

1 ***Clostridioides difficile* exploits toxin-mediated inflammation to alter the host nutritional**
2 **landscape and exclude competitors from the gut microbiota**

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27 **Introductory paragraph**

28 *Clostridioides difficile* is a bacterial pathogen that causes a range of clinical disease from mild to
29 moderate diarrhea, pseudomembranous colitis, and toxic megacolon. Typically, *C. difficile*
30 infections (CDIs) occur after antibiotic treatment, which alters the gut microbiota, decreasing
31 colonization resistance against *C. difficile*. Disease is mediated by two large toxins and the
32 expression of their genes is induced upon nutrient depletion via the alternative sigma factor
33 TcdR. Using *tcdR* mutants in two strains of *C. difficile*, we defined how toxin-induced
34 inflammation alters *C. difficile* metabolism, tissue gene expression, and the gut microbiota to
35 determine how inflammation by the host may be beneficial to *C. difficile*. Here we show that *C.*
36 *difficile* metabolism is significantly different in the face of inflammation, with changes in many
37 carbohydrate and amino acid uptake and utilization pathways. Host gene expression signatures
38 suggest that degradation of collagen and other components of the extracellular matrix by matrix
39 metalloproteinases is a major source of peptides and amino acids that supports *C. difficile* growth
40 *in vivo*. Lastly, the inflammation induced by *C. difficile* toxin activity alters the gut microbiota,
41 excluding members from the genus *Bacteroides* that are able to compete against *C. difficile* for
42 the same essential nutrients released from collagen degradation.

43 **Introduction**

44 *Clostridioides difficile* (*C. difficile*) is a Gram-positive anaerobic gut pathogen that causes
45 diarrhea, with severe cases resulting in significant morbidity and mortality¹. *C. difficile* produces
46 two large toxins, TcdA and TcdB, that glycosylate host Rho and Rac GTPases, leading to a
47 disruption in the actin cytoskeleton and loss of epithelial barrier integrity; the subsequent
48 apoptosis and tissue damage results in significant inflammation². Previous work revealed
49 inflammation can be beneficial for prominent enteric pathogens such as *Salmonella enterica* and

50 *Vibrio cholerae* by providing a metabolic niche for them in the gut. Whether inflammation can
51 benefit *C. difficile* is not clear, yet patients with Inflammatory bowel disease are four times more
52 likely to acquire *C. difficile* infection (CDI) compared to the general population, suggesting *C.*
53 *difficile* may thrive in an inflamed environment³⁻⁷. CDI-mediated inflammation results in drastic
54 shifts to the murine gut metabolome, with alterations in amino acid and peptide metabolite
55 concentrations, indicating that toxin activity induces an altered gut metabolic profile⁸. Although
56 a nutritional generalist, *C. difficile* is auxotrophic and requires multiple amino acids, including the
57 branched chain amino acids and proline that are used in Stickland metabolism for ATP
58 production and regeneration of NAD⁺; thus, *C. difficile* must acquire these nutrients from its
59 environment⁹⁻¹². We therefore hypothesized that *C. difficile* gains access to these nutrients by
60 exploiting the host inflammatory response. We hypothesized that toxin-mediated inflammation
61 alters the host gut environment to benefit *C. difficile* growth and persistence, either through
62 nutrient availability and/or the composition of the gut microbiota, potentially excluding
63 competitors or selecting for allies^{13,14}.

64 We addressed this hypothesis by taking a holistic approach to define the response of the
65 pathogen, the host, and the gut microbiota in the face of inflammation induced by *C. difficile*
66 toxins. Here we show that the toxin producing strain (wild type *C. difficile*) induces a unique *C.*
67 *difficile* transcriptomic signature compared to the toxin null strain (isogenic *tcdR* mutant),
68 indicating that inflammation shapes *C. difficile* metabolism *in vivo*. *C. difficile* transcripts for
69 carbohydrate and branched chain amino acid metabolism genes were increased in response to
70 toxin-induced inflammation, which is a reflection of the nutrients available in the inflamed gut.
71 Host tissue extracellular matrix (ECM)-degrading matrix metalloproteinase (MMP) transcripts,
72 encoding enzymes responsible for breaking down amino acid rich collagen, were also increased
73 in expression during peak inflammation. Additionally, we show that toxin activity leads to a
74 reduction and reorganization in collagen around cells *in vitro*, which provided *C. difficile* a

75 mechanism to acquire essential Stickland reaction substrates, supporting growth. Colonization
76 with toxin producing *C. difficile* also led to alterations in the gut microbial community structure,
77 with inflammation suppressing the return of members from the Bacteroidaceae Family. Our
78 results were conserved across different strains, as toxin activity of the epidemic *C. difficile*
79 R20291 strain elicits similar responses in a mouse model, suggesting that these effects may be
80 conserved across toxigenic *C. difficile* strains from phylogenetically distinct backgrounds.

81 **Materials and methods**

82 **Bacterial strains, growth conditions, and mutagenesis.** *C. difficile* strains 630 Δ *erm* and an
83 isogenic *tcdR::ermB* ClosTron insertion mutant (both kindly gifted by Rita Tamayo), as well as
84 the R20291 strain and its isogenic Δ *tcdR* mutant were routinely grown in and on Brain Heart
85 Infusion (BHI) or Tryptone Yeast (TY) broth and agar; plates and cultures were grown at 37°C
86 in an anaerobic chamber (Coy). For genetic manipulation of *C. difficile*, strains were grown on
87 and in BHI agar and broth supplemented as necessary with 10 μ g/ml of thiamphenicol, 50 μ g/ml
88 of kanamycin, and 16 μ g/ml of cefoxitin to select for transconjugants or thiamphenicol alone for
89 plasmid maintenance. Samples derived from *in vivo* studies were plated onto CCFA (cefloxitin,
90 cycloserine, fructose agar) to select for and enumerate vegetative *C. difficile* CFUs, and TCCFA,
91 containing the germinant taurocholate to enumerate spore CFUs.

92 The R20291 Δ *pyrE* strain was used to construct the Δ *tcdR* mutant¹⁵. Briefly, ~1.2 kb
93 upstream and downstream of the *tcdR* gene were PCR amplified with Phusion High-Fidelity DNA
94 polymerase (NEB M0530S). All primers used for construction of the mutagenesis vector and for
95 PCR screening of transconjugants can be found in Supplemental Table 1. The homology arms
96 were then combined into one single linear fragment via splice overlap extension PCR, then A-
97 tailed with Taq polymerase (NEB M0267S). The A-tailed product was ligated into pCR2.1
98 (Thermo Fisher K202020); the resulting plasmid was digested with BamHI and KpnI and the

99 ~2.4 kb homology arm fragment was ligated into the corresponding sites in pMTL_YN4 using T4
100 DNA Ligase (NEB M0202S). The final plasmid was conjugated into the R20291 Δ *pyrE* strain
101 with *E. coli* SD46, and thiamphenicol resistant large colony variants were screened by PCR for
102 plasmid integration into the chromosome. After confirming plasmid integration, colonies were
103 grown in BHI broth overnight in the absence of selection to allow for plasmid excision, then plated
104 onto minimal media agar supplemented with 5 μ g/ml uracil and 2 mg/ml of 5-fluoroorotic acid to
105 select for bacteria that had excised and lost the mutagenesis vector. Individual colonies were re-
106 streaked twice on the same selective media, then PCR screened for loss of *tcdR*.

107 **Spore preparation.** Spores were prepared as in Edwards and McBride¹⁶. 500 μ L of mid-log
108 phase cultures was spread onto 70:30 agar plates and incubated at 37°C for 4 days, after which
109 time the plates were removed from the anaerobic chamber. The bacterial lawns were scraped
110 off and suspended in 10 mL sterile PBS, mixed 1:1 with 96% ethanol, vortexed vigorously for 30
111 sec, and allowed to sit at room temperature on the benchtop for 1 hr. The suspension was
112 centrifuged at 3,000 rpm for 10 min. The pellet was suspended in 10 ml fresh sterile PBS, and
113 centrifuged again; this was repeated twice. The final pellet was suspended in 1 ml PBS and
114 serial dilutions were plated on BHI agar with 0.1% of the germinant taurocholate for spore CFU
115 enumeration. Spore stocks were also enumerated one day prior to the day of challenge to
116 confirm spore stock CFUs prior to making the inocula for *in vivo* studies; inocula were also diluted
117 and plated the day of challenge.

118 **Mouse model of *C. difficile* infection.** C57BL/6J WT mice (5 to 8 weeks old; n=18 male and
119 n=18 female) were purchased from Jackson Labs. The mice were given 0.5 mg/mL
120 cefoperazone in their drinking water for five days to make them susceptible to *C. difficile*
121 infection^{17,18}. The mice were then given plain water for two days, after which time they (n=12,
122 males and females) received 10⁵ spores of either 630 Δ *erm* or *tcdR::ermB* via oral gavage. One

123 group of mice (n=12, males and females) received no *C. difficile* spores. Mice were weighed
124 daily and monitored for clinical signs of distress (ruffled fur, hunched posture, slow ambulation,
125 etc.). Fecal pellets were collected 1 and 3 days after challenge and diluted 1:10 w/v in sterile
126 PBS, then serially diluted in 96 well PCR plates and plated onto CCFA for vegetative *C. difficile*
127 CFU enumeration. The serially diluted samples were then removed from the anaerobic chamber
128 and heated to 65°C for 20 min to kill vegetative cells; the heat-treated dilution plate was passed
129 back into the anaerobic chamber and the dilutions were plated onto TCCFA to enumerate spore
130 CFUs.

131 At day 2 and 4 post challenge, mice were humanely sacrificed (n=6 per treatment), and
132 necropsy was performed. Cecal content was harvested for enumeration of vegetative *C. difficile*
133 and spore CFUs, as well as for RNA extraction and toxin activity. Cecal tissue was harvested
134 for RNA extraction for gene expression analysis, 16S rRNA sequencing, and histopathology.
135 Colon tissue was harvested for histopathology. Samples for sequencing and toxin activity were
136 immediately flash frozen in liquid nitrogen and stored at -80°C until processing. Toxin activity in
137 the cecal content was quantified using the Vero Cell cytotoxicity assay¹⁸. Briefly, the content was
138 diluted 1:10 w/v in sterile PBS, and 10-fold dilutions were added to Vero cells in a 96-well dish
139 for ~16 hr. The reciprocal of the lowest dilution in which ~80% of the cells have rounded was
140 reported as the titer.

141 The R20291 study was conducted similarly to the one described above with some minor
142 differences. C57BL/6J mice (5 to 8 weeks old, n=14 male and 14 female) were orally gavaged
143 with 10⁵ spores of R20291 or the $\Delta tcdR$ mutant (n=14 per strain). Weight and clinical signs of
144 distress were monitored daily. Fecal pellets were collected at 1, 2, and 4 days after challenge
145 and total *C. difficile* CFUs were enumerated on TCCFA agar; samples were then heat-treated to
146 kill vegetative cells for spore CFU enumeration. Necropsy was performed 2 and 4 days after
147 challenge, and cecal tissue was harvested for 16S rRNA sequencing (n=5 per group on day 4

148 post challenge); cecal tissue RNA was isolated from mice (n=3 per group) on day 2 for gene
149 expression analysis via NanoString.

