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Virulence factors enhance *Citrobacter rodentium* expansion through aerobic respiration

Christopher A. Lopez¹, Brittany M. Miller¹, Fabian Rivera-Chávez¹, Eric Velazquez¹, Mariana X. Byndloss¹, Alfredo Chávez-Arroyo¹, Kristen L. Lokken¹, Renée M. Tsois¹, Sebastian E. Winter², and Andreas J. Bäuml^{1,*}

¹Department of Medical Microbiology and Immunology, School of Medicine, University of California, Davis, One Shields Avenue, Davis, CA, USA

²Department of Microbiology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd, Dallas, TX, USA

Abstract

Citrobacter rodentium uses a type III secretion system (T3SS) to induce colonic crypt hyperplasia in mice, thereby gaining an edge during its competition with the gut microbiota through an unknown mechanism. Here we show that by triggering colonic crypt hyperplasia, the *C. rodentium* T3SS induced an excessive expansion of undifferentiated Ki67-positive epithelial cells, which increased oxygenation of the mucosal surface and drove an aerobic *C. rodentium* expansion in the colon. Treatment of mice with the γ -secretase inhibitor dibenzazepine to diminish Notch-driven colonic crypt hyperplasia curtailed the fitness advantage conferred by aerobic respiration during *C. rodentium* infection. We conclude that *C. rodentium* uses its T3SS to induce histopathological lesions that generate an intestinal microenvironment in which growth of the pathogen is fueled by aerobic respiration.

Attaching and effacing (AE) pathogens are defined by virulence characteristics encoded by a pathogenicity island known as the locus of enterocyte effacement (LEE) (1, 2), which contains genes encoding for a T3SS (3) and an adhesin termed intimin (4). The T3SS injects the intimin receptor Tir into the host cell cytosol (5), resulting in intimate attachment of bacteria to the effaced epithelial surface (6). The LEE encoded T3SS of the AE pathogen *C. rodentium* injects additional effector proteins that are required for causing transmissible colonic crypt hyperplasia in mice (7, 8) (Fig. S1). Following the development of colonic crypt hyperplasia, *C. rodentium* blooms in the lumen of the murine large bowel (9). The LEE encoded T3SS is required for this rapid luminal expansion possibly by allowing *C. rodentium* to compete with the microbiota for carbon sources, because the T3SS provides no benefit in germ-free mice (10). These data suggest that the T3SS places *C. rodentium* in a microenvironment that somehow provides a growth advantage during its competition with the resident microbiota, but it remains obscure which resources might become available in this niche to fuel pathogen expansion.

*To whom correspondence should be addressed. ajbauml@ucdavis.edu.

Electron acceptors, such as nitrate, are produced as a by-product of the inflammatory host response and boost luminal growth of pathogenic *Salmonella enterica* or commensal *Escherichia coli* by anaerobic respiration in mouse models of colitis (11–13). Since *C. rodentium* infection triggers colonic crypt hyperplasia, we wanted to determine whether the inflammatory host response would enable the pathogen to grow by anaerobic respiration. The respiratory reductases for nitrate, dimethyl sulfoxide (DMSO) and trimethylamine N-oxide (TMAO) as well as the formate dehydrogenases FdnGHI and FdoGHI contain a molybdopterin cofactor. Thus, to explore a possible role of anaerobic respiration during *C. rodentium* growth in the mouse gut, we constructed a *C. rodentium* mutant lacking a gene required for molybdopterin cofactor biosynthesis (*moaA* mutant) (Fig. S2A) (14). Mice (C57BL/6) were inoculated with an equal mixture of wild-type *C. rodentium* and an isogenic *moaA* mutant to compare the fitness of both strains. Mice developed intestinal inflammation as indicated by increased transcript levels of pro-inflammatory markers in the colonic mucosa (Fig. S3A and S3B). The *C. rodentium* wild type was recovered in significantly ($P < 0.05$) higher numbers than the *moaA* mutant (Fig. 1A). Similar results were observed with genetically susceptible C3H/HeJ mice that experience more severe intestinal inflammation during *C. rodentium* infection (Fig. S3C and S3D). In contrast, when germ-free mice were inoculated with an equal mixture of the *C. rodentium* wild type and a *moaA* mutant, both strains were recovered in similar numbers (Fig. 1B and S3E), suggesting that either anaerobic respiration or the utilization of formate provided an edge during competition of the pathogen with the resident microbiota.

C. rodentium infection resulted in a markedly increased colonic expression of *Nos2* (Fig. S3B and S3D), the gene encoding inducible nitric oxide synthase (iNOS), an enzyme necessary for generating nitric oxide, which is converted to nitrate in the intestinal lumen (11). To determine whether nitrate respiration enhances growth, we constructed a mutant deficient for the three nitrate reductases encoded by *C. rodentium* (*narG napA narX* mutant) (Fig. S2B and S2F). Remarkably, in mice inoculated with an equal mixture of the *C. rodentium* wild type and a *narG napA narX* mutant, both strains were recovered in equal numbers from colon contents and feces (Fig. 1A, S3C and S3E), suggesting that nitrate respiration did not provide a fitness advantage.

We next examined the possibility that the phenotype of the *moaA* mutant was due to an inability to utilize formate as an electron donor rather than nitrate as an electron acceptor. Since formate dehydrogenases FdnGHI and FdoGHI can couple electron transfer from formate through the quinone pool to nitrate (15) or oxygen (16), respectively, we envisioned that analysis of this pathway would provide indirect information about the electron acceptor used by *C. rodentium* in the gut. To test whether the nitrate-dependent and/or the oxygen-dependent formate dehydrogenase contributed to growth of *C. rodentium* during colitis, mice were inoculated with an equal mixture of the *C. rodentium* wild type and either an *fdnG* mutant or *fdoG* mutant (Fig. S2C and S2D). Consistent with our observation that nitrate respiration did not contribute to fitness (Fig. 1A), inactivation of the nitrate-dependent *fdnG* did not reduce growth of *C. rodentium* (Fig. 1B). However, inactivation of the oxygen-dependent *fdoG* reduced fitness of *C. rodentium* in conventional (C57BL/6) mice ($P < 0.05$), while little benefit was observed in germ-free (Swiss Webster) mice (Fig. 1B and S3E). The finding that *fdoG* was required for pathogen expansion was intriguing, because it suggested

that *C. rodentium* might utilize oxygen as a terminal electron acceptor during luminal growth.

We thus wanted to test the hypothesis that aerobic respiration fuels *C. rodentium* growth during infection. To test this idea, we deleted the *C. rodentium* *cydAB* genes (Fig. S2E), which encode a high-affinity cytochrome *bd* oxidase required for aerobic respiration in microaerobic environments. *In vitro*, the *C. rodentium* wild type was recovered in higher numbers than the *cydAB* mutant when both strains were co-cultured under conditions mimicking hypoxia (1% O₂) or normal tissue oxygenation (4% or 8% O₂), but not under anaerobic conditions (0% O₂) or at atmospheric oxygen levels (21% O₂) (Fig. 1C). Next, we compared the fitness of the *C. rodentium* wild type and a *cydAB* mutant by infecting C57BL/6 mice with a 1:1 mixture of both strains. Remarkably, the *C. rodentium* wild type was recovered in 20,000-fold higher numbers ($P < 0.01$) than the *cydAB* mutant from colon contents seven days after infection (Fig. 1D), suggesting that aerobic respiration contributed to growth of the pathogen. Expression of *Ier*, encoding the master regulator of T3SS synthesis, was reduced in the *cydAB* mutant, but this difference did not reach statistical significance (Fig. S4A). When infection with a 1:1 mixture of the *C. rodentium* wild type and a *cydAB* mutant was repeated in germ-free (Swiss Webster) mice, the fitness advantage provided by *cydAB* was greatly reduced (Fig. 1D). Collectively, these data suggested that aerobic respiration drove an uncontrolled luminal expansion of *C. rodentium* in an environment occupied by the gut microbiota.

