Depletion of Butyrate-Producing Clostridia from the Gut Microbiota Drives an Aerobic Luminal Expansion of Salmonella

Graphical Abstract

Highlights
- Salmonella-induced colitis drives a depletion of butyrate-producing Clostridia.
- Antibiotic-mediated depletion of Clostridia increases colonocyte oxygenation.
- Increased epithelial oxygenation drives an aerobic post-antibiotic pathogen expansion.
- A respiration-driven Salmonella expansion in the gut is required for transmission.

In Brief
Paradoxically, antibiotic treatment can promote relapse of Salmonella gastroenteritis. Rivera-Chávez et al. show that antibiotic treatment lowers colonization resistance by depleting butyrate-producing Clostridia. Decreased butyrate availability increases epithelial oxygenation, thereby fueling aerobic pathogen expansion in the gut lumen. Aerobic respiration synergizes with nitrate respiration to drive fecal-oral transmission of Salmonella.

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Depletion of Butyrate-Producing Clostridia from the Gut Microbiota Drives an Aerobic Luminal Expansion of Salmonella

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SUMMARY

The mammalian intestine is host to a microbial community that prevents pathogen expansion through unknown mechanisms, while antibiotic treatment can increase susceptibility to enteric pathogens. Here we show that streptomycin treatment depleted commensal, butyrate-producing Clostridia from the mouse intestinal lumen, leading to decreased butyrate levels, increased epithelial oxygenation, and aerobic expansion of Salmonella enterica serovar Typhimurium. Epithelial hypoxia and Salmonella restriction could be restored by tributyrin treatment. Clostridia depletion and aerobic Salmonella expansion were also observed in the absence of streptomycin treatment in genetically resistant mice but proceeded with slower kinetics and required the presence of functional Salmonella type III secretion systems. The Salmonella cytochrome bd-II oxidase synergized with nitrate reductases to drive luminal expansion, and both were required for fecal-oral transmission. We conclude that Salmonella virulence factors and antibiotic treatment promote pathogen expansion through the same mechanism: depletion of butyrate-producing Clostridia to elevate epithelial oxygenation, allowing aerobic Salmonella growth.

INTRODUCTION

Non-typhoidal Salmonella enterica serovars, such as S. enterica serovar Typhimurium (S. Typhimurium), are a common cause of human gastroenteritis (Majowicz et al., 2010). Studies using animal models of infection show that upon ingestion, S. Typhimurium uses the invasion-associated type III secretion system (T3SS-1) to enter epithelial cells (Galán and Curtiss, 1989). After crossing the epithelial barrier, the pathogen deploys a second type III secretion system (T3SS-2) to enhance its survival in the underlying tissue (Hensel et al., 1995). T3SS-1-mediated epithelial invasion and T3SS-2-mediated survival in tissue trigger acute intestinal inflammation and diarrhea (Tsolis et al., 1999), the hallmarks of gastroenteritis. Interestingly, intestinal inflammation confers a benefit to the pathogen because it drives its expansion in the lumen of the large bowel (Barman et al., 2008; Stecher et al., 2007), which is required for fecal-oral transmission of S. Typhimurium to the next susceptible host (Lawley et al., 2008).

One mechanism that powers a luminal expansion of S. Typhimurium during colitis is the generation of respiratory electron acceptors as a by-product of the inflammatory host response (Ali et al., 2014; Lopez et al., 2015, 2012; Rivera-Chávez et al., 2013; Thiennimitr et al., 2011; Winter et al., 2010). For example, reactive nitrogen species generated by the inflammatory host response can react to form nitrate, the preferred respiratory electron acceptor of S. Typhimurium under anaerobiosis (Lopez et al., 2015, 2012).

A history of antibiotic usage is a risk factor for developing S. enterica-induced gastroenteritis (Pavia et al., 1990), and antibiotic treatment during convalescence may on occasion produce a bacteriologic and symptomatic relapse (Aserkoff and Bennett, 1969; Nelson et al., 1980). These effects of antibiotics can be modeled in mice, as treatment with streptomycin leads to a marked expansion of S. Typhimurium in the lumen of the murine large intestine (Que and Henges, 1985). The mechanisms contributing to this post-antibiotic pathogen expansion remain poorly understood.