150 **Histopathological examination of the mouse cecum and colon.** At the time of necropsy,
151 tissue from the cecum and colon were prepared for histology by placing the intact tissue into
152 histology cassettes and stored in 10% buffered formalin for 48 hr at room temperature, then
153 transferred to 70% ethyl alcohol for long term storage. Tissues were processed, paraffin
154 embedded, sectioned at 4 μ m thickness, and hematoxylin and eosin stained for
155 histopathological examination (University of North Carolina Animal Histopathology & Lab
156 Medicine core). Histological specimens were randomized and scored in a blinded manner by a
157 board-certified veterinary pathologist (SM). Edema, inflammation (cellular infiltration), and
158 epithelial damage for the cecum and colon were each scored 0-4 based on a previously
159 published numerical scoring scheme¹⁷. Edema scores were as follows: 0, no edema; 1, mild
160 edema with minimal (2x) multifocal submucosal expansion or a single focus of moderate (2-3x)
161 sub-mucosal expansion; 2, moderate edema with moderate (2–3x) multifocal sub-mucosal
162 expansion; 3, severe edema with severe (3x) multifocal sub-mucosal expansion; 4, same as
163 score 3 with diffuse sub-mucosal expansion. Cellular infiltration scores were as follows: 0, no
164 inflammation; 1, minimal multifocal neutrophilic inflammation of scattered cells that do not form
165 clusters; 2, moderate multifocal neutrophilic inflammation (greater submucosal involvement); 3,
166 severe multifocal to coalescing neutrophilic inflammation (greater submucosal \pm mural
167 involvement); 4, same as score 3 with abscesses or extensive mural involvement. Epithelial
168 damage was scored as follows: 0, no epithelial changes; 1, minimal multifocal superficial
169 epithelial damage (vacuolation, apoptotic figures, villus tip attenuation/necrosis); 2, moderate
170 multifocal superficial epithelial damage (vacuolation, apoptotic figures, villus tip
171 attenuation/necrosis); 3, severe multifocal epithelial damage (same as above) +/-
172 pseudomembrane (intraluminal neutrophils, sloughed epithelium in a fibrinous matrix); 4, same

173 as score 3 with significant pseudomembrane or epithelial ulceration (focal complete loss of
174 epithelium). Photomicrographs were captured on an Olympus BX43 light microscope with a
175 DP27 camera using cellSens Dimension software.

176 **RNA extraction from cecal tissue and cecal content.** RNA was extracted from cecal tissue
177 using the PureLink RNA Mini kit (Thermo Fisher, 12183025) following the manufacturer's
178 protocol. The RNA was treated with Turbo DNase (Thermo Fisher, AM2239); the protocol was
179 modified by increasing the amount of enzyme to 5 μ l per sample. After 30 min of incubation in a
180 water bath at 37°C, 2 μ l of Turbo DNase enzyme was added to each sample for a further 30 min
181 of incubation. The RNA was then column purified according to the manufacturer's instructions
182 (Zymo, R1019). PCR with primers specific to intron 1 and exon 3 from the mouse β -actin gene
183 were used to screen samples for genomic DNA after DNase treatment.

184 For extraction of RNA from cecal content for bacterial RNAseq, the content was thawed
185 on ice, then added to 10 mL of TRIzol Reagent (Thermo Fisher, 15596018) in a 15 mL conical.
186 The content was dispersed by vortexing for 10 sec, and then was given 20 min on the benchtop
187 to settle. The TRIzol-cecal content mix was then transferred in 1.2 mL aliquots to eight 1.7 mL
188 centrifuge tubes. 350 μ l of chloroform was added, and the tubes were vigorously inverted for 15
189 sec each, after which they were incubated at room temperature for 20 min. The samples were
190 centrifuged at 14,000 rpm at 4°C for 20 min. The aqueous phase (~650 μ l) was then added to
191 650 μ l of isopropanol that had been supplemented with 5 μ g/mL glycogen. Samples were
192 vortexed and incubated on ice for 20 min, and then were centrifuged at 4°C for 30 min. Pellets
193 were washed three times with 70% ethanol, and then dissolved in sterile deionized water. The
194 samples were then treated with Turbo DNase, with the same augmentation of the protocol that
195 was done for the cecal tissue RNA. For reasons that are unclear, the *in vivo* samples required
196 multiple rounds of Turbo DNase treatment to remove contaminating genomic DNA, resulting in

197 degradation of the RNA in some samples. After column purification, PCR was performed with
198 primers specific to *tcdA* and *rpoC* to confirm removal of genomic DNA.

199 **RNA extraction from *C. difficile* cultures *in vitro*.** Three independent colonies of 630 Δ *erm*
200 and *tcdR::ermB* each were inoculated into 5 ml of TY broth and grown overnight. These were
201 subcultured 1:500 in 5 ml fresh TY and allowed to grow for 18 hr at 37°C in the anaerobic
202 chamber. The cultures were centrifuged and supernatants were decanted. Pellets were
203 dissolved in 1 ml TRIzol Reagent for 20 min on the bench top, after which time 200 μ l of
204 chloroform was added and the cultures were vigorously inverted for 15 sec and incubated on the
205 benchtop for a further 20 min. The RNA was precipitated as described above, with ~500 μ l of
206 the aqueous phase added to 500 μ l of isopropanol with glycogen supplementation. The Turbo
207 DNase treatment as described above was performed once, and RNA was confirmed to be free
208 of genomic DNA with the aforementioned primer sets.

209 **RNA sequencing and transcriptome analysis.** Sequencing of RNA derived from cecal content
210 and *in vitro* cultures was performed at the Roy J. Carver Biotechnology Center at the University
211 of Illinois at Urbana-Champaign. Ribosomal RNA was removed from the samples using the
212 RiboZero Epidemiology Kit (Illumina). RNAseq libraries were prepped with the TruSeq Stranded
213 mRNA Sample Prep Kit (Illumina). Library quantification was done via qPCR, and the samples
214 were sequenced on one lane for 151 cycles from each end of the fragments on a NovaSeq 6000
215 using a NovaSeq S4 reagent kit. The FASTQ files were generated and demultiplexed using the
216 bcl2fastq v2.20 Conversion Software (Illumina). Raw paired Illumina reads were imported into
217 Geneious 10.2.6, where adapters and low-quality reads were removed using BBDuk with a Kmer
218 length of 27, a minimum base quality score of 30, and a minimum average read quality of 30¹⁹.
219 Reads less than 30 bases in length (and their paired read) were also discarded. The filtered
220 reads were mapped to the *C. difficile* 630 Δ *erm* genome (NCBI accession no. NC_009089.1)

221 using BMap with a Kmer length of 10 and no other changes to the default settings. Visual
222 inspection of the data indicated that the majority of reads from three wild type and three *tcdR*
223 samples, each from day 4, mapped to ribosomal RNA genes. These samples were excluded
224 from the analysis, but are included in the SRA submission. The average number of reads that
225 mapped from wild type (n=5) and the *tcdR* mutant (n=6) from day 2 were 7,395,921 and
226 22,768,296, respectively; average reads mapped from day 4 for wild type (n=3) and the *tcdR*
227 mutant (n=3) were 12,959,255 and 4,319,864, respectively. Differential expression analysis was
228 performed using DESeq2 with no changes to the default settings, and genes were considered
229 differentially expression if they had ± 1 log₂ fold change and an adjusted p value of <0.05.²⁰.
230 Gene set enrichment analysis of differentially expressed genes was performed using the GSEA-
231 Pro v3 program (<http://gseapro.molgenrug.nl>) with user defined cutoff values of -1 and 1. Bar plots
232 of enriched Gene Ontology (GO) terms and log₂ fold change values of individual transcripts were
233 generated in GraphPad Prism 8.

234 **Quantitative reverse transcription PCR.** RNA from cecal tissue, cecal content, and *in vitro*
235 bacterial cultures was used as template in reverse transcription reactions using the Murine
236 Moloney Leukemia Virus Reverse Transcriptase (NEB M0253S) following the manufacturer's
237 protocol. The resulting cDNA was diluted 1:5 in deionized water and used as template for
238 quantitative PCR with the SsoAdvanced Universal SYBR Green Supermix (Bio Rad).
239 Quantification of each gene assayed (Supplemental Table 1 for primers) was performed via
240 standard curve and copy number was determined by comparison to the housekeeping genes
241 *tbp* (TATA Binding Protein) for host genes and *rpoC* (RNA polymerase subunit beta) for *C.*
242 *difficile* genes.

243 **NanoString analysis.** RNA from cecal tissue was submitted to the Lineberger Comprehensive
244 Cancer Center Pre-Clinical Genomic Pathology Core at the University of North Carolina at
245 Chapel Hill for quantification of transcripts via NanoString technology²¹. The RNA was hybridized

246 to probes on the Mouse nCounter Immunology Panel, plus custom probes targeting mouse
247 *Mmps* and *Timps*. Raw data was imported into the nSolver Advanced Analysis software for data
248 normalization and differential expression analysis. One mouse (challenged with wild type, day 4
249 post challenge) was excluded after principal components analysis and hierarchical clustering of
250 the data identified it as an outlier with respect to all other samples. The data were normalized
251 and differential expression analysis was performed within the nSolver Advanced Analysis
252 software. Correction for multiple comparisons was performed using the method of Benjamini-
253 Hochberg. Heatmaps of the data were constructed in R using the ‘pheatmap’ package
254 (<https://cran.r-project.org/web/packages/pheatmap/index.html>) and volcano plots were
255 constructed in R with the ‘EnhancedVolcano’ package²². Gene set enrichment analysis was
256 performed using the WebGestalt server (<http://www.webgestalt.org>) with the following changes
257 to the default parameters: the minimum number of genes required for a pathway was lowered to
258 5 and the False Discovery Rate was adjusted to 0.1. Enriched pathways were visualized in Prism
259 8. Cecal tissue RNA from mice infected with R20291 and the $\Delta tcdR$ mutant was also used in a
260 separate run with the NanoString Mouse nCounter Inflammation panel customized to include
261 code sets for the *Mmps* and *Timps*. The data was analyzed identically in the nSolver Advanced
262 program as described above.

263 **Confocal microscopy.** IMR90 human fibroblasts were cultured in Eagle’s Minimum Essential
264 Medium (EMEM) (ATCC, USA) supplemented with 10% fetal bovine serum at 37 °C with 5%
265 CO₂. Cells were seeded on glass coverslips in 24-well plates for 3 days, followed by incubation
266 with 0.5 pM of TcdA and TcdB. After 12 hr or 15 hr, cells were fixed in PBS with 4%
267 paraformaldehyde for 20 min at room temperature and blocked in 10% normal goat serum
268 (Sigma). Collagen was detected using a mix of antibodies against collagen types I, III, and V
269 (Santa Cruz) in a 1:1:1 ratio, and Alexa Fluor 568-conjugated goat anti-mouse secondary
270 antibody (ThermoFisher). Glass coverslips were mounted using VECTASHEIELD mounting

271 media with DAPI (Vector Laboratories). Confocal imaging was performed on Zeiss LSM 880
272 confocal microscope using a 40× Plan-Apochromat objective lens (numerical aperture of 1.4)
273 and operated with ZEN software (Carl Zeiss, Inc).

274 ***C. difficile* growth in defined minimal media supplemented with heat-degraded collagen.**

275 Heat-degraded collagen was generated by heating type I collagen (Advanced BioMatrix) at
276 100°C for 4 hr. Degraded collagen was then concentrated and the pH was adjusted to 7.0. *C.*
277 *difficile* was grown in a well-established defined minimal media (CDMM), from which proline was
278 omitted and 0.5 mg of heat-degraded collagen was substituted. The media was passed into the
279 anaerobic chamber and allowed to reduce for 24 hr before inoculation. At the same time,
280 individual colonies of *C. difficile* were inoculated into TY media for overnight growth, after which
281 they were subcultured 1:100 into fresh TY. After 4 hr of growth, the cultures were centrifuged in
282 a microcentrifuge in the anaerobic chamber and washed three times in 1 ml sterile PBS, then
283 inoculated 1:500 into the CDMM. Immediately after inoculation, the cultures were serially diluted
284 and plated onto BHI agar for enumeration of *C. difficile* at 0 hr and 24 hr time points. Growth was
285 calculated from 4 independent experiments.