To test whether tissue/mucus-associated bacteria have a respiratory metabolism compared to luminal bacteria, we investigated transcription of the *sucA* gene, which encodes a subunit of 2-ketoglutarate dehydrogenase, an enzyme required for the aerobic tricarboxylic acid (TCA) cycle. Under anaerobic conditions, SucA is no longer required as the normally cyclic TCA pathway switches to a non-cyclic series of reactions. Higher expression of *sucA* under aerobic than anaerobic conditions can serve as an indicator for a respiratory central metabolism (17). *C. rodentium* *sucA* was transcribed at significantly ($P < 0.05$) higher levels when bacteria were cultured *in vitro* under microaerobic conditions compared to anaerobic conditions (Fig. 1E and S4B). To investigate *sucA* expression *in vivo*, RNA was isolated from mucus scrapings or colon contents of mice infected with *C. rodentium*. *C. rodentium* residing in close proximity to tissue (i.e. mucus scrapings) transcribed *sucA* at significantly ($P < 0.05$) higher levels than bacteria located in colon contents (Fig. 1F and S4C). These results provided further support for the idea that bacteria in close proximity to the mucosal surface had an oxidative metabolism *in vivo*.

Intestinal inflammation caused by *Salmonella enterica* drives a depletion of *Clostridia*, which in turn increases epithelial oxygenation (18). We thus wanted to determine whether *C. rodentium*-induced colonic crypt hyperplasia would increase oxygen availability in the gut by driving a depletion of *Clostridia*. The numbers of *C. rodentium* recovered from colon contents of mice infected with the *C. rodentium* wild type were 1,000-fold higher ($P < 0.05$) than those recovered from colon contents of mice infected with a *cydAB* mutant (Fig. 1G), which correlated with reduced colonic inflammation ($P < 0.05$) (Fig. 1H and S5). Consistent with a previous report (9), infection of mice with the *C. rodentium* wild type resulted in a shift in the microbial community structure (Fig. 1I and S6). The absolute abundances of

members of the *Enterobacteriaceae* (Fig. S7A) and *Erythrobacteraceae* (Fig. S7B) were significantly ($P < 0.05$) increased in mice infected with the *C. rodentium* wild type, a shift in the community structure previously associated with increased oxygen availability in the gut (19). However, infection with the *C. rodentium* wild type resulted in an increased ($P < 0.05$) abundance of *Clostridia* (Fig. 1I), which was most pronounced for members of the *Lachnospiraceae* belonging to the genus *Dorea* (Fig. S7C) and the genus *Coproccoccus* (Fig. S7D). These changes in the microbial population profile were reduced or absent in mice infected with a *cydAB* mutant (Fig. 1I, S6 and S7). In conclusion, our data were not consistent with the hypothesis that colonic crypt hyperplasia increased oxygen availability by depleting *Clostridia*.

C. rodentium attaches to murine colonic epithelial cells (colonocytes) using a Type IV pilus encoded by the *cfc* operon (20). To test whether this adhesin contributed to establishing a niche in which the pathogen could grow by CydAB-mediated respiration, we constructed a *cfcH* mutant (Fig. S1F). Genetic ablation of Type IV pilus biosynthesis did not change the CydAB-dependent growth advantage seven days after infection of mice (Fig. 2A and Fig. S8A), possibly because a *cfcH* mutant still occupied a niche near the epithelium (Fig. S8B).

C. rodentium intimately attaches to colonocytes in a T3SS-dependent fashion using intimin. To test whether the T3SS and intimin contributed to CydAB-dependent growth, we constructed mutations in *escN*, encoding a component of the T3SS, and *eae*, the gene encoding intimin (Fig. S1G and S1H). Interestingly, the fitness advantage conferred by CydAB-dependent respiration seven days after infection was significantly reduced in *C. rodentium* strains lacking *eae* or *escN* (Fig. 2A and S8A), suggesting that in the absence of intimate attachment or a functional T3SS, oxygen respiration no longer conferred a marked fitness advantage. However, at three days after infection, when intestinal inflammation was just beginning to develop (Fig. S8C and S8D), inactivation of *escN* did not significantly reduce the fitness advantage conferred by the *cydAB* genes (Fig. S8E). Since the *escN* mutation did not reduce the fitness advantage conferred by the *cydAB* genes in the absence of overt crypt hyperplasia (i.e. at three days after infection), we considered the possibility that enhanced access to oxygen might not depend on intimate attachment, but require T3SS-mediated host responses.

Colonocytes mature as they migrate from the bottom of the crypt to the luminal surface. The mature surface colonocyte population functions in water absorption, which is driven by electrolyte transport (21). To energize absorption of electrolytes, mature colonocytes respire butyrate in their mitochondria (22), a process consuming oxygen and rendering the mucosal surface hypoxic (< 7.6 mmHg pO_2 or $< 1\%$ O_2) (23, 24) (Fig. S1). To investigate whether T3SS-induced colonic crypt hyperplasia, which developed by seven days after infection (Fig. S9A and S9B), would alter epithelial oxygenation *in vivo*, we visualized the “physiologic hypoxia” of surface colonocytes using the exogenous hypoxic marker pimonidazole (25) (Fig. 2B). Remarkably, a lack of hypoxia staining at the mucosal surface suggested that infection with the *C. rodentium* wild type significantly ($P < 0.05$) reduced epithelial hypoxia by seven days after infection (Fig. 2B, 2C and S10), indicative of a marked increase in epithelial oxygenation. This increased epithelial oxygenation was T3SS-

dependent, because it was not observed in mice infected with the *escN* mutant (Fig. 2B and 2C), which did not develop inflammation in the colon (Fig. S8).

T3SS-induced colonic crypt hyperplasia is characterized by an excessive intestinal epithelial repair response (reviewed in (26)). To further investigate the mechanism by which the T3SS increases epithelial oxygenation, we deleted the *map*, *cesF* and *espH* genes to reduce damage to colonocytes (Fig. S2H). Map and EspH are T3SS effector proteins that damage mitochondria (which might impair respiration of butyrate) or activate caspase-3 to induce cytotoxicity, respectively (27, 28), while the *cesF* gene encodes a chaperone for the mitochondria-associated T3SS effector EspF (29). Deletion of the *map*, *cesF* and *espH* genes significantly ($P < 0.05$) reduced the fitness advantage conferred by CydAB-dependent respiration seven days after infection of mice (Fig. 2A), but it was not clear whether this was simply due to an overall lower level of colonization (Fig. S8A). When mice were infected with either the *C. rodentium* wild type or an *espH cesF map* mutant, both strains were detected in association with the mucosal surface, although the *espH cesF map* mutant appeared impaired for colonization (Fig. S11A) and the *C. rodentium* wild type was recovered in higher numbers than a *espH cesF map* mutant from colon contents seven days after infection (Fig. 3A). Only infection with the *C. rodentium* wild type significantly reduced epithelial hypoxia, while the colonic surface remained hypoxic in mice infected with a *espH cesF map* mutant (Fig. S11B and S11C).

