, butyrate, lactate, propionate and succinate standards were included in each run to generate standard curves for quantification. The samples were acidified (37% HCl), and SCFAs were extracted (500μl diethyl ether/extraction; 2 cycles). Each sample was derivatized with N-tert-butyldimethylsilyl-N-methyltrifluoracetamide (MTBSTFA; Sigma Aldrich) and quantified using a gas chromatograph (Model 7890A; Agilent Technologies, Santa Clara, CA) coupled to mass spectrometer detector (Model 5975C; Agilent Technologies). Analyses were carried out in a split mode (1:100) on a DB-5MSUI capillary column (30 m×0.25 mm, 0.25 μm film thickness, Agilent Technologies) using electronic impact (70 eV) as ionization mode and scan in m/z 50–550 mass range. The column head-pressure was 12 p.s.i. Injector, source and quadrupole temperatures were 250, 280 and 150 °C, respectively. The GC oven was programmed as follow: 75 °C held for 2 min, increased to 120 °C at 40 °C min^{-1}, 120 °C held for 5 min, increased to 320 °C at 20 °C min^{-1} and held at 320 °C for 7 min.

Competitive index analysis

DNA was extracted from feces using the Powersoil DNA Isolation kit (MoBio). DNA quantification was conducted by Quantitative PCR using SYBR Green (ABgene) in a MX3000P thermocycler (Strategene, San Diego, CA). Levels of wild-type *C. difficile* were quantified by amplifying a 208 base-pair region of the CD2344 gene with the 2344F and 2344R primers (Supplemental Table S2), which included the area targeted for mutagenesis in the *Cd-CD2344*$^{-}$ mutant. Genomic DNA from *Cd-CD2344*$^{-}$ did not produce a band when amplified with these primers due to the insertion of the transposon within the region. The CDEP692 transposon specific sequencing primer and the 2344F primer (Supplemental Table S2) were used to determine the relative concentrations of the mutant, and did not amplify a band against wild-type *C. difficile* genomic DNA. The ratio of wild-type to mutant bacteria was normalized to the inoculum to determine the competitive index.

Statistical analyses

The Student's t-test was used for statistical calculations involving two group comparisons, and * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$. For comparisons

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involving multiple experimental groups, one-way ANOVA with Bonferroni multiple comparisons test was employed, with **** indicating statistical significance at an alpha value of <0.05. Outliers were excluded from by the Grubbs' statistical test. Error bars indicate standard deviation. *n* indicates the number of mice used per condition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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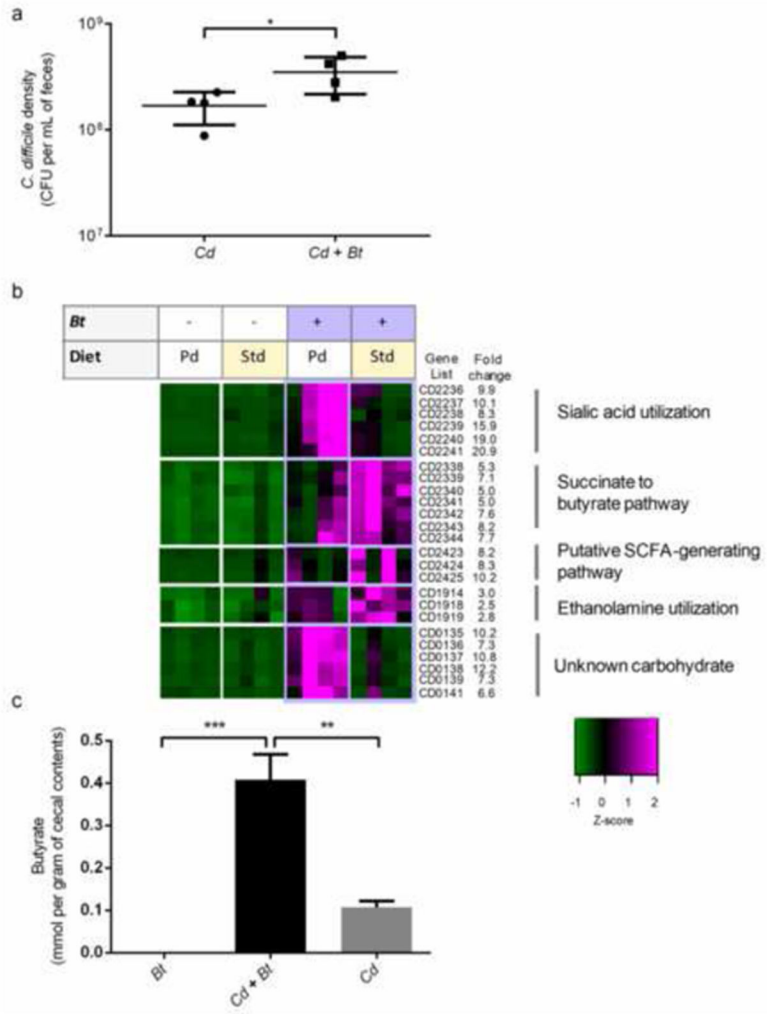