Antibiotic treatment increases epithelial oxygenation in the large intestine (Kelly et al., 2015), which is predicted to elevate diffusion of oxygen into the gut lumen (Espy, 2013). Oxygen is the only respiratory electron acceptor with a higher redox potential than nitrate. Work on oxygen respiration in Escherichia coli suggests that under conditions of high aeration, S. Typhimurium predominantly uses the low-affinity cytochrome bo oxidase encoded by cyoAB (Alexeeva et al., 2003; Cotter et al., 1990; Cotter et al., 1992; Cotter and Gunsalus, 1992; Fu et al., 1991). When the pathogen enters host tissue, it encounters an oxygen partial pressure (pO2) of 23–70 mmHg (3%–10% oxygen), which is considerably lower than the atmospheric pO2 of 160 mmHg (21% O2) (Carreau et al., 2011). S. Typhimurium relies on the high-affinity cytochrome bd oxidase encoded by the cydAB genes to support its growth in tissue during infection of mice (Craig et al., 2013).
Importantly, the S. Typhimurium chromosome encodes another enzyme with homology to cytochrome bd oxidase. This enzyme is termed cytochrome bd-II oxidase and is encoded by the S. Typhimurium cyxAB operon (Atlung and Brendsted, 1994; Dassa et al., 1991; McClelland et al., 2001). Cytochrome bd-II oxidase remains poorly characterized, and its physiological role is not known. Regulation of the cyxAB operon in E. coli suggests that cytochrome bd-II oxidase might have a function under even more oxygen-limiting conditions than cytochrome bd oxidase (Brendsted and Atlung, 1996), but this hypothesis has not been tested. Here we investigated whether cytochrome bd-II oxidase contributes to expansion of S. Typhimurium in the large intestine.

RESULTS

Cytochrome bd and bd-II Oxidases Are Required for Growth in Different Host Niches

We generated S. Typhimurium mutants lacking either a functional cytochrome bd oxidase (cydA mutant) or a functional cytochrome bd-II oxidase (cyxA mutant) to investigate how aerobic respiration affects growth of the pathogen. The S. Typhimurium wild-type exhibited a fitness advantage over the cydA mutant during growth with 8% oxygen, which corresponds to tissue oxygenation (3%–10% oxygen). However, the cydA gene did not confer a fitness advantage during growth with 0.8% oxygen (Figure 1A), which was consistent with repression of the E. coli cydAB operon in low-oxygen environments (Tseng et al., 1996). The cyxA gene was not required for growth with 8% oxygen or with 0% oxygen but provided a fitness advantage during growth with 0.8% oxygen (Figure 1B).

To assess the contribution of cydA and cyxA to growth in host tissue, mice (C57BL/6) were infected intraperitoneally with a 1:1 mixture of the wild-type and the cydA mutant or a 1:1 mixture of the wild-type and the cyxA mutant, respectively (competitive infection). 4 days after infection, the wild-type was recovered in significantly higher numbers from the spleen than the cydA mutant (Figure 1C), confirming that cytochrome bd oxidase contributes to growth in tissue (Craig et al., 2013). In contrast, cyxA was dispensable for growth in tissue.

We next investigated the contribution of cydA and cyxA to post-antibiotic pathogen expansion in the colon contents of mice. To this end, mice (C57BL/6) received a single dose of streptomycin intragastrically and were infected 1 day later with a 1:1 mixture of the wild-type and the cydA mutant or a 1:1 mixture of the wild-type and the cyxA mutant. 4 days after infection, the cydA gene was dispensable for post-antibiotic pathogen expansion, while the cyxA gene conferred a significant ($p < 0.05$) fitness advantage (Figure 1D). Importantly, the cyxA mutant did not confer a fitness advantage in the colon of mice that had not received antibiotics, suggesting that cytochrome bd-II oxidase contributed to post-antibiotic pathogen expansion in the gut (Figure 1D).

Collectively, our data indicated that cydA was exclusively required during growth in host tissue (Figure 1C), while cyxA contributed exclusively to growth under the more oxygen-limited conditions encountered in the antibiotic-treated gut (Figure 1D).

Cytochrome bd-II Oxidase and Nitrate Reductases Synergistically Drive Post-antibiotic Pathogen Expansion

Consistent with previous studies suggesting that nitrate respiration contributes to a post-antibiotic pathogen expansion (Lopez et al., 2015, 2012), the S. Typhimurium wild-type was recovered in approximately 8-fold higher numbers than a nitrate respiration-deficient mutant (narG napA narZ mutant) 4 days after competitive infection of streptomycin-treated mice (C57BL/6) (Figure 1E). To investigate whether nitrate respiration and aerobic respiration cooperated during post-antibiotic pathogen expansion, we constructed a cyxA narG napA narZ mutant and compared its fitness with that of wild-type S. Typhimurium. Recovery of bacteria 4 days after infection revealed a remarkable synergy between nitrate respiration and aerobic respiration, as the wild-type was recovered in approximately 2,000-fold higher numbers than the respiration-deficient cyxA narG napA narZ mutant (Figures 1E and S1A). Importantly, the S. Typhimurium wild-type and cyxA narG napA narZ mutant were recovered in similar numbers from mice that had not received streptomycin, suggesting that nitrate respiration and aerobic respiration cooperated during post-antibiotic pathogen expansion. Similar results were observed when streptomycin-treated mice were infected with individual S. Typhimurium strains (Figure 1F).