While mature colonocytes at the luminal surface respire butyrate, undifferentiated colonocytes in the crypts exhibit the Warburg metabolism of dividing cells, which is characterized by fermenting glucose to lactate (30). *C. rodentium* infection induces epithelial regeneration and repair mechanisms, which drives a marked expansion of undifferentiated colonocytes, resulting in crypt elongation and the presence of undifferentiated colonocytes at the luminal surface (31, 32)(Fig. S1). Since the Warburg metabolism does not consume oxygen, we wanted to determine whether reduced hypoxia of surface colonocytes observed during *C. rodentium* infection (Fig. 2B, 2C, S11B and S11C) was due to an accumulation of undifferentiated colonocytes at the luminal surface. Interestingly, infection with a *espH cesF map* mutant triggered significantly ($P < 0.05$) less mitotic divisions in the colonic epithelium than infection with wild-type *C. rodentium* (Fig. S8C), suggesting that deletion of the *espH*, *cesF* and *map* genes might reduce crypt hyperplasia. To test this idea, we stained colonic sections from mock-infected mice or mice infected with the *C. rodentium* wild type or an *espH cesF map* mutant with Ki67, a cellular marker for proliferation. Infection with the *C. rodentium* wild type induced a marked expansion of Ki67-positive colonocytes, resulting in crypt elongation, a thickening of the colonic mucosa and an accumulation of Ki67-positive colonocytes along the length of the crypt and at the luminal surface (Fig. 3B and 3C). Interestingly, this host response was blunted in mice infected with a *C. rodentium espH cesF map* mutant (Fig. 3B and 3C), which correlated with increased epithelial hypoxia (Fig. S11B and S11C).

We next wanted to determine whether colonic crypt hyperplasia was a driver of CydAB-dependent pathogen expansion. To this end, mice were mock infected or infected with a 1:1 mixture of the *C. rodentium* wild type and *cydAB* mutant and then treated with dibenzazepine (DBZ) or with vehicle control. DBZ is a Notch and Wnt (wingless-related

integration site) signaling pathway inhibitor that prevents colonic crypt hyperplasia during *C. rodentium* infection (31). Ki67 staining suggested that mice infected with the *C. rodentium* strain mixture developed colonic crypt hyperplasia, which was significantly ($P < 0.05$) blunted by DBZ treatment (Fig. 3D and 3E), although DBZ treatment did not reduce the severity of other histopathological changes (Fig. S12A and S12B). Remarkably, inhibition of colonic crypt hyperplasia by DBZ treatment abrogated the fitness advantage conferred by the *cydAB* genes (Fig. 3F), suggesting that crypt hyperplasia was a main driver of aerobic pathogen expansion.

Our finding that the LEE-encoded T3SS provides *C. rodentium* access to oxygen *in vivo* helps explain why the mechanism by which this trademark virulence factor confers a benefit to AE pathogens has not been apparent from *in vitro* studies, because such experiments are commonly performed in a 95% air (79% N₂/21% O₂) atmosphere, supplemented by 5% of carbon dioxide, thus providing 19.95% O₂ (150 mmHg). This oxygenation is considerably higher than normal tissue oxygenation (23–70 mmHg or 3%–10% oxygen) (35) or oxygenation of colonocytes *in vivo* (< 7.6 mmHg or < 1% O₂) (23, 24). While oxygen emanating from the mucosal surface is a very limited resource in the lumen of the large intestine (36), epithelial oxygenation was markedly elevated during T3SS-induced colonic crypt hyperplasia. As a result, T3SS-induced colonic crypt hyperplasia drove growth of *C. rodentium* through CydAB-mediated aerobic respiration, presumably because a respiratory metabolism enables the pathogen to utilize carbon sources more effectively than competing microbes that rely on fermentation for growth. In conclusion, our results revealed how histopathological changes triggered by a trademark virulence factor of AE pathogens create a unique nutrient-niche to fuel an uncontrolled luminal expansion of *C. rodentium*.

Materials and Methods

Bacterial strains and culture conditions

For the *Escherichia coli* and *Citrobacter rodentium* strains used in this study, see Table S1. Strains were routinely grown in Luria-Bertani broth (BD Biosciences #244620) or on LB plates unless otherwise indicated. Antibiotics were used at the following concentrations: carbenicillin (Carb), 0.1 mg/ml; chloramphenicol (Cm), 0.015mg/ml; kanamycin (Kan), 0.1 mg/ml; tetracycline (Tet), 0.02 mg/ml.

In vitro competitive growth assay

Bacteria were grown in 10 ml M9 minimal media (2mM MgSO₄, 0.2mM CaCl₂, M9 salts [Na₂HPO₄, 6.8 g/l; KH₂PO₄, 3 g/l; NaCl, 0.5 g/l; NH₄Cl, 1 g/l]) with 0.4% glycerol, 1% casamino acids (BD Biosciences). Bacteria for the assay were grown overnight at 37°C in an anaerobe chamber (Shellab) with an environment of 5 % CO₂, 5 % H₂, and the remainder N₂. Pre-reduced media was inoculated with a 1:1 ratio of two *C. rodentium* strains at a concentration of 1×10⁵ total CFU in the anaerobe chamber. The inoculated media was then either left in the anaerobe chamber (0% oxygen) or moved to a hypoxia chamber in an airtight container. The hypoxia chamber was set at either 1 %, 4 %, 8 %, or 21 % oxygen, with the remaining atmosphere being composed of 5 % CO₂ and 95 % N₂. Cultures were

left to incubate for 24 hours and then plated on selective MacConkey agar to differentiate between the two strains. Data represents eight replicates per group.

Nitrate reductase assay

Bacterial cultures were grown overnight in LB broth, then diluted 1:1000 into fresh LB broth containing 40 mM NaNO₃. Cultures were grown statically at 37°C for 3 hours. The assay was then performed as described previously (37). Briefly, 50 µl of a washed cell suspension was permeabilized in 0.1% sodium dodecyl sulfate and chloroform. Next methyl viologen (0.5 mg/ml) and a solution of bicarbonate (8 mg/ml), sodium hydrosulfite (8 mg/ml), and nitrate (0.5 M) were added to cells and left to incubate at room temperature for 5 minutes. Assay was terminated with vigorous vortexing, then sulfanilic HCl (1% sulfanilic acid and 20% HCl) and Marshall's reagent (0.13 % N-1-naphthylethylenediamine 2HCl) were added to the solution. Absorbance was next read at 540 nm and 420 nm. To calculate nitrate reductase activity, we used the following equation: $\{[A_{540} - (0.72 * A_{420})] / (t * v * A_{600})\} * 100$ where t is the time (minutes) of the reaction, v is the volume (ml) of the cell suspension in the assay, and A_{600} is the optical density of the original cell suspension.

Construction of *C. rodentium* mutants

To construct a *napA* mutant, upstream and downstream regions of approximately 1kb in length flanking *C. rodentium napA* were amplified via PCR, run on an agarose gel, and purified using the QiaexII kit (Qiagen). These flanking regions were digested with XbaI (NEB), ligated, and PCR amplified to form one fragment of approximately 2kb that was then inserted into pCR2.1. The plasmid was propagated in Top10 *E. coli*. This plasmid was next digested with SphI (NEB) and the fragment was inserted into pRDH10 to form pCAL7 and cloned into *E. coli* DH5α λ pir. To insert a tetracylin resistance cassette, pCAL7 was digested with XbaI while pSPN23 was digested with NheI (NEB). The tetracycline resistance cassette from pSPN23 was ligated with pCAL7 to form pCAL17. Plasmid pCAL17 was then transformed into *E. coli* strain S17-1 λ pir and conjugation performed with *C. rodentium* that was transformed with pWSK129 to have a selectable marker for the recipient. After selection to pick *C. rodentium* colonies containing a single crossover mutation, sucrose selection was performed to select for colonies that lost the vector backbone along with the *napA* gene, but retained the tetracycline resistance cassette. After confirming the mutation via PCR, this strain was named CAL60. To generate a nonpolar *napA* mutant, CAL60 was conjugated with *E. coli* S17-1 λ pir containing pCAL7. After selection for single crossover *C. rodentium* colonies, sucrose selection was performed to select for colonies that lost the tetracycline cassette (CAL77).

