RESEARCH PAPER/REPORT

Opioid use potentiates the virulence of hospital-acquired infection, increases systemic bacterial dissemination and exacerbates gut dysbiosis in a murine model of *Citrobacter rodentium* infection

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ABSTRACT

Opioid analgesics are frequently prescribed in the United States and worldwide. However, serious side effects such as addiction, immunosuppression and gastrointestinal symptoms limit their use. It was recently demonstrated that morphine treatment results in a significant disruption in gut barrier function, leading to an increased translocation of gut commensal bacteria. Further studies have indicated distinct alterations in the gut microbiome and metabolome following morphine treatment, contributing to the negative consequences that are associated with opioid use. However, it is unclear how opioids modulate gut homeostasis in the context of a hospital-acquired bacterial infection. *Citrobacter rodentium* is an ideal murine model of human infections with enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC). In the current study, a mouse model of *C. rodentium* infection was used to investigate the role of morphine in the modulation of gut homeostasis in the context of a hospital-acquired bacterial infection. Morphine treatment resulted in 1) the promotion of *C. rodentium* systemic dissemination, 2) an increase in the expression of the virulence factors of *C. rodentium* colonization in intestinal contents, 3) altered gut microbiome, 4) damaged integrity of gut epithelial barrier function, 5) inhibition of the *C. rodentium*-induced increase in goblet cells, and 6) dysregulated IL-17A immune response. This study demonstrates and further validates a positive correlation between opioid drug use/abuse and an increased risk of infections, suggesting that the overprescription of opioids may increase the susceptibility to hospital-acquired infection.

Introduction

Risk of lethal bacterial infection is a tremendous concern for hospitalized patients. There is higher mortality in opioids-treated patients with a diagnosis of sepsis. High doses of opioids are associated with increased risk of newly acquired infections in patients in critical care units (ICU). Opioids induce immunosuppression and bowel dysfunction, leading to an increased susceptibility to bacterial and opportunistic infections. Chronic morphine has also shown to lower host defense to infections with enteric bacteria such as *Salmonella enterica* and *Pseudomonas aeruginosa*. It has been demonstrated that opioids induce spontaneous sepsis in mice, and increase mortality following *Acinetobacter baumannii* infection or lipopolysaccharide (LPS) treatment in mice. We have recently shown that morphine induces compromised intestinal barrier and sustained systemic inflammation, resulting in gut microbial disruption, bacterial translocation, and bile dysregulation in mice. Further studies in mice have shown that opioids induce alterations of gut microbiome and metabolome. Intensive care unit (ICU) patients treated with opioids demonstrates intestinal dysbiosis and higher risk of infection with antibiotic-resistant bacteria. However, the mechanism under which opioids increase hospital-acquired infection remains unknown. The aim of the study is to investigate if opioids use increases bacterial virulence, intestinal dysbiosis and immune dysfunction resulting in greater susceptibility to a common hospital-acquired infection.

Enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *Escherichia coli* (EHEC) are important causes of diarrheal disease and are human
specific.\textsuperscript{15} \textit{C. rodentium} is an ideal murine model of human EPEC and EHEC infections in that they share pathological features of attaching and effacing (A/E) lesions, colonic hyperplasia, and diarrhea.\textsuperscript{16-18} It has been well demonstrated that virulence factors such as translocated intimin receptor (Tir) and the virulence gene regulator Ler protein are expressed and required for \textit{C. rodentium} colonization and invasion on the gut epithelium surface in the early stage of intestinal infection.\textsuperscript{19,20} \textit{C. rodentium} virulence factor-mediated adhesion dysregulates host immune responses, which further disrupts gut microbiota and promotes the outgrowth of pathogenic bacteria.\textsuperscript{21-23} Other than the bacterial virulence, the interaction between gut microbiota and intestinal epithelial surface plays important roles in preventing the outgrowth of pathogenic organisms and maintaining gastrointestinal homeostasis.\textsuperscript{24-26} The impairment of gut commensal microbiota contributes to pathogenic bacteria colonization.\textsuperscript{25,27} Pathogen bacterial colonization and invasion are always accompanied by the collapse of host homeostasis by dysregulating immune response and disrupting gut barrier function.\textsuperscript{28,29} It has been known that wild type mice are able to clear \textit{C. rodentium} infection.\textsuperscript{20} Regulation of the virulence factor-mediated colonization and commensal-driven pathogen eradication may be potential therapeutic strategies.

Opioids use is associated with greater risk of hospital-acquired infections.\textsuperscript{3,4} However, it is unclear whether and how morphine modulates the pathophysiologically important functional changes in bacterial virulence and host defense. Here, by using a morphine mouse model in the context of infection with \textit{C. rodentium}, we determined the effects of morphine on \textit{C. rodentium} infection and host gut homeostasis.