Cytochrome bd-II Oxidase-Dependent Aerobic Growth Is Driven by an Antibiotic-Mediated Depletion of Clostridia

The finding that cyxA only conferred a growth advantage upon S. Typhimurium in streptomycin-treated mice suggested that aerobic pathogen expansion required depletion of a component of the gut-associated microbial community (gut microbiota). Analysis of DNA isolated from feces by quantitative real-time PCR with class-specific primers suggested that streptomycin treatment caused a marked depletion of members of the class Clostridia from the gut microbiota within a day (Figure 1G) (Sekirov et al., 2008). By 5 days after streptomycin treatment, the Clostridia population had recovered to levels observed prior to antibiotic treatment. Clostridia are credited for producing the lion’s share of the short-chain fatty acid butyrate, an important fermentation product produced by the gut microbiota (Louis and Flint, 2009; Vital et al., 2014). Cecal butyrate concentrations were diminished by four orders of magnitude 1 day after treatment with streptomycin (Figure 1H). In contrast, streptomycin treatment lowered the concentrations of acetate and propionate in the cecal contents by only one or two orders of magnitude, respectively (Figures S1B and S1C).

We next tested the hypothesis that antibiotic-mediated depletion of Clostridia was responsible for an aerobic post-antibiotic pathogen expansion. To this end, streptomycin-treated mice (C57BL/6) were infected with the wild-type and a cyxA mutant and inoculated 1 day later with chloroform-treated cecal contents of treatment-naive mice. Since chloroform kills vegetative bacterial cells but not spores, this treatment enriches for Clostridia, the dominant group of spore-forming bacteria present in cecal contents (Iloh and Freter, 1989). Inoculation of streptomycin-treated mice with chloroform-treated cecal contents
increased the abundance of *Clostridia* (Figure 1I) and annulled the fitness advantage conferred by cytochrome *bd*-II oxidase (Figure 1E).

**Clostridia Depletion Increases Oxygenation of Colonocytes to Drive a Cytochrome *bd*-II Oxidase-Dependent Aerobic Pathogen Expansion**

We next wanted to investigate how the prevalence of *Clostridia* could alter oxygen availability in the gut. *Clostridia* are the main producers of butyrate (Louis and Flint, 2009; Vital et al., 2014), which serves as the preferred energy source for colonocytes (enterocytes of the colon). Colonocytes oxidize butyrate to carbon dioxide (CO2) (Donohoe et al., 2012), thereby rendering the epithelium hypoxic (<7.6 mmHg or <1% O2) (Kelly et al., 2015). However, in germ-free mice, where butyrate is absent,
colonocytes obtain energy by fermenting glucose to lactate (Donohoe et al., 2012), which is accompanied by an increased oxygenation of the epithelium (Kelly et al., 2015). Since increased oxygenation of the epithelium is predicted to elevate oxygen diffusion into the gut lumen (Espey, 2013), we hypothesized that a depletion of Clostridia-derived butyrate would increase the oxygenation of colonocytes and increase diffusion of oxygen into the gut lumen to fuel a cyxA-dependent S. Typhimurium expansion.

To test this hypothesis, we first investigated whether an antibiotic-mediated depletion of butyrate-producing Clostridia would elevate the oxygenation of colonocytes by using the exogenous hypoxia marker pimonidazole. Under hypoxic conditions, nitroreductase enzymes reduce pimonidazole to hydroxylamine intermediates, which bind irreversibly to nucleophilic groups in proteins or DNA (Kizaka-Kondoh and Konse-Nagasawa, 2009).

Colonocytes of conventional mice (C57BL/6) exhibited hypoxia as indicated by pimonidazole staining (Figure 2A) (Colgan and Taylor, 2010; Kelly et al., 2015). Streptomycin treatment eliminated staining of colonocytes with pimonidazole (Figure 2B), indicative of a marked increase in colonocyte oxygenation. Next, we supplemented streptomycin-treated mice with 1,2,3-tributyrylglycerol (tributyrin), a food additive (butter flavoring) that exhibits delayed absorption in the proximal gut compared to butyrate, which renders it more effective for increasing butyrate concentrations in the large intestine (Kelly et al., 2015).

Remarkably, tributyrin supplementation restored epithelial hypoxia in streptomycin-treated mice (Figure 2C) and significantly (p < 0.001) increased the concentration of cecal butyrate (Figure 2D), while the concentrations of propionate and acetate remained unchanged (Figures 2E and 2F). Thus, a streptomycin-mediated depletion of Clostridia-derived butyrate increased colonocyte oxygenation.