To construct the *narZ* mutation, upstream and downstream regions of approximately 1kb in length flanking *C. rodentium narZ* were amplified via PCR, run on an agarose gel, and purified using the QiaexII kit. These flanking regions were digested with XbaI, ligated, and PCR amplified to form one fragment of approximately 2kb that was then inserted into pCR2.1 and propagated in *E. coli* Top10. This plasmid was next digested with SphI and the fragment was inserted into pRDH10 to form pCAL8, which was propagated in DH5α λ pir *E. coli*. To insert a tetracycline resistance cassette, pCAL8 was digested with XbaI while pSPN23 was digested with NheI. The tetracycline resistance cassette was ligated with

pCAL8 to form pCAL15. Plasmid pCAL15 was transformed into *E. coli* S17-1 λ pir and conjugation performed with CAL77. After selection to pick *C. rodentium* colonies containing a single crossover mutation, sucrose selection was performed to select for colonies that lost the vector backbone and *narZ* gene, but retained the tetracycline resistance cassette. After confirming the mutation via PCR, the *napA narZ* mutant was named CAL92.

To construct the *narG* mutation, a region within *C. rodentium narG* was amplified via PCR, inserted into pCR2.1, and propagated in Top10 *E. coli*. This plasmid and the plasmid pGP704 were then digested with XbaI and SphI. The *narG* fragment was ligated into pGP704 to produce the plasmid pCAL6 and propagated in *E. coli* DH5 α λ pir. Plasmid pCAL6 was then transformed into *E. coli* S17-1 λ pir conjugation performed with CAL92. *C. rodentium* colonies with a single crossover were selected and PCR confirmed the mutation in *narG*. The *napA narZ narG* mutant was named CAL93. To construct a *moaA* mutant, a region within *C. rodentium moaA* was amplified via PCR, inserted into pCR2.1, and propagated in *E. coli* Top10. This plasmid and the plasmid pEP185.2 were then digested with XhoI (NEB) and SacI (NEB). The *moaA* fragment was ligated into pEP185.2 to generate the plasmid pCAL34, which was subsequently propagated in *E. coli* DH5 α λ pir. To aid in selection during conjugation, wild type *C. rodentium* was transformed with the temperature-sensitive plasmid pSW172. Plasmid pCAL34 was then introduced into *E. coli* S17-1 λ pir and conjugation performed with *C. rodentium* (SW172) at 30°C. Since *C. rodentium* exhibits low-level resistance to carbenicillin naturally, a higher concentration of this antibiotic (0.2 mg/ml) was used to select for *C. rodentium* colonies that retained the Carb^R plasmid pSW172 and also contained a single crossover event at the *moaA* locus. The plasmid pSW172 was cured after growth at 40°C and PCR was used to confirm the *moaA* mutation. The *moaA* mutant was named CAL142.

To construct a non-polar *cydAB* mutant, upstream and downstream regions of approximately 1kb in length flanking the *C. rodentium cydAB* operon were amplified via PCR, run on an agarose gel, and purified using the QiaexII kit. These flanking regions were digested with XbaI, ligated, and PCR amplified to form one fragment of approximately 2kb that was then inserted into pCR2.1 and propagated in *E. coli* Top10. While cloning in *C. rodentium*, we observed that sucrose selection does not efficiently prevent the growth of *Citrobacter* colonies that retained a suicide vector encoding *sacRB*. To improve screening for double crossover events, we amplified the *phoN* gene of *Salmonella enterica* serovar Typhimurium via PCR and inserted it into pCR2.1 followed by propagation in *E. coli* Top10. This plasmid was subsequently digested with EcoRI (NEB) and *phoN* was inserted into the EcoRI-digested pRDH10 plasmid to generate pCAL52. *C. rodentium* colonies with this plasmid will turn blue when grown on plates containing X-phos (5-bromo-4-chloro-3-indolyl phosphate). The plasmid pCR2.1 containing the *cydAB* flanking regions was next digested with SalI (NEB) and the fragment was inserted into pCAL52 to generate pCAL59. To insert a kanamycin resistance cassette, pCAL59 and pUC4 KSAC were first digested with XbaI. The kanamycin resistance cassette from pUC4 KSAC was then ligated with pCAL59 to form pCAL60 and propagated in *E. coli* DH5 α λ pir. Plasmid pCAL60 was then transformed into *E. coli* strain S17-1 λ pir and conjugation performed with *C. rodentium* (SW172). *C. rodentium* colonies containing a single crossover mutation were then grown overnight in LB broth at 30°C and plated on LB agar plates containing X-phos (40mg/l). White colonies

were screened for resistance to kanamycin, but sensitivity to chloramphenicol, confirming loss of the pCAL52 vector backbone but retaining the kanamycin resistance cassette. After confirming the *cydAB* deletion via PCR, the plasmid pSW172 was cured after growth at 40°C and the strain named CAL247. To generate a non-polar *cydAB* mutant, CAL247 was conjugated with *E. coli* S17-1 λ pir containing pCAL59. After selection for single crossover *C. rodentium* colonies, we performed blue-white screening on LB agar plates containing X-phos as described above to select for colonies that lost the kanamycin cassette and generated CAL267.

To construct a non-polar *fdnG* mutant, upstream and downstream regions of approximately 1kb in length flanking the *C. rodentium fdnG* gene were amplified via PCR, run on an agarose gel, and purified using the QiaexII kit. These flanking regions were digested with XbaI, ligated, and PCR amplified to form one fragment of approximately 2kb in size that was then inserted into pCR2.1 and propagated in *E. coli* Top10. This plasmid was next digested with SphI and inserted into pCAL52 to generate pCAL55 and propagated in *E. coli* DH5 α λ pir. Plasmid pCAL55 was transformed into *E. coli* S17-1 λ pir, conjugation performed with *C. rodentium* (SW172) and single crossover events were selected for using the appropriate antibiotics. Blue-white screening with X-phos was performed as described above. A double crossover event was confirmed via PCR. The plasmid pSW172 was cured after growth at 40°C and the non-polar *fdnG* mutant was named CAL210. To aid in selection during mouse experiments, CAL210 was transformed with pWSK129.

To construct the *fdoG* mutant, upstream and downstream regions of approximately 1kb in length flanking the *C. rodentium fdoG* gene were amplified via PCR, run on an agarose gel, and purified using the QiaexII kit. These flanking regions were digested with XbaI, ligated, and PCR amplified to form one fragment of approximately 2kb that was then inserted into pCR2.1 and propagated in *E. coli* Top10. This plasmid, containing the *fdoG* flanking regions, was next digested with SphI and the fragment was inserted into pCAL52 to generate pCAL72. To insert a kanamycin resistance cassette, pCAL72 and pUC4 KSAC were first digested with XbaI. The kanamycin resistance cassette from pUC4 KSAC was then ligated with pCAL72 to form pCAL74 and propagated in *E. coli* DH5 α λ pir. Plasmid pCAL74 was then transformed into *E. coli* strain S17-1 λ pir and conjugation performed with *C. rodentium* (SW172). Single crossover events were selected for by growing on the appropriate antibiotics. Blue-white screening with Xphos was performed as described above to identify a colony that lost the vector backbone and the *fdoG* gene, but retained the kanamycin resistance cassette. Plasmid pSW172 was cured after growth at 40°C. After PCR confirmation of the *fdoG* mutation, this strain was named CAL261.