286 ***C. difficile* and *Bacteroides* growth in defined minimal media supplemented with Pro-Gly
287 or Gly-Pro dipeptides, and other collagen degradation substrates.**

288 *C. difficile* to acquire the essential amino acid proline from dipeptides, we utilized a well-
289 established defined minimal media (CDMM), from which proline was omitted and Proline-Glycine
290 or Glycine-Proline dipeptides were substituted. The media was passed into the anaerobic
291 chamber and allowed to reduce for 24 hr before inoculation. At the same time, individual colonies
292 of *C. difficile* were inoculated into TY media for overnight growth, after which they were
293 subcultured 1:100 into fresh TY for 4 hr. After the 4 hr growth, the cultures were centrifuged in a
294 microcentrifuge in the anaerobic chamber and washed three times in 1 ml sterile PBS, then
295 inoculated 1:500 into the CDMM. Immediately after inoculation, the cultures were serially diluted

296 and plated onto BHI agar for enumeration of *C. difficile* at the 0 hr time point. 24 hr later, the
297 cultures were serially diluted and plated for CFU enumeration again. Growth was calculated from
298 3 independent experiments.

299 *Bacteroides thetaiotaomicron* VPI-5482 and *Bacteroides fragilis* Bf NCTC 9343 were
300 cultured anaerobically at 37°C from glycerol stocks into tryptone-yeast extract-glucose (TYG)
301 medium and grown overnight²³. Cultures were back diluted to an OD 600 nm of ~0.1 the next
302 day into minimal media (MM) containing 0.25% glucose, proline, or hydroxyproline²⁴. CFUs
303 were enumerated on BHI-blood agar plates at 0 hr and after 16 hr of growth. Fold change in
304 growth was calculated from 3 independent experiments.

305 **16S rRNA Illumina sequencing and microbiome analysis.** DNA was isolated from cecal snips
306 at the University of Michigan Microbial Systems Molecular Biology Laboratory. The Mag Attract
307 Power Microbiome kit (Mo Bio Laboratories, Inc.) was used to isolate DNA from cecal snips.
308 Dual-indexing sequencing approach was used to amplify the V4 region of the 16S rRNA gene.
309 Each PCR mixture contained 2 µl of 10X Accuprime PCR buffer II (Life Technologies, CA, USA),
310 0.15 µl of Accuprime high-fidelity *polymerase* (Life Technologies, CA, USA), 5 µl of a 4.0 µM
311 primer set, 1 µl DNA, and 11.85 µl sterile nuclease free water. The template DNA concentration
312 was 1 to 10 ng/µl for a high bacterial DNA/host DNA ratio. The PCR conditions were as follows:
313 2 min at 95°C, followed by 30 cycles of 95°C for 20 sec, 55°C for 15 sec, and 72°C for 5 min,
314 followed by 72°C for 10 min. Libraries were normalized using a Life Technologies SequelPrep
315 normalization plate kit as per manufacturer's instructions for sequential elution. The
316 concentration of the pooled samples was determined using the Kapa Biosystems library
317 quantification kit for Illumina platforms (Kapa Biosystems, MA, USA). Agilent Bioanalyzer high-
318 sensitivity DNA analysis kit (Agilent CA, USA) was used to determine the sizes of the amplicons
319 in the library. The final library consisted of equal molar amounts from each of the plates,
320 normalized to the pooled plate at the lowest concentration. Sequencing was done on the Illumina

321 MiSeq platform, using a MiSeq reagent kit V2 (Illumina, CA, USA) with 500 cycles according to
322 the manufacturer's instructions, with modifications²⁵. Sequencing libraries were prepared
323 according to Illumina's protocol for preparing libraries for sequencing on the MiSeq (Illumina, CA,
324 USA) for 2 or 4 nM libraries. PhiX and genomes were added in 16S amplicon sequencing to add
325 diversity. Sequencing reagents were prepared according to the Schloss SOP
326 (https://www.mothur.org/wiki/MiSeq_SOP#Getting_started), and custom read 1, read 2 and
327 index primers were added to the reagent cartridge. FASTQ files were generated for paired end
328 reads.

329 Raw reads were processed in QIIME2, with DADA2 used for de-noising and generating
330 amplicon sequence variants (ASVs)^{26,27}. Taxonomic assignment of the ASVs was done using
331 the Silva reference database (silva-132-99-nb-classifier.qza)²⁸. The code used to process the
332 reads can be found in Supplemental File 7 for 630 Δ *erm* and Supplemental File 8 for R20291.
333 Percent relative abundances of Family level ASVs were calculated for each sample in Excel,
334 averaged across treatment groups, and visualized in GraphPad Prism 8.

335 Principal components analysis (PCA) (Fig. 5b) was performed in the R statistical
336 programming environment (<https://www.r-project.org>). ASVs from the V4 region that differ by just
337 one base pair may come from the different 16S copies in the same genome²⁹. We therefore
338 aggregated ASVs into 99%-identity OTUs using complete-linkage clustering on the Levenshtein
339 edit distances between ASV sequences. Principal components analysis was then performed on
340 Hellinger-transformed OTU abundances³⁰ and the first two principal components plotted with
341 sample scaling (scaling 1). The R code can be found in Supplemental File 9. The essential R
342 packages used were biomformat, Biostrings, phyloseq, vegan, ggplot2, and data importing and
343 manipulation packages from the tidyverse package collection^{31,32}.

344 **Statistical analysis.** With the exception of the RNAseq analysis, all statistical tests were
345 performed in GraphPad Prism 8. Kruskal-Wallis One-Way ANOVA with Dunn's correction for

346 multiple comparisons was used to test for significance when comparing *C. difficile* CFUs, mouse
347 weights, and cecal toxin activity. A mixed effects model with Tukey's multiple comparison's test
348 was used to test for significance on qRT-PCR data. Histopathology summary scores were tested
349 for significance using a Geissner-Greenhouse corrected ordinary Two-Way ANOVA with Tukey's
350 multiple comparisons test. A Mann-Whitney test was used to compare the fluorescence
351 quantification of IMR90 cells treated with vehicle or toxins. DESeq2 identified statistically
352 significant differentially expressed genes in the RNAseq study. A p-value of < 0.05 was
353 considered statistically significant, with *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

354 **Results**

355 **Wild type *C. difficile* induces significantly more inflammation and tissue damage than a**
356 ***tcdR* mutant.** Antibiotic treated mice were challenged with 10^5 spores of *C. difficile* 630 Δ *erm*
357 (wild type, or wild type mice hereafter) or an isogenic *tcdR::ermB* (*tcdR*, or *tcdR* mice) mutant
358 on day 0 and clinical signs of disease were monitored for four days post challenge (Fig. 1a).
359 Mutation of *tcdR* has been reported to significantly reduce levels of both toxin gene expression
360 and toxin protein production *in vitro*^{33,34}. Nearly five-fold fewer *tcdR* vegetative cells were
361 recovered in the feces relative to wild type at day 1 (p=0.0314, Kruskal-Wallis with Dunn's
362 correction for multiple comparisons); however, there was no difference by day 3, nor were
363 significant differences detected in fecal spores between the two strains at either day (Fig. 1b and
364 1c). Cecal content from day 2 and 4 did not harbor significantly different vegetative cells or
365 spores, however nearly 10^5 -fold more toxin activity was detected in wild type mice compared to
366 *tcdR* mice at both days, indicating that the *tcdR* mutant behaves similarly *in vivo* as it does *in*
367 *vitro* with respect to toxin production (Fig. S1a-c, Fig. 1d). Accordingly, histopathological analysis
368 of cecal tissue found significantly increased inflammation in wild type mice when compared to
369 uninfected controls (no *C. difficile*) at day 2 (p=0.006; Two-Way ANOVA with Tukey's multiple
370 comparisons test), as well as significantly more epithelial damage when compared to both

371 uninfected controls and *tcdR* mice at day 4 ($p=0.024$ for both; Two-Way ANOVA with Tukey's
372 multiple comparisons test) (Fig. 1e-f). While cecal inflammation, epithelial damage, and edema
373 were lower in *tcdR* mice at day 2 relative to wild type mice, it was not statistically significant.
374 Tissue damage in wild type mice was even more pronounced in colonic tissue (Fig. S1d).
375 Together, these data show that the *tcdR* mutant fails to produce much detectable toxin activity
376 *in vivo*, and consequently does not elicit significant inflammatory damage to host gut tissue.

377 **Toxin-mediated inflammation significantly alters the *C. difficile* transcriptome *in vivo*.** As
378 colonization with wild type *C. difficile* leads to significant increases in inflammation and damage
379 to the cecal epithelium, we hypothesized that *C. difficile* would shift its transcriptome to reflect
380 such dramatic differences in the inflammatory environment³⁵. However, a *tcdR* mutant in the
381 R20291 strain has numerous differentially expressed genes *in vitro*³⁴. To assess whether the
382 *tcdR* mutation is pleiotropic in 630 Δ *erm*, we performed RNAseq on wild type and the *tcdR* mutant
383 grown for 18 hours in TY media as an *in vitro* control. We found that other than the genes of the
384 Pathogenicity Locus (PaLoc), only two genes were differentially expressed (\log_2 fold change ± 1
385 and adjusted p value < 0.05) between the strains *in vitro* (*CD1917*, encoding *eutE*, and *CD3087*,
386 encoding a transcription factor of the RpiR family) (Supplemental File 3). When comparing *in*
387 *vivo* expression profiles between the two strains, the majority of differentially expressed genes
388 were detected at day 2, with 86 transcripts increased and 82 decreased in wild type relative to
389 *tcdR* (Supplemental File 3). After four days, wild type had 15 transcripts increased and 4
390 decreased. Consistent with the cecal content toxin activity assay, among the most significantly
391 increased transcripts in wild type relative to the *tcdR* mutant were of the PaLoc. Interestingly,
392 time was a more important variable with respect to the number of differentially expressed genes
393 *in vivo* in both wild type and the *tcdR* mutant. When comparing wild type at day 4 to wild type at
394 day 2, 249 genes had increased transcript levels and 155 decreased; the *tcdR* mutant at day 4
395 relative to day 2 had even more dramatic changes in gene expression, with 380 transcripts

396 increased and 338 decreased. A subset of genes identified as differentially expressed between
397 wild type and the *tcdR* mutant were selected for quantitative real-time PCR (qRT-PCR)
398 validation, which confirmed trends in expression from the RNAseq (Fig. S3a-d). Together, these
399 data show that inflammation is an important environmental determinant of the *C. difficile*
400 transcriptome *in vivo*.

401 **Toxin-induced inflammation alters *C. difficile* metabolism.** We used prokaryotic gene set
402 enrichment analysis to summarize the main patterns in differential gene expression (Fig. 2a, Fig.
403 S2, and Supplemental File 4). Gene Ontology (GO) terms related to carbohydrate metabolism
404 were enriched in wild type relative to *tcdR* at both days post challenge, suggesting that wild type
405 *C. difficile* may have had access to different carbohydrate nutrient sources. An operon encoding
406 a phosphoenolpyruvate:carbohydrate phosphotransferase (PTS) system annotated to be
407 specific to mannose/fructose/sorbose was significantly increased in wild type *C. difficile* at day
408 2 (Fig. 2b). PTS systems are typically induced by the presence of the carbohydrate that they
409 import (and absence of a repressing carbohydrate) via a transcriptional antitermination
410 mechanism³⁶. Expression of this operon normalized between wild type and the *tcdR* mutant by
411 day 4, though at this time point the wild type had increased expression of genes predicted to be
412 involved in fructose/mannitol and tagatose metabolism, as well as genes involved in extracellular
413 polysaccharide production (Fig. 2b).