Since streptomycin treatment resulted in depletion of Clostridia and increased oxygenation of colonocytes within 1 day, we...
hypothesized that a cyxA-dependent fitness advantage might already be apparent at early time points after antibiotic treatment. A significant (p < 0.05) fitness advantage conferred by the cyxA gene was apparent just 1 day after infection of streptomycin-treated mice; however, no fitness advantage was observed in the absence of streptomycin treatment (Figure 2G). Remarkably, tributyrin supplementation abrogated the fitness advantage conferred by the cyxA gene in streptomycin-treated mice (Figure 2G). Similar results were obtained with a cyxA napA narZ narG mutant (Figure 2H). Since a benefit of nitrate respiration is not observed 1 day after S. Typhimurium infection of streptomycin-treated mice (Lopez et al., 2015), these results point to cyxA as the main factor driving an early post-antibiotic expansion of S. Typhimurium in the lumen (i.e., 1 day after infection).

At 4 days after infection of streptomycin-treated mice, tributyrin supplementation reduced the fitness advantage conferred by the cyxA napA narZ narG genes to that conferred by the napA narZ narG genes in the absence of tributyrin supplementation (Figure 1E). This outcome was consistent with the idea that tributyrin supplementation only reduced the fitness advantage conferred by cyxA.

**T3SS-1 and T3SS-2 Drive a Cytochrome bd-II Oxidase-Mediated Aerobic Growth at Later Stages of Infection**

To investigate whether virulence factors were required to support luminal growth by aerobic respiration, T3SS-1 and T3SS-2 were inactivated using mutations in the invA and spiB genes, respectively. Similar to what was observed in the wild-type background, a fitness advantage of the avirulent invA spiB mutant over the invA spiB cyxA mutant was observed after 1 day of infection in the fecal contents of streptomycin-treated mice (C57BL/6), but not in mice that had not received antibiotics (Figures 3A and S1A). Thus, virulence factors were not required for cytochrome bd-II oxidase-mediated growth 1 day after infection (corresponding to 2 days after streptomycin treatment). By 4 days after infection with virulent S. Typhimurium strains (i.e., a mixture of wild-type and cyxA mutant), mice developed acute inflammation in the cecal mucosa, while no marked inflammatory changes were observed in mice infected with avirulent S. Typhimurium strains (i.e., a mixture of invA spiB mutant and invA spiB cyxA mutant) (Figures 3B, 3C, and S2). Interestingly, while the virulent S. Typhimurium wild-type exhibited a fitness advantage over a cyxA mutant in colon contents 4 days after infection (corresponding to 5 days after streptomycin treatment), no fitness advantage was observed at this time point when streptomycin-treated mice were inoculated with a 1:1 mixture of the avirulent invA spiB mutant and a invA spiB cyxA mutant (Figure 3A). Thus, virulence factors were required for cytochrome bd-II oxidase-mediated growth at later times after antibiotic treatment.

We next investigated whether the degree of Clostridia depletion differed at later time points after infection between mice inoculated with virulent or avirulent S. Typhimurium strains. Strikingly, by 4 days after infection of streptomycin-treated mice with avirulent S. Typhimurium strains, the abundance of Clostridia had recovered to levels similar to those observed in mock-treated mice (Figure 1), and butyrate levels in the colon were significantly (p < 0.001) elevated compared to concentrations measured 1 day after streptomycin treatment (Figure 3D). In stark contrast, a marked depletion of Clostridia DNA was still observed 4 days after infection with virulent S. Typhimurium strains (Figure 1I), and colonic butyrate concentrations remained at levels observed 1 day after treatment with streptomycin (Figure 3D). While virulence factors reduced butyrate levels by two orders of magnitude (Figure 3D), acetate levels remained unchanged (Figure 3E), and a 5.5-fold change in propionate levels was seen (Figure 3F).

Collectively, these data suggested that virulence factors were unessential for aerobic S. Typhimurium growth during early stages of infection when antibiotics maintained a depletion of Clostridia. However, once the effect of antibiotics wore off, Clostridia levels began to rebound and butyrate concentrations started to rise again. During this recovery from antibiotic treatment, virulence factors contributed to maintaining Clostridia depletion and thus became essential for cyxA-dependent aerobic pathogen expansion.