To construct a *escN* mutant, the Gibson Assembly method (New England Biolabs) was used to generate plasmid pCAL81 with a pEP185.2 vector backbone and an insert of an internal amplified region of *escN*. Plasmid pCAL81 was first propagated in *E. coli* DH5 α λ pir, then transformed into *E. coli* S17-1 λ pir and conjugation performed with *C. rodentium* (SW172) at 30°C. After selection for single crossover events, the plasmid pSW172 was cured after growth at 40°C and PCR was used to confirm the *escN* mutation. This *escN* mutant was named CAL286.

To construct a *cydAB escN* double mutant, the *E. coli* S17-1 λ pir strain containing pCAL81 was conjugated with CAL247. After selection for single crossover events, PCR was used to confirm the *escN* mutation. The *cydAB escN* mutant was named CAL287.

For generation of a *cfcH* mutant, a region within the *C. rodentium* *cfcH* was amplified via PCR. Fragments were run on an agarose gel and purified using the Zymoclean Gel DNA Recovery kit (Zymo Research). Creation of pBMM1 was accomplished by insertion of the purified fragment into pEP185.2 via the Gibson Assembly (New England Biolabs), and propagation in *E. coli* DH5 α λ pir. pBMM1 was then transformed into *E. coli* S17-1 λ pir, followed by conjugation with *C. rodentium* (SW172). *C. rodentium* colonies with a single crossover were selected, and PCR was used to confirm the mutation in *cfcH*. The *cfcH* mutant was named BMM12.

For construction of a *cydAB cfcH* double mutant, the *E. coli* S17-1 λ pir strain containing pBMM1 was conjugated with a *cydAB* mutant. *C. rodentium* colonies carrying a single crossover were selected, and PCR was used to confirm the *cfcH* mutation. This *cydAB cfcH* double mutant was then named BMM11.

To construct an *eae* mutant, primers were designed using NEBuilder Assembly Tool (New England Biolabs) to amplify an *eae* intragenic region with overlapping regions with the plasmid pEP185.2. The intragenic region was amplified using Q5 high-fidelity master mix (New England Biolabs) and ligated via Gibson Assembly with pEP185.2 after the vector was digested with SacI. The assembled plasmid, named pCAL82, was transformed into *E. coli* DH5 α λ pir then into *E. coli* S17-1 λ pir. *C. rodentium* (SW172) or the *cydAB::Kan^R* mutant (CAL247) were next conjugated with *E. coli* S17-1 λ pir containing pCAL82. A *C. rodentium* colony resistant to Cm was verified by PCR to contain a single crossover at the *eae* locus. The strain was then cured of the temperature sensitive plasmid after growth at 40°C and designated CAL290. A *C. rodentium cydAB* mutant resistant to Kan and Cm was verified by PCR to contain a single crossover at the *eae* locus in addition to the deletion of *cydAB* and this strain was named CAL291.

To construct a non-polar *espH cesF map* mutant, regions of approximately 400bp in length flanking the *espH*, *cesF*, and *map* genes were amplified via PCR using Q5 high fidelity master mix, run on an agarose gel, and purified using Zymoclean Gel DNA Recovery kit (Zymo Research). Fragments were ligated into pRDH10 via Gibson Assembly after the vector was digested with SphI. The resulting plasmid pBMM3 was transformed into *E. coli* S17-1 λ pir and conjugated with *C. rodentium* wild type or a *cydAB* mutant (CAL247), which had been transformed with the temperature-sensitive plasmid pSW172. Exconjugants were selected for loss of the plasmid using sucrose selection and screened via PCR to confirm deletion of the target genes. The strains were then cured of the temperature sensitive plasmid after growth overnight at 42°C and were named BMM30 and BMM40, respectively.

Animal experiments

The Institutional Animal Care and Use Committee at the University of California, Davis, approved all animal experiments. For experiments involving mice colonized with

conventional specific pathogen free microbiota, either C57BL/6J or C3H/HeJ strains (Jackson Laboratories) aged 6–8 weeks were inoculated with 1×10^9 colony forming units (CFU)/mouse of a single *C. rodentium* strain in LB broth intragastrically. In competitive infections, a 1:1 ratio of two *C. rodentium* strains were given intragastrically at a combined final concentration of 1×10^9 CFU/mouse. Fecal pellets were collected on days 3, 7, or 10 and mice were sacrificed between 3 and 14 days after infection. Colon contents were stored in phosphate-buffered saline (PBS) on ice. Colon tissue for histopathology was fixed in 10% buffered formalin phosphate while colon sections for murine RNA analysis were flash frozen and stored at -80°C . *C. rodentium* from feces and colon contents were enumerated by plating serial ten-fold dilutions of samples on LB agar or MacConkey agar (BD Biosciences) containing the appropriate antibiotics based on the resistance markers listed for each strain in Table S1. Plates were incubated overnight at 37°C under atmospheric oxygen conditions.

Germ-free Swiss Webster mice were bred and housed at the Teaching and Research Animal Care Services facilities at UC Davis. These mice were infected and euthanized as described above.

To inhibit hyperplasia, mice were first mock infected or infected with a 1:1 mixture of the indicated *C. rodentium* strains as described above. Dibenazepine (DBZ) was initially dissolved in DMSO and then suspended in a solution of hydroxypropyl methylcellulose (0.5 %)(Sigma) and Tween80 (0.1 %). Mice were given 0.1ml of the DBZ solution or DMSO vehicle control starting at day 2 after infection (approximately $10 \mu\text{mol/kg}$) and continuing daily up to day 6 post-infection, or a total of 5 doses. On day 7 after infection, mice were given pimonidazole (PMDZ) one hour before euthanasia was performed.

Microbiota analysis

Seven days after treatment of mice, DNA from the colon content was extracted using the PowerSoil DNA Isolation kit (Mo-Bio, Carlsbad, CA) according to the manufacturer's protocol. Bacterial DNA was amplified via a PCR enrichment of the 16S rDNA (V4 region) using primers 515F and 806R modified by addition of barcodes for multiplexing. Libraries were sequenced with an Illumina MiSeq system. Sample sequences were demultiplexed and trimmed, followed by filtering for quality. QIIME open-source software (<http://qiime.org>) (38) was used for initial identification of operational taxonomic units (OTU), clustering, and phylogenetic analysis. Samples containing less than 1000 quality reads were removed from dataset. Subsequent data transformation was performed using MEGAN 4 software (<http://ab.inf.uni-tuebingen.de/software/megan4/>) (39) and then further analyzed using Explicet version 2.10.5 (<http://www.explicet.org/>) (40). Data analyzed using METAGENassist (<http://www.metagenassist.ca/>) was first normalized by logarithmically transforming the reads to yield a more normal distribution (41).

Histopathology

Fixed colon sections were stained with hematoxylin and eosin. A veterinary pathologist scored histopathological changes of blinded samples using the scoring scheme described in Figure S4A. For mitotic divisions, the data represent the number of mitotic figures per high power field (40X), with the average of five fields per slide displayed.

RNA isolation

For murine RNA isolation, colon tissue sections were homogenized in a Mini-Beadbeater (BioSpec Products, Bartlesville, OK) and RNA was isolated by the TRI-Reagent method (Molecular Research Center, Inc.) following the manufacturer's protocol. Contaminating DNA was removed using the DNA-free kit (Applied Biosystems) and RNA was stored at -80°C .

Upon *C. rodentium* infection of mice, bacterial RNA was isolated from two compartments: the intestinal colon contents and mucus layer. During necropsy, a section of the middle colon containing a fecal pellet was removed and cut longitudinally. The fecal pellet was carefully removed to avoid pellet disintegration and stored in RNeasy (Qiagen) overnight at 4°C . Forceps were next used to gently scrape off the mucus layer from the underlying tissue. The mucus layer was also stored in RNeasy overnight at 4°C . Sample isolation was performed at atmospheric oxygen tensions. Excess solution was subsequently removed from the pellet and Trizol (Invitrogen) was added. Next chloroform was added and the solution was transferred to 2 ml phase-lock tubes (5 Prime) and centrifuged at $14000g$. The upper phase was removed and RNA was precipitated with 100% ethanol at -20°C overnight. RNA was collected after centrifugation and washed with 70% ethanol. Contaminating DNA was removed as described above.