Figure 1. Metabolic strategies employed by *C. difficile* in response to a human gut commensal

a. *C. difficile* density in feces at 1 day post-infection of germ free (*Cd*) or *Bt*-associated mice (*Cd* + *Bt*) and fed standard diet (Std diet) or polysaccharide-deficient diet (Pd diet) (n=4/group).

b. *C. difficile* catabolic pathways with significant differences in gene expression levels *in vivo* in the presence and absence of *Bt*. Mice were fed standard diet (Std) or polysaccharide-deficient diet (Pd). Colors indicate the deviation of each gene's signal above (purple) and below (green) its mean (black) expression value across all sixteen *in vivo* samples (n=4/group) at 5 days post-infection in cecal contents. Fold change values correspond to gene expression in the *Bt*-biassociation relative to the monoassociation state.

c. Concentration of the short-chain fatty acid butyrate in cecal contents of standard diet-fed mice colonized with *Bt*, *C. difficile* and *Bt* (*Cd* + *Bt*), or *C. difficile* (*Cd*) at 5 days post-infection (n=4/group).

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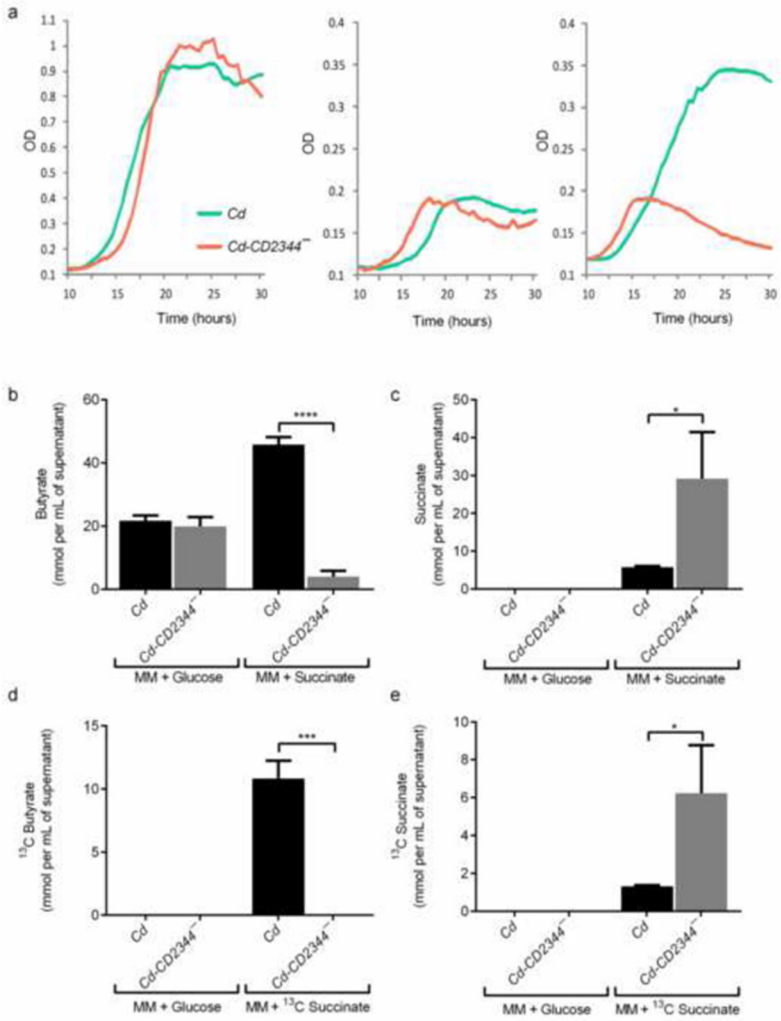