**Infection with Virulent S. Typhimurium Triggers Dysbiosis Characterized by a Depletion of Clostridia**

Post-antibiotic pathogen expansion is not likely to represent the true physiological role of cytochrome bd-II oxidase, since the cyxAAB operon is conserved among serovars of S. enterica, a species that formed long before the advent of antibiotic therapy. While streptomycin-treatment was solely responsible for cyxA-dependent growth of S. Typhimurium 1 day after infection, a contribution of virulence factors was apparent at 4 days after infection (Figure 3A). We thus reasoned that at later time points after S. Typhimurium infection, the physiological role of cyxA might be apparent even in the absence of antibiotic treatment. Since genetically susceptible C57BL/6 mice become moribund at 5 days after infection, we switched to a genetically resistant mouse lineage (CBA mice) to test this idea.

To investigate whether S. Typhimurium virulence factors could deplete Clostridia in the absence of antibiotic treatment, CBA mice were mock infected or infected with either the S. Typhimurium wild-type or an invA spiB mutant. Measurement of fecal lipocalin 2, an inflammatory marker, suggested that intestinal inflammation peaked between days 10 and 17 after infection and required intact virulence factors (Figure 4A). Transcript levels of Kc (encoding the neutrophil chemoattractant CXCL1), Mip2 (encoding the neutrophil chemoattractant CXCL2), and Il17a (encoding the pro-inflammatory cytokine interleukin [IL]-17A) were significantly elevated in the cecal mucosa at days 10 and 17 after infection with the S. Typhimurium wild-type compared to mock-infected and invA spiB mutant-infected mice (Figures S3A–S3F). Mice infected with the S. Typhimurium wild-type shed numbers of the pathogen in their feces approximately three orders of magnitude higher than mice infected with the invA spiB mutant (Figure 4B). Importantly, intestinal inflammation induced by the S. Typhimurium wild-type was accompanied by a significant (p < 0.05) depletion of Clostridia (Figure 4C).

To get a more detailed view of how S. Typhimurium infection alters the composition of the microbiota in the cecum, its composition was analyzed by 16S rRNA gene sequencing (16S profiling) at 10 and 17 days after infection. Compared to mock-infected mice, both the relative abundance (Figures 4D, S4A, S5, and S6) and the normalized abundance (Figure 4E) of Clostridia were significantly reduced in CBA mice at both 10
and 17 days after infection with the S. Typhimurium wild-type strain (p < 0.0001), but not in invA spiB mutant-infected mice. Conversely, the abundance of members from the highly represented phylum Bacteroidetes remained unchanged during infection with the S. Typhimurium wild-type strain compared to mock-treated mice (Figures 4D, S4B, S5, and S7). Principle coordinate analysis revealed marked changes in the microbial community structure caused by infection with the S. Typhimurium wild-type. Concentrations of butyrate (D), acetate (E), and propionate (F) in colon contents were determined at the indicated time points. For (A)–(C) and (D)–(F), bars represent geometric means ± SE. *p < 0.05; **p < 0.01; ***p < 0.005; ****p < 0.001; ns, not statistically significantly different; wt, S. Typhimurium wild-type.

Figure 3. Virulence Factors Drive a Cytochrome bd-II Oxidase-Dependent Expansion of S. Typhimurium at Later Time Points after Infection (A–C) Groups of C57BL/6 mice (n is shown in Figure S1A) were mock treated or treated with streptomycin (Strep) and infected 1 day later with the indicated strain mixtures. (A) At 1 and 4 days after infection (Days p.i.), the competitive index (CI) in colon contents was determined. (B) Cecal histopathology was scored using tissue collected 4 days after infection from four mice per group using criteria listed in Table S1 (see also Figure S2). Each bar represents data from one individual animal. (C) Representative images of H&E-stained cecal sections scored in (B). All images were taken at the same magnification. (D–F) Groups of C57BL/6 mice were treated with streptomycin or mock treated. 1 day after streptomycin treatment organs, were collected for analysis or mice were mock infected or infected with the indicated S. Typhimurium strain mixtures. Concentrations of butyrate (D), acetate (E), and propionate (F) in colon contents were determined at the indicated time points. For (A)–(C) and (D)–(F), bars represent geometric means ± SE. *p < 0.05; **p < 0.01; ***p < 0.005; ****p < 0.001; ns, not statistically significantly different; wt, S. Typhimurium wild-type.

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A Luminal Aerobic S. Typhimurium Expansion Is Observed in Genetically Resistant Mice in the Absence of Antibiotic Treatment

We next wanted to investigate whether a respiration-dependent expansion could also be observed in the absence of antibiotic treatment. The cyxA narG napA narZ genes did not provide a fitness advantage 5 days after competitive infection of CBA mice; however, at days 10 and 17 after infection, the wild-type displayed a significant fitness advantage over the cyxA narG bacteria (Figure S3D), the latter of which was due to an increased normalized abundance of Enterobacteriaceae (Figure S3E). However, there was no correlation between abundances of Gammaproteobacteria and Bacteroidia (Figure S3D).
napA narZ mutant, and by day 28 after infection the cyxA narG napA narZ mutant had been cleared from colon contents of most mice (Figure 5A).