414 Among the most abundant GO terms in the set of transcripts that were decreased in wild
415 type were those for oxidation-reduction and catalytic processes, as well as those for leucine
416 biosynthesis. The transcript levels for *ilvD*, involved in isoleucine and valine biosynthesis, were
417 also decreased in wild type relative to *tcdR* (Fig. 2b). Both the *leu* operon and *ilvD* in *C. difficile*
418 are transcriptionally repressed by CodY, whose repressive activity is high when bound by
419 branched chain amino acids and/or GTP^{37,38}. Similar patterns of decreased expression in wild
420 type were seen in the CodY-regulated operon encoding the machinery for metabolism of

421 succinate to butyrate (Fig. 2b). CodY has also been shown to positively regulate the expression
422 of some genes *in vitro*, including *pflB*³⁸. We found that *pflB* expression *in vivo* was increased at
423 day 2 in wild type *C. difficile* (Fig. 2b). These data, when combined with previous metabolomic
424 studies, suggest that the metabolomic environment of the inflamed gut may be enriched for
425 specific carbohydrates and oxidative Stickland reaction substrates in the form of branched chain
426 amino acids.

427 Wild type *C. difficile* also had increased transcript levels of a number of genes involved in
428 amino acid acquisition and metabolism compared to *tcdR* *in vivo*. One such gene, *CD3442*,
429 encodes a putative M24 family Xaa-Pro prolidase. Eukaryotic prolidases are intimately linked to
430 collagen metabolism, while prokaryotic prolidases are often involved in protein turnover and
431 proline recycling^{39,40}. In contrast, the gene encoding 4-hydroxyphenylacetate decarboxylase
432 (*hpdB*) was decreased in wild type at day 4; the transcript from the *hpdC* gene immediately
433 downstream was also decreased ($p=0.08$) (Fig. 2b). HpdB is involved in the fermentation of
434 tyrosine to p-cresol, which has been shown to affect fitness *in vivo* in a murine relapse model
435 and to modulate gut microbial community structure; the decreased *hpdB* transcript levels we
436 observed may be consistent with lower levels of its substrate in the ceca of wild type mice⁴¹⁻⁴³.

437 **Gene expression for multiple aspects of *C. difficile* physiology is altered in the presence**
438 **of inflammation.** Multiple genes encoding structural components of the flagella, including some
439 that have been shown to induce inflammatory responses from host cells *in vitro* and *in vivo*, were
440 decreased in expression in wild type (Fig. 2b). In contrast to flagellar genes, wild type had
441 increased expression of the *cdd* operon, comprised of three genes (*cdd4*, *cdd3*, and *cdd2*) that
442 are divergently transcribed from a two-component system (TCS) response regulator and
443 histidine kinase (Fig. 2b). The *cdd* genes are annotated to encode the components of a
444 multidrug/antibiotic ABC transport system, and given their genomic association with a TCS, it is
445 tempting to speculate that it may encode an undescribed defense mechanism against

446 antimicrobial peptides that could function semi-analogously to the CprK-CprR/CprABC system
447 that has been described for *C. difficile*^{44,45}.

448 **Wild type *C. difficile* induces a robust inflammatory and proteolytic gene expression**
449 **profile in host gut tissue.** To determine how the host responds to *C. difficile* toxin-induced
450 inflammation we compared the cecal tissue gene expression between three groups of mice
451 (uninfected or no *C. diff*, wild type, and *tcdR*) with the NanoString Mouse Immunology panel
452 modified to include probes targeting transcripts encoding matrix metalloproteinases (MMPs) and
453 tissue inhibitors of metalloproteinases (TIMPs) (Fig. 3, Fig. S4-S6, and Supplemental File 1).
454 The top 50 differentially expressed transcripts (in terms of significance) from the wild type relative
455 to *tcdR* comparisons from both days were combined and plotted in a heatmap with hierarchical
456 clustering of samples, and two distinct clusters were observed (Fig. 3a). All samples from *tcdR*
457 mice and uninfected controls formed one large cluster, while all wild type mice formed their own
458 distinct cluster; neither cluster showed sub-clustering based on time points. Only 42% of the
459 differentially expressed genes in wild type mice relative to *tcdR* mice were significant at both day
460 2 and 4, suggesting that the nature of the immune response to wild type *C. difficile* changed over
461 the course of infection (Fig. S5). In contrast to wild type mice, *tcdR* mice had no significant
462 differentially expressed genes in their cecal tissue when compared to uninfected controls (data
463 not shown). A number of transcripts were selected for further analysis via qRT-PCR, which
464 confirmed expression patterns observed via the NanoString approach (Fig.S6a-e). These data
465 are consistent with the histopathological analysis, and show that in the absence of toxin activity,
466 the *tcdR* mutant is relatively inert *in vivo* with respect to stimulating a host immune response.

467 We next performed gene set enrichment analysis for each group of differentially
468 expressed genes, using GO biological process terms and considering the direction of expression
469 for each transcript (Fig. 3b and Supplemental File 2). The GO term for regulation of inflammatory
470 processes was enriched in transcripts with increased abundance in cecal tissue from wild type

471 mice at both days. The second most enriched at day 2 was regulation of peptide secretion,
472 consistent with the role of host-derived antimicrobial peptides being produced as an arm of the
473 innate immune response. Of particular interest was the enrichment of genes involved in the
474 positive regulation of proteolysis, as peptide fragments derived from these processes may serve
475 as nutrient sources for *C. difficile*, which is well-known for using amino acid fermentation as an
476 energy source *in vitro* and *in vivo*^{8,9,35,46–50}. In addition to the upstream regulators of various
477 proteolytic processes, cecal tissue from wild type mice had significant increases in transcripts
478 from genes encoding multiple MMPs including *Mmp3*, *Mmp10*, *Mmp12*, and *Mmp13*; probes
479 targeting *Mmp3* were not included in the NanoString custom panel, so fold change for this
480 transcript is reported based on qRT-PCR results (Fig. 3c, Fig S6c). Taken together, these data
481 show that *C. difficile* toxin activity induces a highly inflammatory gut environment, and implicate
482 MMP substrates, such as collagen and other ECM components, as reservoirs of Stickland
483 substrate amino acids *in vivo*.

484 **Toxin activity induces collagen degradation which supports *C. difficile* growth *in vitro*.**

485 The increase in numerous *Mmp* transcripts during toxin-induced inflammation suggested the
486 ECM may be altered during CDI. We chose to examine whether toxin activity on cells *in vitro*
487 affected collagen integrity, as it is highly abundant and an excellent source of proline,
488 hydroxyproline, glycine, and alanine, all of which are amino acids that *C. difficile* can ferment via
489 the Stickland reaction¹¹. Confluent ECM-producing IMR90 human fibroblast monolayers were
490 cultured on 24-well plates for three days prior to toxin treatment. Collagen remodeling was then
491 visualized using immunofluorescence (Fig. 4a). By confocal microscopy, we observed toxins
492 caused a notable disruption of the collagen network over a 12 hour period. Extensive networks
493 of collagen fibrils were apparent in untreated cells, whereas collagen in toxin-treated cells
494 appeared fragmented and condensed into globular structures. Moreover, we observed a

495 significant decrease in collagen fluorescence over a 15 hour period, validating that collagen was
496 being degraded in the presence of toxins (Mann-Whitney test $p < 0.0001$) (Fig. 4b).

497 Given that toxins induced the degradation of collagen in IMR90 cells, we next speculated
498 that *C. difficile* can acquire nutrients from degraded collagen. To test this, *C. difficile* was grown
499 in a minimal media with proline, without proline, or without proline and supplemented with heat-
500 degraded collagen for 24 hours (Fig. 4c). Although not statistically significant ($p = 0.233$, Kruskal
501 Wallis one-way ANOVA), *C. difficile* grew approximately ten-fold higher in media supplemented
502 with degraded collagen compared to media with no proline, indicating collagen can provide a
503 source of proline for *C. difficile* growth. Additionally, when host collagen rich dipeptides Pro-Gly
504 and Gly-Pro, were substituted for collagen degradation products, *C. difficile* grew to levels
505 comparable to the standard minimal media control after 24 hours ($p = 0.0015$ for both conditions
506 compared to their respective 0 hour time point CFUs, two-way ANOVA with Sidak's multiple
507 comparisons test) (Fig. 4d). This suggests that *C. difficile* can exploit host collagen degrading
508 activity as a means to acquire nutrients.

509 **Toxin-mediated inflammation suppresses the return of the Bacteroidaceae in the gut**
510 **microbiota.** Given the importance of the microbiota in rendering the gut an inhospitable
511 environment for *C. difficile*, we hypothesized that toxin-mediated inflammation may exclude or
512 suppress members of the gut microbiota that contribute to colonization resistance, and/or select
513 for microbes that may benefit *C. difficile* through further niche modification/preservation, or other
514 mechanisms like cross-feeding. We performed 16S rRNA amplicon sequencing on cecal DNA
515 from mice challenged with wild type, the *tcdR* mutant, and uninfected controls or no *C. diff*
516 (Supplemental File 5 for ASV relative abundances and taxonomy). As expected, a significant
517 driver of community similarity was *C. difficile* colonization status, however a number of mice from
518 the uninfected controls (no *C. diff*) and the *tcdR* mice had community structures with an
519 increased abundance of ASVs from the Akkermansaceae (at day 2) and Bacteroidaceae (both

520 days) Families, which were low or undetected in the cecal microbiota of wild type mice (Fig. 5a).
521 Additionally, a number of low abundance Family members, including the Coriobacterales,
522 Paenibacillaceae, and Burkholderiaceae, were present in either uninfected controls, *tcdR* mice,
523 or both, but undetected in the cecal microbiota of wild type mice (Supplemental File 5). Amplicon
524 sequence variants (ASVs) from the Staphylococcaceae Family were detected at relatively high
525 abundance in uninfected controls at both days, but were in low abundance or undetected in mice
526 challenged with either strain of *C. difficile* (Fig. 5a). ASVs generated by DADA2 were clustered
527 into operational taxonomic units (OTUs) at 99% sequence identity, and Hellinger-transformed
528 OTU abundances were analyzed by principal components analysis (PCA) to determine the
529 similarity of each cecal community (Fig. 5b). At day 2, both wild type mice and all but one of the
530 uninfected controls formed tight, distinct clusters, while *tcdR* mice showed no specific clustering.
531 By day 4, no group clustered very closely. Some wild type and *tcdR* mice community structures
532 were driven by ASVs from various Lachnospiraceae and Erysipelotrichaceae Families, while
533 community structures of other *tcdR* and uninfected mice were driven by ASVs from the
534 Bacteroidaceae, Akkermansaceae, and Staphylococcaceae Families. These data show that
535 colonization with *C. difficile* and toxin-mediated inflammation can significantly impact the return
536 of the gut microbial community structure.

537 The ASV from the Bacteroidaceae Family identified in this study classified as genus
538 *Bacteroides*. Since the Bacteroidaceae were more abundant in some mice in the absence of
539 inflammation, and hydroxyproline utilization genes are enriched in this Family, we sought to
540 characterize the growth of two representative members, *Bacteroides thetaiotaomicron* and
541 *Bacteroides fragilis*, in a minimal media with and without supplementation of collagen rich
542 substrates, proline or hydroxyproline (Fig. 5c-d)⁵¹. Supplementation of the minimal media with
543 either amino acid led to approximately ten-fold increased growth for both species over 16 hours
544 relative to un-supplemented media. Glucose was required for robust growth, however when

545 minimal media with glucose was supplemented with either amino acid there was almost two-fold
546 higher growth of *B. thetaiotaomicron* and *B. fragilis* relative to glucose alone, though this was
547 not statistically significant. This trend of increased growth in supplemented media indicates that
548 both proline and hydroxyproline can be utilized by members of the genus *Bacteroides*, and that
549 these amino acids may be valuable nutrients to compete for in the gut.