Results

1) Morphine treatment increases \textit{C. rodentium} systemic dissemination

A high risk of bacterial infection in hospitalized patients is a significant concern, especially in patients who are on opioids for pain management.\textsuperscript{2,30} To determine whether morphine treatment can increase the dissemination of \textit{C. rodentium} following infection, we infected mice with \textit{C. rodentium} at day 1 post morphine treatment, mimicking bacterial infections in opioids users. C57/Bl6j wild type mice were subcutaneously implanted with 25 mg morphine or placebo pellet. Mice were infected with 200 \(\mu\)l (~10\(^9\)) \textit{C. rodentium} through oral gavage right after morphine treatment. Mesenteric lymph nodes (MLNs), spleen, and liver were collected and homogenized in 1 ml, 2 ml, and 5 ml PBS, respectively, at day 5 post-infection. After this, 100 \(\mu\)l suspensions and 100 \(\mu\)l collected blood were cultured on LB agar plates with 50 \(\mu\)g/ml nalidixic acid overnight at 37°C. The colony forming units (CFUs) were counted. At day 5 post \textit{C. rodentium} infection, placebo-implanted mice showed very few colonies growing on the antibiotic-selective LB agar plates, indicating no systemic dissemination. However, mice receiving morphine revealed an increased number of CFUs, indicating the systemic dissemination of \textit{C. rodentium} into the mesenteric lymph nodes, spleen, liver and blood circulation (Figure 1).

2) Morphine treatment facilitates bacterial adherence

Bacterial adherence to the gut epithelial surface is required in the early stage of intestinal bacterial infection.\textsuperscript{31,32} To determine whether bacterial adherence to the intestines was increased by morphine treatment, we determined commensal bacterial adherence to the intestines following morphine treatment. C57/Bl6j wild type mice were subcutaneously implanted with 25 mg morphine or placebo pellet. After 24 h, the duodenum, jejunum, ileum, and colon were collected and washed then homogenized in 5 ml PBS. After this, 100 \(\mu\)l tissue suspensions were cultured on blood agar plates overnight at 37°C. Bacterial colonies were quantified and described as colony forming units (CFUs). Compared with placebo treatment, morphine increased the adherence of commensal bacterial to the jejunum, ileum, and colon but not to the duodenum (Figure 2(a)). To visualize the bacterial adherence to intestinal epithelium, we determined bacterial adherence through fluorescence \textit{in situ} hybridization (FISH) on frozen tissue sections by using the FITC-labeled universal probe EUB-388. The small intestines were excised and processed to 5 \(\mu\)m thick cryostat sections for FISH staining. Blue color indicates the
staining of nuclei with DAPI, and the green color indicates bacterial staining with FITC (Figure 2(b, c)). The FISH results revealed that morphine treatment increased the adherence of C. rodentium to the small intestines and colon when compared to that of the placebo treatment (Figure 3).

(3) Morphine treatment increases C. rodentium virulence

Pathogen colonization in the intestine has been shown to be controlled by bacterial virulence and to compete with the gut commensal microbiota.\(^{20,33}\) To investigate whether morphine treatment increases C. rodentium virulence, we determined the mRNA levels of the virulence factors ler and tir at day 5 post-infection by quantitative polymerase chain reaction in the fecal pellets of mice that were infected with C. rodentium. The mRNA expression of each gene was normalized to that of the 16S rRNA genes. The expression levels of the virulence factors Ler and Tir were significantly increased in the morphine treatment group when compared to those of the placebo. Similar effects were seen in fecal samples that were obtained from the small intestines, cecum, and colon (Figure 4).

(4) Morphine increases C. rodentium load in fecal matter and shifts gut microbiome.

Since the expression of ler and tir is essential for pathogen colonization in the intestines of mice, we next asked the question of whether morphine can increase C. rodentium growth in the intestinal contents and modulate the gut microbiome in the context of C. rodentium infection. To determine the C. rodentium load, we collected the fecal matter and homogenized it in PBS at day 5 post-infection. Then, 100 μl suspensions were cultured on LB agar plates with kanamycin overnight at 37°C. Colony forming units (CFUs) were quantitated. The results revealed that morphine treatment increases C. rodentium growth in intestinal contents (Figure 5). Fecal bacterial DNA was isolated and purified for the microbiome analysis. The results of the alpha diversity analysis revealed that, when compared to placebo, C. rodentium infection decreased the alpha diversity
However, morphine treatment did not further decrease the alpha diversity of the gut microbiome in the context of infection (Figure 6(a)). The results of the beta diversity analysis revealed that morphine treatment shifts the gut microbiome in the context of *C. rodentium* infection, indicating that the relative bacterial abundance and composition were changed (Figure 6(b)).