Infection of CBA mice with individual S. Typhimurium strains revealed that the wild-type was shed in significantly higher numbers in the feces on days 10 and 17 after infection compared to a cyxA mutant (p < 0.05 and p < 0.01, respectively), a narG napA narZ mutant (p < 0.01 and p < 0.05, respectively), or a cyxA narG napA narZ mutant (p < 0.01 and p < 0.0001, respectively) (Figure 5B). At the peak of fecal shedding (day 17 after infection), the cyxA narG napA narZ mutant was shed in significantly lower numbers with the feces than either the cyxA mutant (p < 0.05) or the narG napA narZ mutant (p < 0.05).

To test the hypothesis that a depletion of Clostridia was responsible for a cyxA-dependent pathogen expansion, CBA mice were infected intragastrically with a 1:1 mixture of the S. Typhimurium wild-type and a cyxA mutant. At days 5, 7, and 10 after infection, mice were mock treated or inoculated intragastrically with a community of 17 human Clostridia isolates. The cyxA gene conferred a significant (p < 0.005) fitness advantage at 11 days after infection of mock-treated mice (Figure 6A), which was abrogated in mice inoculated with a community of 17 human Clostridia isolates. Furthermore, S. Typhimurium infection significantly (p < 0.001) diminished cecal butyrate concentrations. While inoculation with a community of 17 human Clostridia isolates resulted in a significant (p < 0.05) increase in butyrate levels (Figure 6B), cecal levels of acetate and propionate remained unchanged (Figures 6C and 6D). Importantly, colonocytes of mock-infected mice exhibited hypoxia (Figure 6E), which was eliminated during S. Typhimurium infection.

**Figure 4. S. Typhimurium Virulence Factors Induce Intestinal Inflammation and Are Required for Clostridia Depletion in the Absence of Antibiotic Treatment**

Groups of CBA mice (n = 6) were mock infected or infected intragastrically with 1 × 10⁹ CFU/animal of the virulent S. Typhimurium wild-type (wt) or the avirulent invA spiB mutant (invA spiB).

(A) Fecal lipocalin-2 levels were determined by ELISA (see also Figure S3).

(B) S. Typhimurium CFU was determined in the feces over time.

(C) Clostridia 16S rRNA gene copy numbers were determined by quantitative real-time PCR.

For (A)–(C), data points represent geometric means ± SE.

(D) Average relative abundances of phylogenetic groupings at the class level determined by 16S profiling of the microbial community present in colon contents on day 10 after infection.

(E) Normalized abundances of members of the class Clostridia in colon contents at the indicated days after infection (days p.i.). Boxes in whisker plots represent the second and third quartiles, while lines indicate the first and fourth quartiles.

(F) Weighted principal coordinate analysis of 16S profiling data in which all reads identified as Enterobacteriaceae were excluded from analysis. For additional analysis of 16S profiling data, see Figures S4–S7.

Each dot represents data from one animal. *p < 0.05; **p < 0.01; ***p < 0.001.
Cytochrome bd-II Oxidase and Nitrate Reductases Are Required for Transmission by the Fecal-Oral Route

Maximal bacterial shedding is relevant because *S. Typhimurium* numbers in the feces need to exceed a threshold of $10^8$ bacteria per gram feces for transmission (Lawley et al., 2008). Interestingly, only the *S. Typhimurium* wild-type always exceeded this critical threshold during infection of CBA mice (Figure 5B). To test the prediction that respiration is required for transmission, we studied transmission of *S. Typhimurium* from infected CBA mice to naive CBA mice. All naive mice cohoused with *S. Typhimurium* wild-type-infected mice started to shed the pathogen within 7 days of cohousing (Figure 7). In contrast, the pathogen could not be isolated from any of the naive mice cohoused with either *cyxA* mutant-infected mice or with *cyxA napG narZ* mutant-infected mice after 7 days of cohousing. After 18 days of cohousing, 33% of naive mice cohoused with *cyxA* mutant-infected mice became colonized with the pathogen. However, none of the naive mice cohoused for 18 days with *cyxA napG narZ* mutant-infected mice became colonized with the pathogen. Thus, aerobic respiration contributed to transmission, but an inability to respire both oxygen and nitrate resulted in complete loss of *S. Typhimurium* transmissibility by the fecal-oral route.