550 **Toxin-mediated alterations of host *Mmp* expression and the gut microbiota are conserved**
551 **in mice challenged with epidemic R20291 *C. difficile* strain.** As the wild type strain used in
552 this study (630 Δ *erm*) is a multi-passaged, erythromycin sensitive lab strain, we sought to
553 replicate our findings in the clinically relevant R20291 strain. A Δ *tcdR* mutant was constructed
554 via allelic replacement, and the parent strain was used to challenge antibiotic treated mice (Fig.
555 6). No differences in total fecal *C. difficile* load (vegetative cells + spore) were observed between
556 the strains at any day post challenge, however, significantly fewer Δ *tcdR* spores were recovered
557 from feces at day 2 ($p < 0.0001$, Kruskal-Wallis with Dunn's correction for multiple comparisons),
558 consistent with a previous report on a *tcdR* Clostron mutant in R20291 showing decreased
559 sporulation efficiency *in vitro* (Fig. 6a-b)³⁴. Similar to what was observed for 630 Δ *erm* and its
560 *tcdR::ermB* derivative, cecal content from mice challenged with wild type R20291 had
561 significantly higher toxin titers than that from Δ *tcdR* at both days (Fig. 6c). Importantly,
562 comparable patterns of increased expression of the same *Mmps* was observed in cecal tissue
563 from wild type R20291 mice relative to the Δ *tcdR* mice (Fig. 6d). Given the pleiotropic nature of
564 the *tcdR* mutation in the R20291 strain, we elected not to perform *C. difficile* gene expression
565 studies *in vivo*. 16S rRNA amplicon sequencing on cecal tissue isolated at day 4 showed similar
566 patterns in the microbial community structures as observed in mice challenged with the 630 Δ *erm*
567 strains. Wild type R20291 mice had very low or undetectable Bacteroidaceae ASVs, while Δ *tcdR*
568 mice had considerably higher levels (2% average relative abundance vs. 32%, respectively) (Fig.

569 6e and Supplemental File 6). The ASV designated as *Bacteroides* in this study is identical to the
570 *Bacteroides* ASV that was identified in the cecal communities of uninfected controls and *tcdR*
571 mice from the 630 Δ *erm* study in Fig. 5a-b. Collectively, these data show that induction of host
572 *Mmp* gene expression is a conserved component of the immune response to *C. difficile* toxin
573 activity, and that the decreased levels of Bacteroidaceae in the inflamed gut may be biologically
574 significant and an additional mechanism where *C. difficile* is able to exploit a niche and thrive
575 due to host inflammation.

576 **Discussion**

577 *C. difficile* is a major nosocomial pathogen and cases of CDI are beginning to be diagnosed in
578 individuals who lack the classic predisposing traits of recent antibiotic use or compromised
579 immune status⁵². While effective treatments exist for *C. difficile*, some patients require fecal
580 microbiota transplants (FMTs) to resolve their infections, highlighting the need to better
581 understand how this pathogen creates a niche for persistence in a host. CDI is highly
582 inflammatory; therefore, we leveraged the power of bacterial genetics with *tcdR* mutants in two
583 strains of *C. difficile* to define how toxin-mediated inflammation alters the gut environment *in*
584 *vivo*, with a focus on bacterial metabolism and the gut microbiome. We observed changes in
585 numerous *C. difficile* metabolic genes *in vivo*, consistent with the hypothesis that toxin-induced
586 inflammation alters the nutrient landscape in the host gut environment. In particular, we found
587 time-dependent increases and decreases in the expression of multiple PTS carbohydrate import
588 genes, and decreased expression of CodY-regulated genes for branched chain amino acid
589 biosynthesis and butyrate production in wild type *C. difficile*. On its face, this represents a
590 paradox: CodY de-repression of *tcdR*, *tcdA*, and *tcdB* leads to extremely high levels of the toxins,
591 yet in our study we found that a number of CodY regulated genes were repressed *in vivo* in the
592 presence of inflammation. Two possible mechanisms may explain this discrepancy: phase
593 variation in the expression of *sigD*, a positive regulator of *tcdR*, and bimodal expression of *tcdR*

594 and the toxin genes^{33,53}. Our transcriptomic approach captured average expression of genes
595 within large populations of bacteria in each sample, but cannot detect differences in expression
596 between individual cells. Future studies examining the per-cell expression levels of the CodY-
597 regulated genes identified in our RNAseq studies are warranted. Regardless, our RNAseq
598 approach has identified numerous targets for further study via mutagenesis with respect to *C.*
599 *difficile* metabolic requirements *in vivo*, or how it resists the deleterious effects of host
600 inflammation. Further, it highlights the need to fully understand the spectrum of behavior of
601 individual bacterial cells across a population during the infection process. This may open novel
602 avenues for therapeutic targeting of specific subsets of pathogens within a metabolically
603 heterogeneous population.

604 Our data support a model where the activity of the toxins stimulate an inflammatory host
605 response that includes gene expression signatures consistent with degradation of collagen and
606 other components of the ECM. Collagen is rich in Stickland reaction substrates like proline (and
607 hydroxyproline, which *C. difficile* can dehydrate to proline) and glycine, amongst other amino
608 acids that *C. difficile* can ferment^{10,51,54}. Hence, the ECM and collagen may serve as a reservoir
609 of preferred amino acid nutrients that sustain the metabolic burden of large bacterial populations
610 and production of the toxins over time within the host gut environment. Aberrant MMP activity
611 has been reported as a factor in the pathogenesis of Inflammatory bowel disease (IBD), and IBD
612 patients may be more likely to contract CDI than the non-IBD population, suggesting that ECM
613 remodeling could contribute to creating a niche in humans that *C. difficile* can more readily
614 colonize and thrive³⁻⁷. The gastrointestinal pathogens, *Salmonella enterica* and *Vibrio cholerae*,
615 have also been shown to benefit from inflamed host tissue, gaining access to nutrients that
616 increase their fitness as pathogens^{13,14,55-57}. Future studies are needed to determine the extent
617 to which MMP activity contributes to the peptides and amino acids that *C. difficile* has access to
618 in the inflamed gut.

619 Lastly, we show that the community structure of the gut microbiota is altered by the
620 presence of *C. difficile* and the activity of its toxins, supporting the hypothesis that inflammation
621 can benefit *C. difficile* by selecting against competitors and for potential allies. In particular, we
622 found that the Bacteroidaceae tended to bloom in the uninflamed ceca of antibiotic treated
623 uninfected controls, or mice challenged with two *tcdR* mutants from phylogenetically divergent
624 *C. difficile* strains⁵⁸. In the latter case, the experiments were conducted over a year apart,
625 suggesting that the inflammation induced by wild type *C. difficile* in our mouse model selects
626 against a Bacteroidaceae population expansion. In support of this, negative associations
627 between *C. difficile* and members of the Bacteroidaceae have been reported in human studies,
628 as well as *in vitro*, and in mouse models of CDI^{59–63}. Further work is necessary to identify which
629 mediator(s) of host inflammation are responsible for the restriction of the Bacteroidaceae in our
630 model of CDI. *B. thetaiotaomicron* has been shown to cross-feed *C. difficile* with succinate in a
631 co-colonization model and our RNAseq studies support this, in that *tcdR* mice colonized with the
632 Bacteroidaceae had increased expression of the succinate to butyrate operon. Interestingly,
633 homologs of the *C. difficile* hydroxyproline dehydratase gene, *hypD*, are enriched in the
634 Bacteroidaceae, and *C. difficile* can satisfy its proline requirements through utilization of
635 hydroxyproline *in vitro*⁵¹. Given the high levels of hydroxyproline in collagen, it is possible that
636 toxin-mediated exclusion or suppression of the Bacteroidaceae removes a competitor for a vital
637 nutrient. Additional studies are needed to determine if members of this Family produce
638 antagonistic effects against *C. difficile in vitro* and *in vivo*.

639 Use of a toxin null or *tcdR* mutant combined with omic technologies and *in vivo* models
640 represents a powerful approach for asking how *C. difficile* toxin-induced inflammation alters the
641 host gastrointestinal environment in ways that may create or preserve a niche during
642 colonization and disease. These results provide multiple avenues for future study of the basic
643 biology of CDI at the level of host response, pathogen response to inflammation, and

644 manipulation of the host gut microbiota. While it was outside the scope of this work, we think the
645 approach of querying the gut microbiota of mice colonized with wild type and mutant strains of
646 *C. difficile*, in particular mutants in key metabolic pathways, may be fruitful for identifying bacterial
647 taxa that bloom in the presence of a nutrient(s) that a mutant *C. difficile* population can no longer
648 use. This approach may, with enough mutants in important metabolic pathways, contribute to a
649 rationally designed consortium of bacteria that could compete with *C. difficile* for essential
650 nutrients in models of colonization and disease.

651 **Data availability.**

652 Raw sequences have been deposited in the Sequence Read Archive (SRA) with SRA
653 accession number SUB6663505 and BioProject ID PRJNA612095.

654 **Biological materials availability.**

655 Available upon request.

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665 **Author contributions.**

666 J.R.F., C.M.P., R.J.P., A.J.R., and M.H.F. performed the experiments. S.A.M. conducted blinded
667 histology scoring, imaging, and analysis of murine cecal and colonic tissue. J.R.F., C.M.P.,
668 M.H.F., and C.M.T. designed the experiments and J.R.F., C.M.P., M.H.F., and M.R.M. analyzed

669 and interpreted the data. J.R.F., C.M.P., M.H.F., M.R.M., and C.M.T. wrote the paper. All authors
670 edited the manuscript.

671 **Competing interests.**

672 C.M.T. is an advisor of Locus Biosciences and also consults for Vedanta Biosciences, Inc. and
673 Summit Therapeutics.

674 **Figure legends.**

675 **Fig. 1. Inflammation is attenuated in *tcdR* mice in a mouse model of *C. difficile* infection.**

676 A) Schematic depicting experimental design. All mice (n=36) received the antibiotic
677 cefoperazone in their drinking water. Subsets were orally gavaged with wild type (n=12) or *tcdR*
678 (n=12) after antibiotic treatment. B) *C. difficile* vegetative cell CFUs in feces (n=6-8 per strain).
679 C) *C. difficile* spore CFUs in the feces (n=6-8 per strain). D) Toxin activity in the cecal content of
680 mice (n=4-6). E) Histopathological summary scores of the cecum. F) Representative images of
681 H&E stained ceca; scale bar, 500 μ m. Kruskal-Wallis test with Dunn's correction for multiple
682 comparisons was used to test for statistical significance in B, C, and D. Geissner-Greenhouse
683 corrected ordinary Two-Way ANOVA with Tukey's multiple comparisons test was used in E. *p
684 < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

685 **Fig. 2. Metabolic gene expression in *C. difficile* is significantly altered by toxin-mediated**

686 **inflammation.** A) Gene set enrichment analysis of the differentially expressed genes *in vivo*
687 from wild type *C. difficile* relative to the *tcdR* mutant from both days 2 and 4. GO terms that had
688 transcripts with decreased levels are depicted in black bars and GO terms containing transcripts
689 with increased levels are shown as red bars. B) Heatmap of the log₂ fold change of key operons
690 and transcripts that were differentially expressed in wild type *C. difficile* (n=5 on day 2, n=3 on
691 day 4) relative to *tcdR* (n=6 on day 2, n=3 on day 4). The labels of known CodY regulated
692 transcripts are color-coded in red if they increased in expression in a *codY* mutant and green if
693 they decreased.