For bacterial RNA isolation from cultures, 1 ml of bacterial culture was processed using the Aurum Total RNA mini kit (BioRad) following the manufacturer's protocol. Contaminating DNA was removed as described above.

Quantitative real-time PCR

Isolated RNA was reverse transcribed using random hexamers and Moloney murine leukemia virus reverse transcriptase (Applied Biosystems). Quantitative real-time PCR was performed using SYBR green (Applied Biosystems) PCR mix and the appropriate primer sets (see Table S2) at a final concentration of 0.25 mM. Absolute values were calculated using a series of standards and a standard curve. To generate standards, the above mentioned primer sets were used in standard PCR reaction to amplify the target genes, which were subsequently inserted into pCR2.1 using the TOPO cloning kit (Life Technologies). The resulting plasmids were sequenced for accuracy and the concentration quantified to create a set of standards ranging from 10^8 to 10^1 copies/ μl diluted in a 0.02 mg/ml yeast RNA (Sigma) solution.

To quantify transcription of the regulator of the LEE pathogenicity island, *Ier*, RNA was isolated from the colon contents (see above) and qRT-PCR was applied with a published primer pair (42). To quantify transcription of *sucA* expression *in vivo*, RNA was isolated from both the mucus layer and colon contents (see above) and qRT-PCR was applied using the primers in Table S2.

Flow cytometry

Flow cytometry analysis was performed for detection of PMDZ staining on whole colon epithelial cells isolated from mock, *C. rodentium*-infected and *C. rodentium escN* mutant-

infected mice. Single cell suspensions of colonocytes were obtained as described previously (43). Cells were suspended in 2 ml of dPBS and stained with Aqua Live/Dead cell discriminator (Invitrogen) according to the manufacturer's protocol. After Live/Dead staining, cells were washed with dPBS and re-suspended in 100 μ l of PBS containing 3.6% of M.O.M.TM mouse Ig blocking reagent (Vector Laboratories) and incubated in the dark at 4°C for 20 min. Cells were washed with 2 ml of dPBS containing 1% bovine serum albumin and 2 mM EDTA (fluorescence-activated cell sorter [FACS] buffer) and then re-suspended in 50 μ l of FACS. Cells were stained with a FC receptor blocking antibody, anti-CD16/32 (Biolegend), for 5 minutes at 4°C and then stained for 30 min at 4°C with a cocktail of anti-CD45 PerCp-Cy5.5 (Biolegend) and anti-EpCam FITC (Biolegend). Cells were washed in FACS buffer, fixed and permeabilized with BD Cytfix/CytopermTM (BD Biosciences) for 20 minutes at 4°C and then washed with BD Perm/Wash bufferTM (BD Biosciences). Cells were re-suspended in 100 μ l of BD Perm/Wash bufferTM containing 3.6% of M.O.M.TM mouse Ig blocking reagent and incubated in the dark at 4°C for 20 min. Cells were washed with BD Perm/Wash bufferTM and then stained with an anti-PMDZ PE antibody (Hypoxypore), diluted in BD Perm/Wash bufferTM for 30 min at 4°C. Cells were washed in BD Perm/Wash bufferTM and re-suspended in FACS. For quantification of cell populations, 50 μ l of SPHERO AccuCount Fluorescent Particles 10.1 μ m (Spherotech) were added to each sample prior to analysis. Calculation of absolute counts was performed according to manufacturer's protocol. Flow cytometry analysis was performed using a BD (Becton Dickinson) LSRII, and 4.0×10^5 events were collected per mouse. Data were analyzed using FlowJo software (Treestar, inc. Ashland, OR) and gates were based on fluorescence-minus-one (FMO) controls.

Fluorescent Imaging

For mouse colon imaging, 10–15 mm sections of mid-colon were frozen in Optimal Cutting Temperature (OCT) compound (Fisher HealthCare) and cut in transverse sections to a thickness of 7 μ m. Sections were fixed with 4% PFA and permeabilized with 0.2% Triton-X 100. *C. rodentium* cells were stained with a rabbit anti-*Citrobacter* primary antibody (Abcam, ab37056) and a goat anti-rabbit Alexafluor 647 conjugate (Abcam, ab150079). Proliferating cells were visualized with a primary rabbit antibody targeting the nuclear protein Ki67 (Abcam, ab15580) and a goat anti-rabbit Alexafluor 647 conjugate. Nuclei were stained with DAPI in SlowFade mounting media (Invitrogen). Actin was stained using phalloidin-tetramethylrhodamine B isothiocyanate (Sigma Aldrich).

For imaging of intestinal hypoxia, at the day of euthanasia mice were treated intraperitoneally with 20 mg per mouse of pimonidazole HCl (Hypoxypore). One hour after treatment, mice were euthanized and colon sections were frozen and cryosectioned as stated above. These PMDZ adducts were detected with a mouse monoclonal antibody MAb1 (Hypoxypore) followed by a secondary goat anti-mouse IgG antibody (Alexafluor 546, ThermoFisher). After fixation and permeabilization sections were incubated first with Universal Block (KPL) then with an IgG blocking reagent from a Mouse on Mouse (M.O.M) kit (Vector Laboratories). Sections were finally incubated with the anti-PMDZ primary antibody and the secondary anti-mouse Alexafluor 546 antibody.

PMDZ and Ki67 staining was quantified using ImageJ 1.49v software (44). For PMDZ quantification, staining intensity of the outer colonocytes was normalized to the staining intensity of DAPI. This ratio was then expressed as fold change over the mock control group. For Ki67 quantification, staining intensity of all the cells within a colonic crypt was normalized to the staining intensity of DAPI. This ratio was then expressed as fold change over the mock control group. For both PMDZ and Ki67 quantification, three to four non-overlapping images were used per mouse.

***In vitro* bacterial RNA expression**

To measure gene expression *in vitro*, wild type *C. rodentium* was grown in M9 media supplemented with 1% casamino acids and 0.4% mannose for 16 hours at 37°C under either microaerobic or anaerobic conditions. A microaerobic environment was created by growing bacteria without shaking under environmental oxygen tension while anaerobiosis was achieved by growing bacteria in pre-reduced media in an anaerobe chamber. Experiments were performed in triplicate. RNA was then isolated as described above.