**DISCUSSION**

The intestine is home to a large microbial community that confers benefit by preventing pathogen expansion (Bohnhoff et al., 1954). Streptomycin treatment of mice depletes populations of spore-forming bacteria belonging to the class *Clostridia* (Sekirov et al., 2008), which are the members within the community that are most effective in preventing expansion of commensal *E. coli* in the mouse intestine (Itoh and Freter, 1989). *Clostridia* become depleted at later stages of an *S. Typhimurium* infection in streptomycin-treated mice through a neutrophil-dependent mechanism (Gill et al., 2012). Here, we show that inflammation induced by *S. Typhimurium* virulence factors leads to a depletion of *Clostridia* in genetically resistant mice even in the absence of antibiotic treatment.

Changes in the microbiota composition induced either by streptomycin treatment or by *S. Typhimurium* infection were accompanied by a depletion of the short-chain fatty acid butyrate. Butyrate production by the gut microbiota proceeds through the acetyl-CoA pathway, the glutarate pathway, the 4-aminobutyrate pathway, or the lysine pathway, and the majority of bacteria encoding these pathways are members of the class *Clostridia* (Louis and Flint, 2009; Vital et al., 2014). An antibiotic-induced depletion of short-chain fatty acids has been previously implicated in the loss of colonization resistance against *S. Typhimurium* (Garner et al., 2009; Meynell, 1963). However, the mechanism by which short-chain fatty acids limit pathogen expansion is not fully resolved. Butyrate has a marked influence on host cell physiology because it serves as the main energy source for colonocytes (Donohoe et al., 2012). The main function of the colon is to absorb water by generating an osmotic gradient through the absorption of sodium (Na$^+$). Na$^+$ diffuses along an electrochemical gradient through channels located in the apical membrane of surface colonocytes and is then actively extruded by a Na$^+$ pump (Na$^+$ K$^+$ ATPase) located in their basolateral membrane (Sandle, 1998). The ATP required by surface colonocytes to energize Na$^+$ transport comes from the oxidation of microbiota-derived butyrate to carbon dioxide (CO$_2$) (Velázquez et al., 1997). The oxidation of butyrate to CO$_2$, consumes considerable quantities of oxygen within the host cell, thereby rendering the epithelium hypoxic (<7.6 mmHg or <1% O$_2$).
Importantly, a streptomycin-mediated depletion of Clostridia-derived butyrate changes the energy metabolism of colonocytes toward fermentation of glucose, thereby conserving oxygen within the host cell and increasing oxygenation of the epithelium (Kelly et al., 2015). Since oxygen diffuses freely across biological membranes, increased oxygenation of the epithelium is predicted to elevate oxygen availability in the intestinal lumen (Espy, 2013). Consistent with this idea, we found that streptomycin treatment fueled a cyxA-dependent aerobic expansion of S. Typhimurium, which could be blunted by increasing the abundance of Clostridia or by restoring the physiologic hypoxia of colonocytes through tributyrin supplementation.

Streptomycin treatment depleted Clostridia within a day, while S. Typhimurium infection alone reduced the prevalence of Clostridia within the community more slowly. It has been reported previously that streptomycin treatment can rescue the ability of an S. Typhimurium mutant lacking both T3SS-1 and T3SS-2 to colonize the lumen of the large bowel at early stages of infection (Barthel et al., 2003). Our data indicate that this effect is at least in part due to increased oxygenation of the intestinal epithelium driving a cyxA-dependent aerobic expansion of S. Typhimurium. Interestingly, our data suggest that virulence factors and antibiotic treatment drive pathogen expansion through the same mechanism, namely a depletion of Clostridia. The biological significance of this pathogen expansion is that shedding of the pathogen in high numbers with the feces is required for its successful transmission by the fecal-oral route (Lawley et al., 2008). Consistent with this idea, we found that a respiration-dependent expansion of S. Typhimurium was essential for transmission of the pathogen in the mouse model.

The picture emerging from this study is that S. Typhimurium uses its virulence factors to deplete butyrate-producing Clostridia from the gut-associated microbial community. The resulting increase in epithelial oxygenation drives a cytochrome bd-II oxidase-dependent aerobic expansion of S. Typhimurium within the gut lumen, which synergizes with a nitrate respiration-driven expansion to enhance transmission. This pathogenic strategy is exacerbated by oral antibiotic therapy since it enhances and accelerates Clostridia depletion, which might explain why treatment with oral antibiotics often precedes infection

Figure 6. Depletion of Clostridia Increases Colonocyte Oxygenation and Drives a Cytochrome bd-II Oxidase-Dependent Expansion of S. Typhimurium

Groups of CBA mice were mock infected or infected intragastrically with a 1:1 mixture of the S. Typhimurium wild-type (wt) and a cyxA mutant. At 5, 7, and 10 days after infection, mice were mock inoculated or inoculated with 17 human Clostridia isolates (C17).

(A) The competitive index (CI) in feces or colon contents was determined at the indicated time points.