694 **Fig. 3. *C. difficile* induces expression of numerous transcripts associated with**
695 **inflammation and ECM degradation.** A) Heatmap of the top 50 differentially regulated
696 transcripts (by adj. p value) in the ceca of uninfected controls, wild type mice, and *tcdR* mice
697 (n=5-6 per treatment and time point). B) Gene set enrichment analysis of the differentially
698 expressed genes in wild type mice relative to *tcdR* mice. C) Log₂ fold changes of various *Mmps*
699 and associated transcripts from wild type vs. *tcdR* mouse ceca. Significance for all transcripts
700 except *Mmp3* was determined using differential expression analysis within the NanoString
701 nSolver Advanced analysis software. *Mmp3* expression levels were determined via qRT-PCR
702 on cDNA generated from the same RNA used in the NanoString analysis.

703 **Fig. 4. Toxin-mediated degradation of collagen supports *C. difficile* growth *in vitro*.** A)
704 Representative images of collagen (red) produced by IMR90 cells. Confluent cell monolayers
705 were treated with 0.5 pM TcdA and TcdB and images were collected 12 hours later. Collagen
706 was stained with a mix of antibodies against collagen types I, III and V in a 1:1:1 ratio; scale bar,
707 10 μm. B) Mean fluorescent intensity of Alexa Fluor 568 stained collagen produced by IMR90
708 cells cultured in the presence or absence of 0.5 pM TcdA and TcdB for 15 hours calculated using
709 ImageJ software. Statistical significance was determined by Mann-Whitney rank-sum test. C) *C.*
710 *difficile* was grown in complete CDMM, CDMM lacking proline, or CDMM lacking proline and
711 supplemented with heat-degraded collagen. CFUs/ml were enumerated at 0 and 24 hours. D)
712 Similar to the scheme in C, except that purified Pro-Gly or Gly-Pro dipeptides were added to
713 CDMM lacking proline. Statistical significance was determined by two-way ANOVA with Sidak's
714 multiple comparisons test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

715 **Fig. 5. *C. difficile* toxin activity suppresses the Bacteroidaceae that are able to compete**
716 **with *C. difficile* for amino acids.** A) Averaged percent relative abundance of Family-level ASVs
717 in each treatment group per timepoint. ASVs with less than 1% relative abundance in all samples
718 were not included. B) PCA biplot of 16S rRNA amplicon sequences derived from cecal tissue

719 from uninfected or no *C. diff* controls (n=5 on day 2, n=6 on day 4), and wild type mice (n=4 on
720 day 2, n=6 on day 4) or *tcdR* mice (n=5 on day 2, n=6 on day 4). Each colored symbol represents
721 an individual mouse's cecal microbiome, with circles being those from day 2 and triangles from
722 day 4. 99% of OTUs are shown as gray crosses; the 10 OTUs furthest from the origin are labeled
723 by the finest taxonomic rank identified (family, genus, or species). C) 16 hour fold change in
724 CFUs of *B. thetaiotaomicron* in minimal media with or without glucose, supplemented with either
725 proline or hydroxyproline. D) 16 hour fold change growth of *B. fragilis* in identical media
726 conditions as in C.

727 **Fig. 6. *C. difficile* R20291 toxin activity similarly shapes the host gut transcriptome and**
728 **microbiota community structure in mice.** A) Total *C. difficile* CFUs (vegetative and spores) in
729 feces over time (n=6-8 per strain). B) Fecal spore CFUs over time (n=6-8 per strain). C) Toxin
730 activity in the cecal content of R20291 or $\Delta tcdR$ mice, as assessed by the Vero cell cytotoxicity
731 assay (n=5 per strain on day 2, n=4 per strain on day 4). D) Log₂ fold change of *Mmp* and *Timp*
732 transcripts (n=3 per strain). E) Averaged percent relative abundances of 16S rRNA amplicon
733 sequences from cecal tissue isolated at day 4 (n=5 per strain). Kruskal-Wallis test with Dunn's
734 correction for multiple comparisons was used to test for statistical significance. *p < 0.05, **p <
735 0.01, ****p < 0.0001.

736 **Fig. S1. Extended data from the mouse model of *C. difficile* infection.** A) Mouse weights
737 over the course of the experiments (n=12 on day 2, n=6 on day 4). B) Vegetative cell CFUs in
738 the cecal content on day 2 and 4 (n=6 per strain). C) Spore CFUs in cecal content from B (n=6
739 per strain). D) Histopathological summary scores of the colon. Geissner-Greenhouse corrected
740 ordinary Two-Way ANOVA with Tukey's multiple comparisons test was used to test for
741 significance. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

742 **Fig. S2. *C. difficile* transcriptome shifts over the course of infection.** A) Enriched GO terms
743 in the differentially expressed genes in wild type *C. difficile* and B) *tcdR* at day 4 (n=3 per strain)

744 relative to day 2 (n=5-6 per strain). GO terms for transcripts with decreased or increased levels
745 are shown in black and red bars, respectively.

746 **Fig. S3. Validation of differentially expressed *C. difficile* transcripts by qRT-PCR. A-D)**

747 Expression was quantified from cDNA generated from the same RNA that was used in the
748 RNAseq experiment, including samples from *in vitro* *C. difficile* cultures.

749 **Fig. S4. Volcano plots depicting log₂ fold change in expression and the adjusted p-value**

750 **for mouse cecal tissue transcriptomes. A and B) Gene expression in wild type ceca relative**

751 **to *tcdR* ceca at days 2 and 4, respectively. C and D) Gene expression in wild type ceca relative**

752 **to ceca from uninfected, cefoperazone treated controls at 2 and 4 days, respectively. E and F)**

753 **Gene expression at day 4 relative to day 2 in wild type ceca and *tcdR* ceca, respectively. The**

754 **number of genes increased in expression are in red font and decreased are in green font.**

755 **Fig. S5. Shared and unique differentially expressed genes in ceca of wild type relative to**

756 ***tcdR* mice over time. A) Transcripts that increased in expression. B) Transcripts that**

757 **decreased in expression. Venn diagrams were created using Venny 2.1**

758 **(<https://bioinfogp.cnb.csic.es/tools/venny/>).**

759 **Fig. S6. Validation of transcripts from NanoString cecal tissue analysis by qRT-PCR. A-E)**

760 **Expression was quantified in cDNA generated from the same cecal RNA that was used in the**

761 **NanoString analysis. F) Unscaled heatmap depicting the log₂ fold change of each *Mmp* and**

762 ***Timp* in every differential expression analysis. The heatmap was created using the R package**

763 **pheatmap (<https://cran.r-project.org/web/packages/pheatmap/index.html>).** A mixed effects

764 **model with Tukey's multiple comparison's test was used to test for significance. *p < 0.05, **p <**

765 **0.01.**

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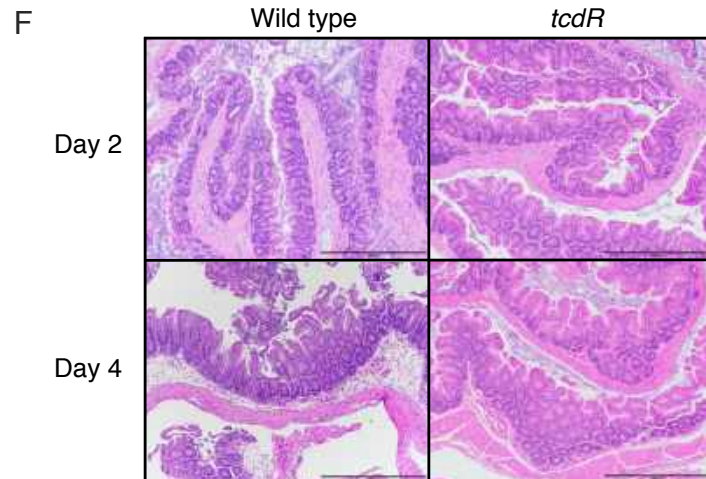
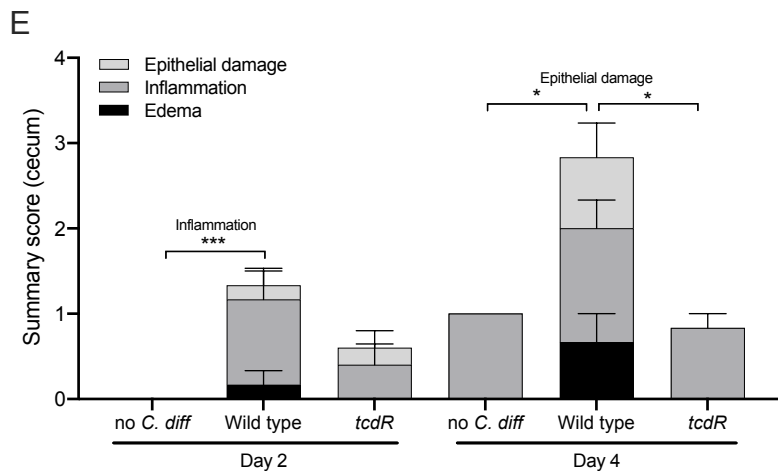
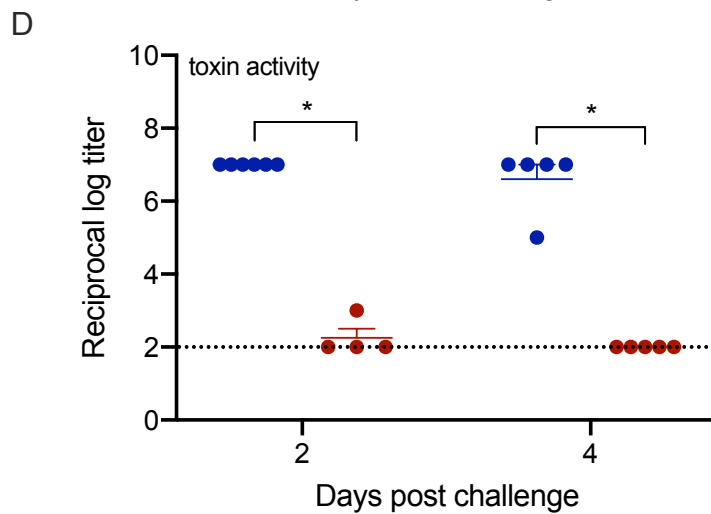
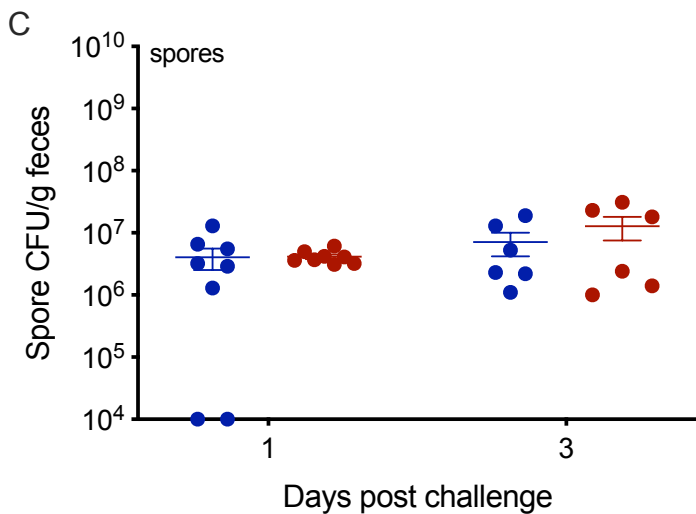
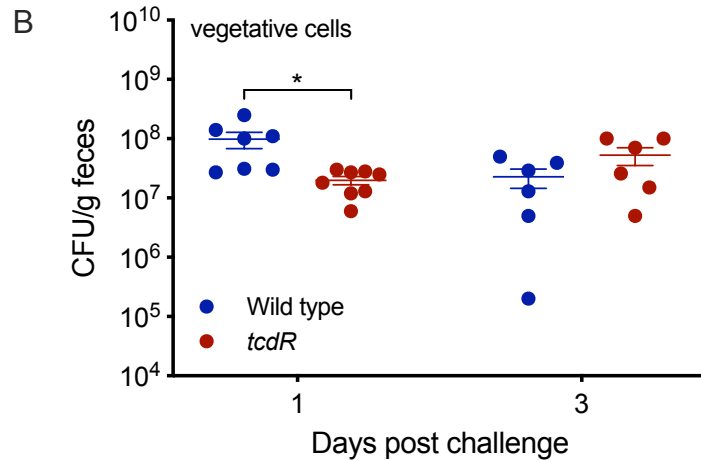
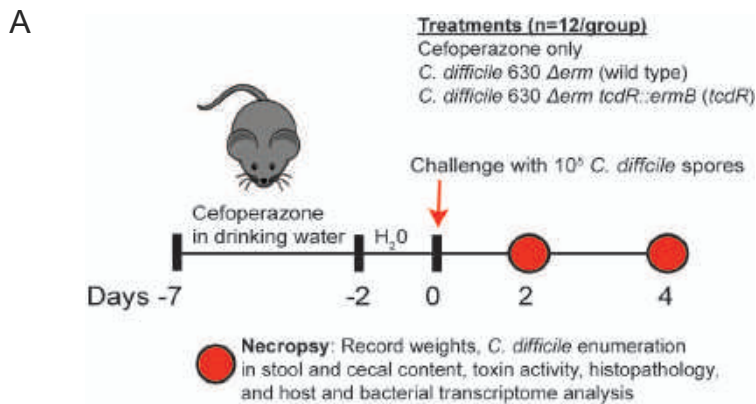