Statistical analysis

Data for our experiments displayed as bar graphs represent the geometric mean and the standard error of the mean. For most experiments, data points were first log transformed and differences between experimental groups were determined on the transformed data using a Student's T-test (for comparing two groups) or ANOVA followed by Fisher's LSD post hoc test (for comparison of more than two groups). If appropriate, outliers were removed using Grubb's test. Data representing the percent of PMDZ positive colonocytes were first arc-sine transformed before applying the ANOVA test. A one-way ANOVA using METAGENassist online software was used to determine statistical significance on the changes in the proportion of microbial taxa from the 16S sequencing reads. A *P*-value of less than 0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References and notes

1. Staley TE, Jones EW, Corley LD. Attachment and penetration of *Escherichia coli* into intestinal epithelium of the ileum in newborn pigs. *The American journal of pathology*. 1969; 56:371–392. [PubMed: 4898367]

2. Moon HW, Whipp SC, Argenzio RA, Levine MM, Giannella RA. Attaching and effacing activities of rabbit and human enteropathogenic *Escherichia coli* in pig and rabbit intestines. *Infection and immunity*. 1983; 41:1340–1351. [PubMed: 6350186]
3. Jarvis KG, et al. Enteropathogenic *Escherichia coli* contains a putative type III secretion system necessary for the export of proteins involved in attaching and effacing lesion formation. *Proceedings of the National Academy of Sciences of the United States of America*. 1995; 92:7996–8000. [PubMed: 7644527]
4. Jerse AE, Yu J, Tall BD, Kaper JB. A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. *Proceedings of the National Academy of Sciences of the United States of America*. 1990; 87:7839–7843. [PubMed: 2172966]
5. Kenny B, et al. Enteropathogenic *E. coli* (EPEC) transfers its receptor for intimate adherence into mammalian cells. *Cell*. 1997; 91:511–520. [PubMed: 9390560]
6. Deng W, Vallance BA, Li Y, Puente JL, Finlay BB. *Citrobacter rodentium* translocated intimin receptor (Tir) is an essential virulence factor needed for actin condensation, intestinal colonization and colonic hyperplasia in mice. *Mol Microbiol*. 2003; 48:95–115. [PubMed: 12657048]
7. Nataro JP, Kaper JB. Diarrheagenic *Escherichia coli*. *Clin Microbiol Rev*. 1998; 11:142–201. [PubMed: 9457432]
8. Luperchio SA, Schauer DB. Molecular pathogenesis of *Citrobacter rodentium* and transmissible murine colonic hyperplasia. *Microbes Infect*. 2001; 3:333–340. [PubMed: 11334751]
9. Lupp C, et al. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. *Cell Host Microbe*. 2007; 2:204. [PubMed: 18030708]
10. Kamada N, et al. Regulated virulence controls the ability of a pathogen to compete with the gut microbiota. *Science*. 2012; 336:1325–1329. [PubMed: 22582016]
11. Lopez CA, et al. Phage-mediated acquisition of a type III secreted effector protein boosts growth of salmonella by nitrate respiration. *MBio*. 2012; 3
12. Winter SE, et al. Host-derived nitrate boosts growth of *E. coli* in the inflamed gut. *Science*. 2013; 339:708–711. [PubMed: 23393266]
13. Winter SE, et al. Gut inflammation provides a respiratory electron acceptor for *Salmonella*. *Nature*. 2010; 467:426–429. [PubMed: 20864996]
14. Rivers SL, McNairn E, Blasco F, Giordano G, Boxer DH. Molecular genetic analysis of the *moa* operon of *Escherichia coli* K-12 required for molybdenum cofactor biosynthesis. *Molecular microbiology*. 1993; 8:1071–1081. [PubMed: 8361352]
15. Berg BL, Stewart V. Structural genes for nitrate-inducible formate dehydrogenase in *Escherichia coli* K-12. *Genetics*. 1990; 125:691–702. [PubMed: 2168848]
16. Sawers G, Heider J, Zehelein E, Bock A. Expression and operon structure of the *sel* genes of *Escherichia coli* and identification of a third selenium-containing formate dehydrogenase isoenzyme. *Journal of bacteriology*. 1991; 173:4983–4993. [PubMed: 1650339]
17. Amarasingham CR, Davis BD. Regulation of alpha-ketoglutarate dehydrogenase formation in *Escherichia coli*. *J Biol Chem*. 1965; 240:3664–3668. [PubMed: 5319784]
18. Rivera-Chavez F, et al. Depletion of Butyrate-Producing Clostridia from the Gut Microbiota Drives an Aerobic Luminal Expansion of *Salmonella*. *Cell Host Microbe*. 2016; 19:443–454. [PubMed: 27078066]
19. Albenberg L, et al. Correlation between intraluminal oxygen gradient and radial partitioning of intestinal microbiota. *Gastroenterology*. 2014; 147:1055–1063. e1058. [PubMed: 25046162]
20. Mundy R, et al. Identification of a novel type IV pilus gene cluster required for gastrointestinal colonization of *Citrobacter rodentium*. *Molecular microbiology*. 2003; 48:795–809. [PubMed: 12694622]
21. Lomax RB, McNicholas CM, Lombes M, Sandle GI. Aldosterone-induced apical Na⁺ and K⁺ conductances are located predominantly in surface cells in rat distal colon. *Am J Physiol*. 1994; 266:G71–82. [PubMed: 8304460]
22. Velazquez OC, Lederer HM, Rombeau JL. Butyrate and the colonocyte. Production, absorption, metabolism, and therapeutic implications. *Adv Exp Med Biol*. 1997; 427:123–134. [PubMed: 9361838]

23. Furuta GT, et al. Hypoxia-inducible factor 1-dependent induction of intestinal trefoil factor protects barrier function during hypoxia. *J Exp Med*. 2001; 193:1027–1034. [PubMed: 11342587]
24. Kelly CJ, et al. Crosstalk between Microbiota-Derived Short-Chain Fatty Acids and Intestinal Epithelial HIF Augments Tissue Barrier Function. *Cell Host Microbe*. 2015; 17:662–671. [PubMed: 25865369]
25. Terada N, Ohno N, Saitoh S, Ohno S. Immunohistochemical detection of hypoxia in mouse liver tissues treated with pimonidazole using “in vivo cryotechnique”. *Histochem Cell Biol*. 2007; 128:253–261. [PubMed: 17680263]
26. Collins JW, et al. *Citrobacter rodentium*: infection, inflammation and the microbiota. *Nat Rev Microbiol*. 2014; 12:612–623. [PubMed: 25088150]
27. Wong AR, Clements A, Raymond B, Crepin VF, Frankel G. The interplay between the *Escherichia coli* Rho guanine nucleotide exchange factor effectors and the mammalian RhoGEF inhibitor EspH. *MBio*. 2012; 3
28. Ma C, et al. *Citrobacter rodentium* infection causes both mitochondrial dysfunction and intestinal epithelial barrier disruption in vivo: role of mitochondrial associated protein (Map). *Cell Microbiol*. 2006; 8:1669–1686. [PubMed: 16759225]
29. Nougayrede JP, Donnenberg MS. Enteropathogenic *Escherichia coli* EspF is targeted to mitochondria and is required to initiate the mitochondrial death pathway. *Cell Microbiol*. 2004; 6:1097–1111. [PubMed: 15469437]
30. Fan YY, et al. A bioassay to measure energy metabolism in mouse colonic crypts, organoids, and sorted stem cells. *American journal of physiology Gastrointestinal and liver physiology*. 2015; 309:G1–9. [PubMed: 25977509]
31. Ahmed I, et al. Critical roles of Notch and Wnt/beta-catenin pathways in the regulation of hyperplasia and/or colitis in response to bacterial infection. *Infect Immun*. 2012; 80:3107–3121. [PubMed: 22710872]
32. Chandrakesan P, et al. Novel changes in NF- κ B activity during progression and regression phases of hyperplasia: role of MEK, ERK, and p38. *J Biol Chem*. 2010; 285:33485–33498. [PubMed: 20710027]
33. Caron E, et al. Subversion of actin dynamics by EPEC and EHEC. *Curr Opin Microbiol*. 2006; 9:40–45. [PubMed: 16406772]
34. Frankel G, et al. Enteropathogenic and enterohaemorrhagic *Escherichia coli*: more subversive elements. *Molecular microbiology*. 1998; 30:911–921. [PubMed: 9988469]
35. Carreau A, El Hafny-Rahbi B, Matejuk A, Grillon C, Kieda C. Why is the partial oxygen pressure of human tissues a crucial parameter? Small molecules and hypoxia. *J Cell Mol Med*. 2011; 15:1239–1253. [PubMed: 21251211]
36. Espey MG. Role of oxygen gradients in shaping redox relationships between the human intestine and its microbiota. *Free radical biology & medicine*. 2013; 55:130–140. [PubMed: 23127782]
37. Stewart V, Parales J Jr. Identification and expression of genes narL and narX of the nar (nitrate reductase) locus in *Escherichia coli* K-12. *Journal of bacteriology*. 1988; 170:1589–1597. [PubMed: 2832370]
38. Caporaso JG, et al. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods*. 2010; 7:335–336. [PubMed: 20383131]
39. Huson DH, Mitra S, Ruscheweyh HJ, Weber N, Schuster SC. Integrative analysis of environmental sequences using MEGAN4. *Genome Res*. 2011; 21:1552–1560. [PubMed: 21690186]
40. Robertson CE, et al. Explicet: graphical user interface software for metadata-driven management, analysis and visualization of microbiome data. *Bioinformatics*. 2013; 29:3100–3101. [PubMed: 24021386]
41. Arndt D, et al. METAGENassist: a comprehensive web server for comparative metagenomics. *Nucleic Acids Res*. 2012; 40:W88–95. [PubMed: 22645318]
42. Kamada N, et al. Humoral Immunity in the Gut Selectively Targets Phenotypically Virulent Attaching-and-Effacing Bacteria for Intraluminal Elimination. *Cell Host Microbe*. 2015; 17:617–627. [PubMed: 25936799]
43. Godinez I, et al. Interleukin-23 orchestrates mucosal responses to *Salmonella enterica* serotype Typhimurium in the intestine. *Infect Immun*. 2009; 77:387–398. [PubMed: 18955477]

44. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods*. 2012; 9:671–675. [PubMed: 22930834]
45. Barthold SW, Coleman GL, Bhatt PN, Osbaldiston GW, Jonas AM. The etiology of transmissible murine colonic hyperplasia. *Lab Anim Sci*. 1976; 26:889–894. [PubMed: 1018473]
46. Pal D, Venkova-Canova T, Srivastava P, Chatteraj DK. Multipartite regulation of *rctB*, the replication initiator gene of *Vibrio cholerae* chromosome II. *Journal of bacteriology*. 2005; 187:7167–7175. [PubMed: 16237000]
47. Simon R, Priefer U, Puhler A. A Broad Host Range Mobilization System for *Invivo* Genetic-Engineering - Transposon Mutagenesis in Gram-Negative Bacteria. *Bio-Technology*. 1983; 1:784–791.
48. Wang RF, Kushner SR. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. *Gene*. 1991; 100:195–199. [PubMed: 2055470]
49. Miller VL, Mekalanos JJ. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *Journal of bacteriology*. 1988; 170:2575–2583. [PubMed: 2836362]
50. Kingsley RA, et al. Ferrioxamine-mediated Iron(III) utilization by *Salmonella enterica*. *Appl Environ Microbiol*. 1999; 65:1610–1618. [PubMed: 10103258]
51. Kinder SA, Badger JL, Bryant GO, Pepe JC, Miller VL. Cloning of the *YenI* restriction endonuclease and methyltransferase from *Yersinia enterocolitica* serotype O8 and construction of a transformable R-M⁺ mutant. *Gene*. 1993; 136:271–275. [PubMed: 8294016]
52. Raffatellu M, et al. Lipocalin-2 resistance confers an advantage to *Salmonella enterica* serotype Typhimurium for growth and survival in the inflamed intestine. *Cell Host Microbe*. 2009; 5:476–486. [PubMed: 19454351]

Figure 1. Oxygen respiration supports *C. rodentium* expansion in the mouse colon
(A) C57BL/6 (C57) mice were infected with *C. rodentium* wild type (wt, DBS100) and either a *moaA* mutant (CAL142) or a *napA narG narZ* mutant (CAL93). **(B)** Conventional C57 or germ-free Swiss Webster (SW) mice were infected with wt *C. rodentium* and either a *moaA* mutant, a *fdnG* mutant (CAL210 [pWSK129]), or a *fdoG* mutant (CAL261). *N* is indicated in Fig. S3E. **(C)** Competitive *in vitro* growth (*N*=8) of *C. rodentium* wild type (wt) and a *cydAB* mutant (CAL247) for 16 hours in minimal medium in the presence of the indicated oxygen levels (% O₂). **(D)** Conventional or germ-free mice were infected with an equal mixture of the *C. rodentium* wild type (wt) and a *cydAB* mutant. **(A and D)** *N*=4. **(E)** *C. rodentium* was grown in minimal medium supplemented with mannose as a carbon source under either microaerobic or anaerobic conditions. **(F)** Bacterial RNA was isolated from either mucus scrapings or colon contents of *C. rodentium*-infected mice. **(E–F)** The transcript levels of *sucA* were quantified by real-time PCR, normalized to 16S rRNA levels and shown as fold-changes. *N* is shown in Fig. S4B and S4C. **(G–I)** Mice (*N* indicated in **I**) were either mock-treated, infected with the *C. rodentium* wild type (wt) or with a *cydAB* mutant (CAL247). **(G)** Enumeration of *C. rodentium* by plating on selective media. **(A–G)** Bars represent geometric mean $\pm$ standard error. **(H)** Boxes in Whisker plots represent the second and third quartiles of combined histopathology scores, while lines indicate the first and fourth quartiles. **(I)** Microbial representation at the class-level based on 16S rRNA gene sequencing of colon contents 7 days after infection. Color-coding for classes is shown on the right. *, *P* < 0.05; **, *P* < 0.01; ns, not statistically significantly different.

Figure 2. A functional T3SS increases epithelial oxygenation and drives a CydAB-dependent *C. rodentium* expansion

(A) Mice (*N* is indicated in Fig. S8A) were infected with the indicated *C. rodentium* strain mixtures. Bars represent geometric means of the competitive index (CI) $\pm$ standard error. (B) Representative images of colonic sections stained to detect pimonidazole hypoxia stain (red fluorescence) and counterstained with DAPI nuclear stain (blue fluorescence). Arrow heads point to the mucosal surface. E, epithelial cell; L, lumen. (C) Pimonidazole staining (PMDZ⁺) was quantified by flow cytometry in colonic epithelial (CD45⁻ CD326⁺) cells. White bars indicate the level of background staining observed in mice that were not injected with PMDZ. Bars represent geometric mean $\pm$ standard error. **, $P < 0.01$; *, $P < 0.05$; ns, not statistically significantly different; wt, DBS100; *cydAB*, CAL247; *cfcH*, BMM12; *cfcH cydAB*, BMM11; *eae*, CAL290; *eae cydAB*, CAL291; *escN*, CAL286; *escN cydAB*, CAL287; *espH cesF map*, BMM30; *espH cesF map cydAB*, BMM40.

Figure 3. Colonic crypt hyperplasia drives a CydAB-dependent *C. rodentium* expansion
 (A–C) C57BL/6 mice were mock infected or infected with the *C. rodentium* wild type (wt, DBS100) or an *espH cesF map* mutant (BMM30). (D–F) C57BL/6 mice were mock infected or infected with a 1:1 mixture of the *C. rodentium* wild type (wt) and a *cydAB* mutant (CAL247). Mice were either treated with dibenzazepine (DBZ) or with vehicle control. (A–F) Organs were collected seven days after infection. (A) CFU recovered from colon contents. (B and D) Representative images of colonic sections were stained to detect Ki67 (yellow fluorescence) and counterstained with DAPI nuclear stain (blue fluorescence). (C and E) Ki67 staining was quantified by image analysis. (F) Enumeration of wt and *cydAB* mutant recovered from colon contents was used to calculate the competitive index (CI). (A, C, E and F) Bars represent geometric mean $\pm$ standard error. **, $P < 0.01$; *, $P < 0.05$; ns, not statistically significantly different.