Figure 2. A *C. difficile* operon encodes succinate-to-butyrate conversion

a. Growth of wild-type *C. difficile* (*Cd*) and *CD2344* mutant *C. difficile* (*Cd-CD2344⁻*) in minimal media (MM) containing 1% glucose (left), water (center), or 0.1% succinate (right). OD_{600} measurements were taken every 30 minutes (n=3/group). Representative of at least 3 replicates.

b. Concentrations of butyrate produced after growth of *Cd* or *Cd-CD2344⁻* in minimal media containing 0.5% glucose (MM + glucose) or 0.1% succinate (MM + succinate) (n=3/group).

c. Concentrations of succinate remaining after growth of *Cd* or *Cd-CD2344⁻* in minimal media containing 0.5% glucose (MM + glucose) or 0.1% succinate (MM + succinate) (n=3/group).

d. Levels of ^{13}C-butyrate produced by *Cd* or *Cd-CD2344⁻* in minimal media containing 0.5% glucose (MM + glucose) or 0.1% ^{13}C-succinate (MM + succinate) (n=3/group).

e. Levels of ^{13}C-succinate remaining after growth of *Cd* or *Cd-CD2344⁻* in minimal media containing 0.5% glucose (MM + glucose) or 0.1% ^{13}C-succinate (MM + succinate) (n=3/group).

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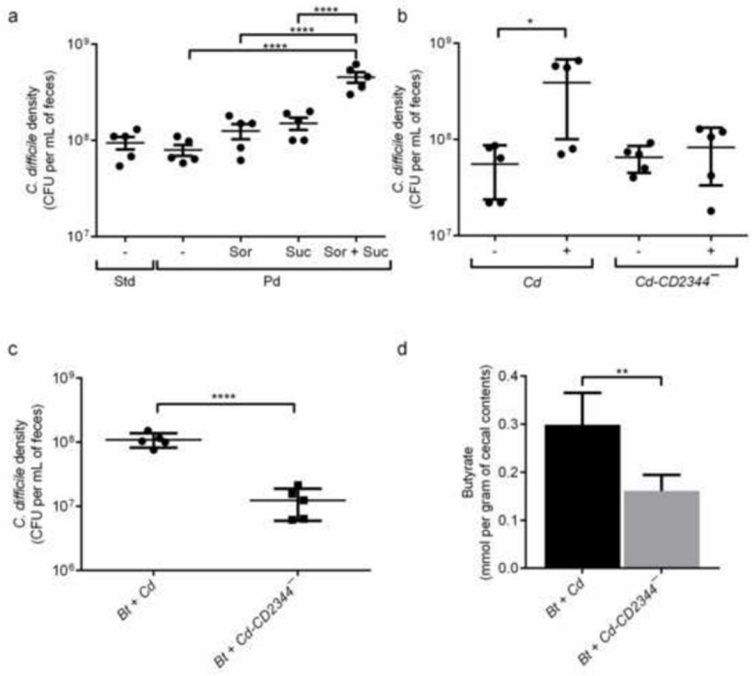

Figure 3. *C. difficile* consumes the *Bt* fermentation product succinate to expand in the intestinal ecosystem

a. *C. difficile* density in feces at 2 days post-infection of germ-free mice standard diet (Std) or polysaccharide deficient diet (Pd), supplemented with water containing 1% sorbitol (Sor), 1% succinate (Suc) or both 1% sorbitol and 1% succinate (Sor + Suc) (n=5/group). **** indicates statistical significance at an alpha value of <0.05 by one-way ANOVA with Bonferroni multiple comparisons test.