Figure 1

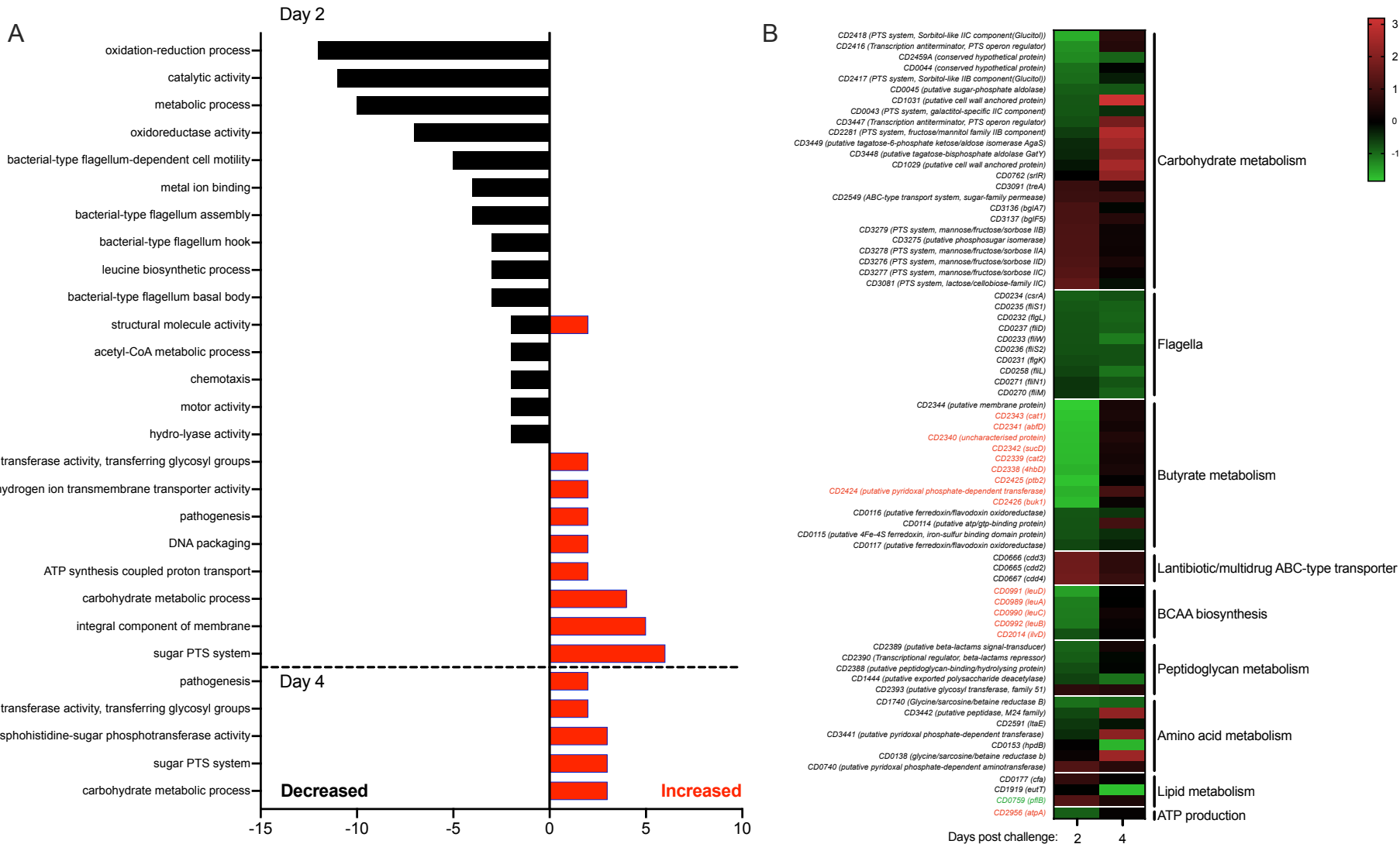


Figure 2

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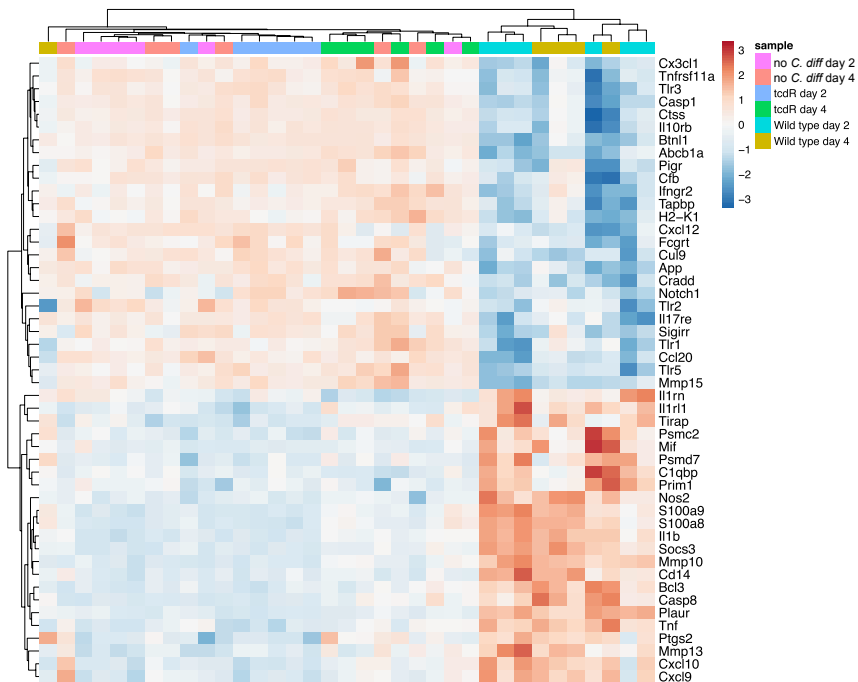
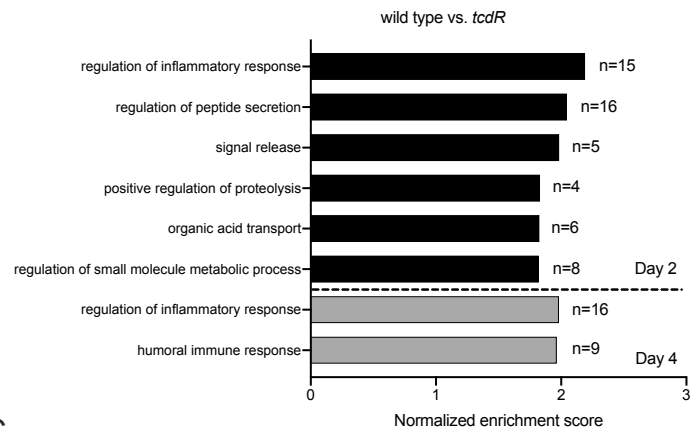
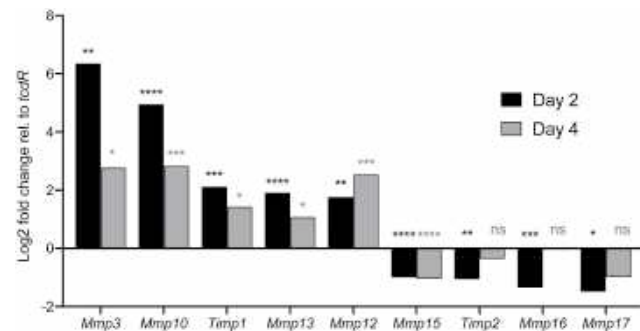