(B–D) Concentrations of butyrate (B), acetate (C), and propionate (D) in cecal contents are shown. For (A)–(D), bars represent geometric means ± SE.

(E–G) Binding of pimonidazole (red fluorescence) was detected in colonic sections counter-stained with DAPI nuclear stain (blue fluorescence). Representative images are shown. *p < 0.05; ***p < 0.005; ****p < 0.001; ns, not statistically significantly different.
with antibiotic-sensitive *S. enterica* serovars causing human gastroenteritis (Pavia et al., 1990).

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**

The 17 human *Clostridia* isolates were kindly provided by K. Honda (Atarashi et al., 2011; Narushima et al., 2014) and were cultured individually as described previously (Atarashi et al., 2013). *S. Typhimurium* and *E. coli* strains used in this study are listed in Table S1, and culture conditions are described in the Supplemental Experimental Procedures. The plasmids and primers used in this study are listed in Tables S2 and S3, respectively. Construction of *S. Typhimurium* mutants and plasmids is described in the Supplemental Experimental Procedures.

**Animal Experiments**

All experiments in this study were approved by the Institutional Animal Care and Use Committee at the University of California at Davis.

Female C57BL/6 mice, aged 8–12 weeks, and female CBA mice, aged 6–8 weeks, were obtained from The Jackson Laboratory (C57BL/6J mice; CBA/J mice). C57BL/6 mice were treated with 20 mg/animal streptomycin or mock treated and orally inoculated 24 hr later with *S. Typhimurium* strains as described in the Supplemental Experimental Procedures. For tributyrin supplementation, mice were mock treated or received tributyrin (5 g/kg) by oral gavage 3 hr after infection. CBA mice were inoculated with *S. Typhimurium* strains as described in the Supplemental Experimental Procedures. For microbiota analysis, mice were euthanized at 10 and 17 days after infection, and DNA from the cecal contents was extracted using the PowerSoil DNA Isolation kit (Mo-Bio) according to the manufacturer’s protocol. Generation and analysis of sequencing data are described in detail in the Supplemental Experimental Procedures. Transmission was studied as described previously (Lawley et al., 2008) and is described in detail in the Supplemental Experimental Procedures. Inoculation of mice with a community of 17 *Clostridia* strains or with spore preparations is described in the Supplemental Experimental Procedures. In some experiments Pimonidazole hydrochloride (PMDZ) (Hypoxyprobe) was administered by intraperitoneal injection at a dosage of approximately 60 mg/kg body weight 60 min prior to euthanasia.

**Analysis of Animal Samples**

Hypoxia staining was performed as described previously (Kelly et al., 2015) and is described in detail in the Supplemental Experimental Procedures. Blinded evaluation of histopathological changes was performed as described previously (Spees et al., 2013) using the criteria listed in Table S4. Short-chain fatty acid concentrations in cecal and colon contents were determined by mass spectrometer as described in the Supplemental Experimental Procedures. RNA was isolated from tissue using standard methods (see Supplemental Experimental Procedures for details) and 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 (Table S2) at a final concentration of 0.25 mM. Absolute values were calculated using a plasmid carrying the cloned gene to generate a standard curve using concentrations ranging from 10^8 to 10^1 copies/ml diluted in a 0.02 mg/ml yeast RNA (Sigma) solution.

**Statistical Analysis**

Fold changes of ratios (bacterial numbers or mRNA levels) were transformed logarithmically prior to statistical analysis. An unpaired Student’s t test was used to determine whether differences in fold changes between groups were statistically significant (p < 0.05). Significance of differences in histopathology scores was determined by a one-tailed non-parametric test (Mann-Whitney).

**ACCESSION NUMBERS**

The accession numbers for the data reported in this paper are NCBI BioSample: SAMN04543794, SAMN04543795, SAMN04543796, SAMN04543797, SAMN04543798, SAMN04543799, SAMN04543800, SAMN04543801, SAMN04543802, SAMN04543803, SAMN04543804, SAMN04543805, SAMN04543806, SAMN04543807, SAMN04543808, SAMN04543809, SAMN04543810, SAMN04543811, SAMN04543812, SAMN04543813, SAMN04543814, SAMN04543815, SAMN04543816, SAMN04543817, SAMN04543818, SAMN04543819, SAMN04543820, SAMN04543821, SAMN04543822, SAMN04543823, SAMN04543824, SAMN04543825, SAMN04543826, SAMN04543827, SAMN04543828.
SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2016.03.004.

AUTHOR CONTRIBUTIONS

F.R.-C., L.F.Z., F.F., C.A.L., M.X.B., and E.E.O. performed and analyzed the experiments. G.X., C.B.L., and S.E.W. performed experiments. F.R.-C. and A.J.B. were responsible for the overall study design and for writing the manuscript.

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