b. *C. difficile* density in feces of germ-free mice consuming polysaccharide-deficient diet and infected with wild-type *C. difficile* (*Cd*) or *Cd-CD2344*⁻ mutant *C. difficile* (*Cd-CD2344*⁻) and administered water (−) or water containing 1% sorbitol and 1% succinate (+) at 1 day post-infection (n=5/group).

c. *C. difficile* density in feces of *Bt*-associated mice infected with wild-type *C. difficile* (*Bt* + *Cd*) or *Cd-CD2344*⁻ mutant *C. difficile* (*Bt* + *Cd-CD2344*⁻) at day 1 post-infection and under standard dietary conditions (n=5/group).

d. Concentration of butyrate in cecal contents of *Bt*-associated mice infected with wild-type *C. difficile* (*Cd*) or *Cd-CD2344*⁻ mutant *C. difficile* (*Cd-CD2344*⁻) at day 3 post-infection and under standard dietary conditions (n=5/group).

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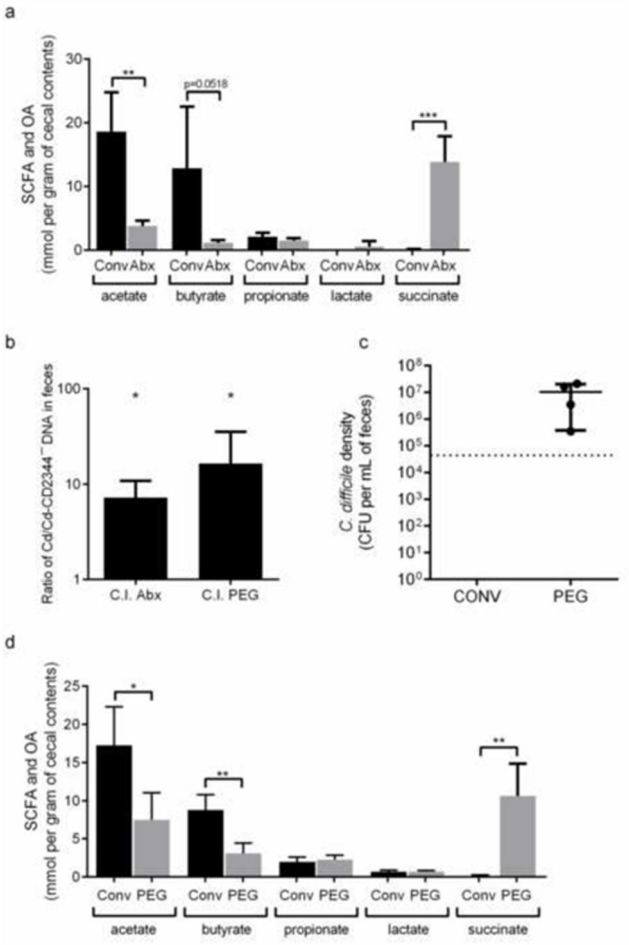

Figure 4. *C. difficile* exploits a spike in succinate availability after microbiota perturbations

a. Concentrations of acetate, butyrate, propionate, lactate and succinate detected in cecal contents of streptomycin-treated conventional mice before treatment (Conv) and 3 days after antibiotic treatment (Abx) (n=5/group).

b. DNA of wild-type *C. difficile* (*Cd*) versus *Cd-CD2344*⁻ mutant *C. difficile* (*Cd-CD2344*⁻) in feces of conventional mice treated with antibiotics (Abx) or PEG 3 days after infection with a 1:1 mixture of the two strains (n=5/group). *, $p<0.05$, one-sample t-test vs. a theoretical mean of 1.0.

c. *C. difficile* density in feces of conventional (Conv) or PEG-treated mice infected with wild-type *C. difficile* at day 3 post-infection (n=5 in Conv and n=4 in PEG). Dotted line represents the detection limit.

d. Concentrations of acetate, butyrate, propionate, lactate and succinate detected in cecal contents of PEG-treated conventional mice before treatment (Conv) and 3 days after treatment (PEG) (n=5/group).