Figure 3

B



C



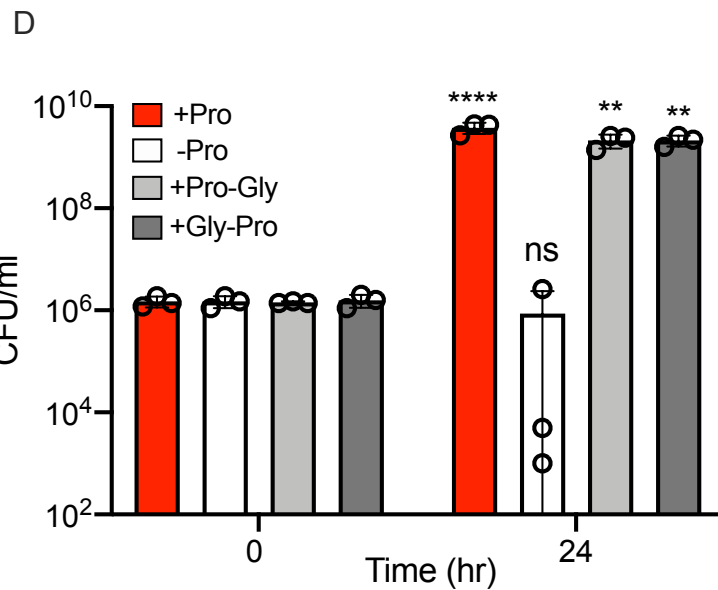
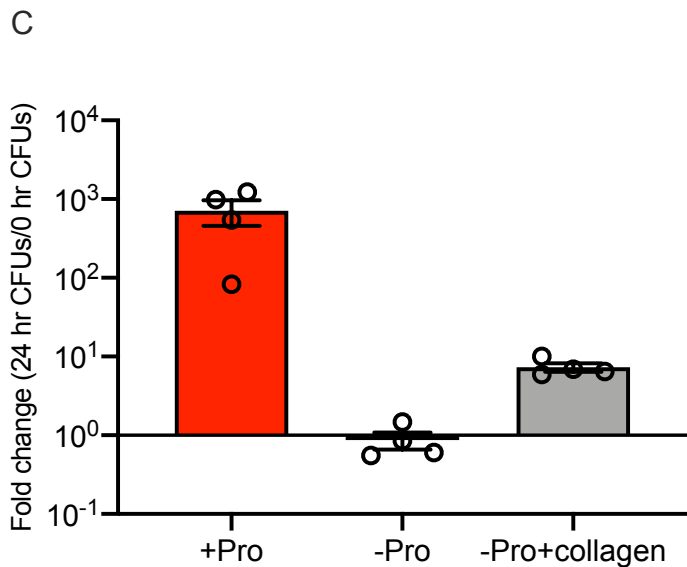
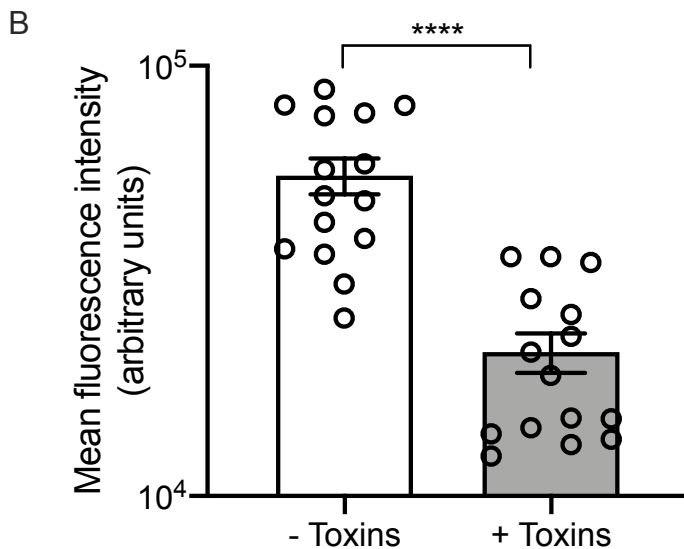
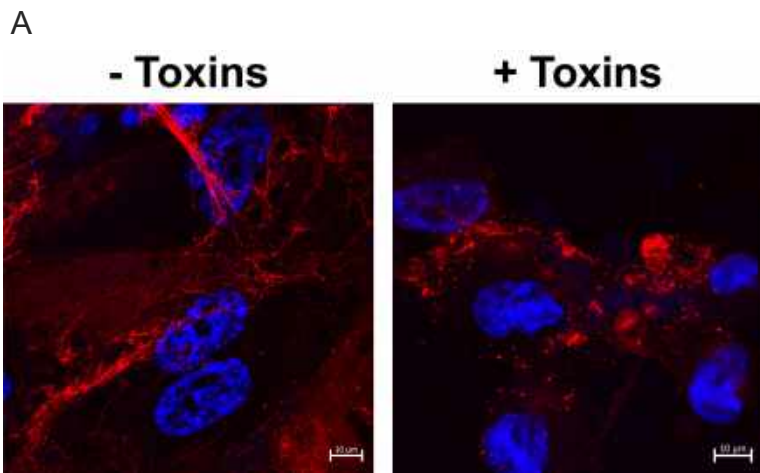


Figure 4

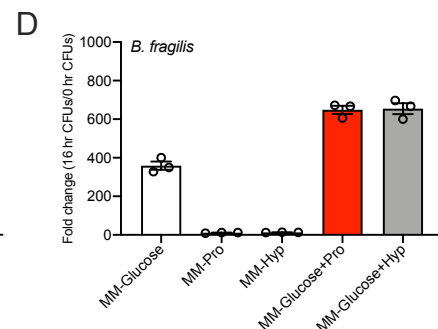
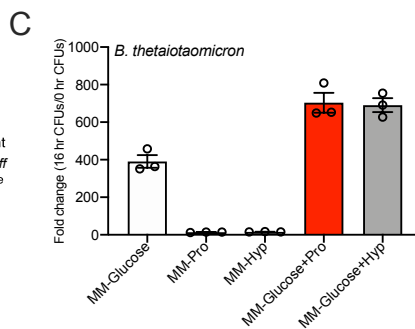
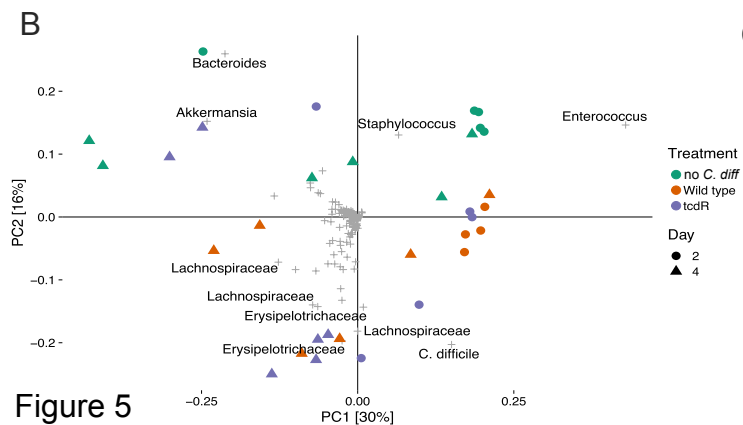
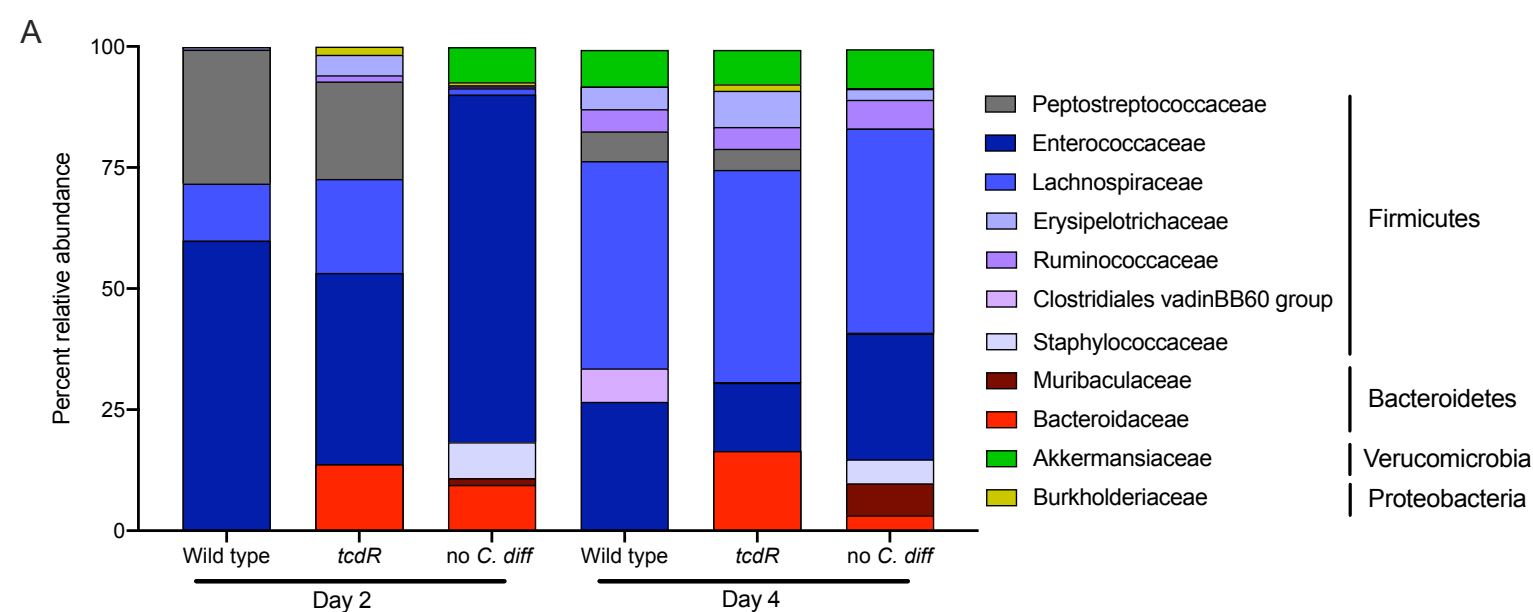


Figure 5

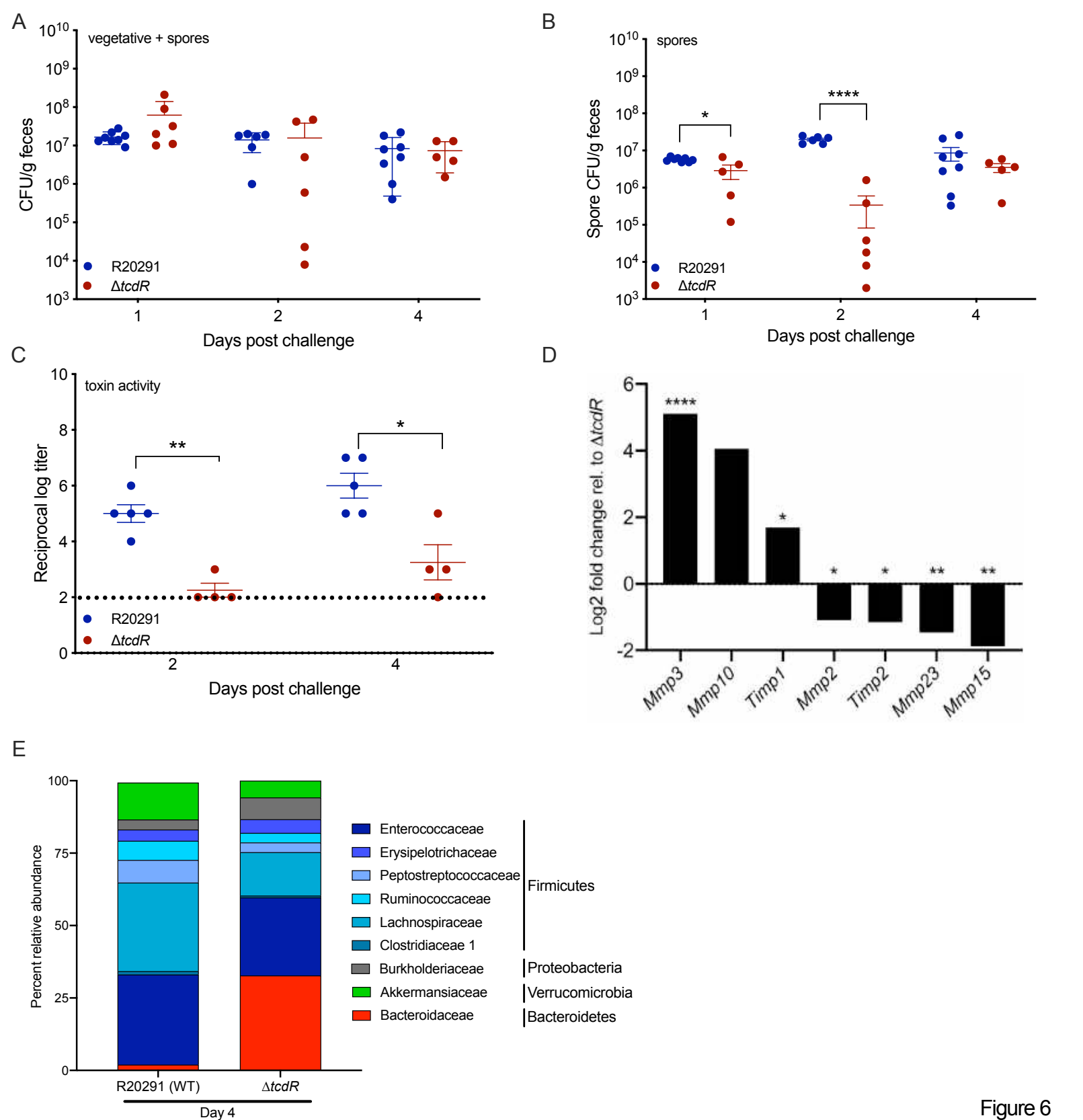


Figure 6