(5) Morphine treatment disrupts the integrity of epithelial barrier function in the context of *C. rodentium* infection

The gut epithelial barrier contains only a single layer of epithelium, which is necessary for it to absorb nutrients. Thus, intestinal immune defense is designed to allow for the immune surveillance of pathogens while operating with minimal disruption to the absorptive function of the gut. To understand early events that occur in morphine-treated mice that can precipitate an increased *C. rodentium* infection, we evaluated the extent of inflammation and epithelial damage at day 5 post-infection. A histologic evaluation of the extent of epithelial damage revealed that...
morphine treatment disrupts the morphological structure of the intestinal mucosal surface (Figures 7 and 8). A histological evaluation of the tight junction protein ZO-1 on sections of jelly rolled small intestines and colons revealed that morphine treatment decreased the organization of tight-junctions between the intestinal epithelial cells (Figure 9). However, C. rodentium infection alone did not result in any histological damage to the intestinal mucosal epithelial integrity or to barrier function.

(6) Morphine treatment inhibits the C. rodentium-induced increase of goblet cells.

Goblet cells secrete mucus to protect the lining of the intestine. To determine whether morphine modulates goblet cells in the mucosal lining of the small intestine and large intestines, we evaluated goblet cells in intestinal sections with Alcian staining on jelly rolled intestinal sections. In each section, goblet cells were counted on 20 microvilli on the epithelial surface of the small and large intestines. Following Alcian blue staining, an intensely blue material, interpreted as mucus, is evident within the goblet cells lining intestinal epithelium. A faint blue material (mucus) is also present within the adjacent intestinal lumen. The results of the Alcian staining revealed that morphine treatment can inhibit the C. rodentium induced increase in the number of goblet cells in the small intestines and large intestines, although morphine alone does not decrease the number of goblet cells when compared to those of the placebo (Figures 10 and 11).

(7) Morphine treatment disrupts C. rodentium induced IL-17A immune response

At the early phase of infection, C. rodentium induces an IL-17 dependent bacterial clearance in wild type mice. To determine whether morphine treatment modulates C. rodentium induced IL-17 immune response in mice, we determined the IL-17 immune response by flow cytometry. Cells from the mesenteric lymph nodes (mLNs) were isolated and fixed for flow cytometry. CD4+, CD17A+, and CD17F+ were stained for analysis. The flow cytometry results revealed that morphine treatment disrupts the C. rodentium induced IL-17A
immune response at day 5 post-infection (Figure 12 (a,c,d)). Morphine treatment also decreased the percentage of CD4+ cells in the mesenteric lymph nodes in the context of C. rodentium infection (Figure 12(c)). Neither morphine treatment nor C. rodentium infection regulated the IL-17F immune response (Figure 12(b)).

Discussion

Opioid users and abusers are at a higher risk of infectious diseases.3,30 Humans and animals that are treated with opioids exhibit a greater susceptibility to enteric infections by strains such as C. difficile, Listeria monocytogenes, Pseudomonas aeruginosa, Salmonella enterica, and Vibrio cholerae.5,7,39-41 To date, opioid analgesics are the most commonly prescribed medications for pain management.3 As such, the correlation between opioids use and the increased susceptibility to pathogenic strains needs to be well investigated. When orally inoculated into wild type mice, C. rodentium is unable to colonize their hosts and cause pathologic lesions.20 In wild type mice, commensal gut microbiota can outcompete the invading C. rodentium and inhibit their colonization in the host. An intact intestinal barrier function and host immune response to pathogens may also contribute to the eradication of C. rodentium from wild type mice. Virulence factor-mediated adhesion to gut epithelium plays an important role in the early stage of infection.42,43 In the present study, we demonstrate for the first time that morphine increases C. rodentium virulence and potentiates its systemic dissemination in wild type mice.

In this study, we utilize the C. rodentium mouse model of hospital-acquired bacterial infection to determine whether morphine treatment increases C. rodentium virulence factors, Tir and Ler.27 While establishing colonization, C. rodentium delivers a variety of virulence factors, such as

![Figure 4. Morphine increases the mRNA expression of the virulence factors of C. rodentium. (a) ler and (b) tir mRNA levels were determined by quantitative polymerase chain reaction in the fecal pellets of mice that were infected with C. rodentium at day 5 post-infection. Data represent mRNA expression relative to that of the 16S rRNA gene. The results are the means ± SEM of individual mice (n = 4). The results are representative of at least two experiments. *P<.05, **P<.01.](image)

![Figure 5. Morphine increases the bacterial load of C. rodentium in the intestinal contents at day 5 post-infection. WT mice (n = 7) were infected with C. rodentium (~10^9), and the pathogen loads in feces were determined over the indicated time. Mice were subcutaneously treated with placebo or 25 mg morphine pellet for 24 hr prior to infection. Data points are the means ± SEM. The results are representative of at least two independent experiments. **P < .01, Student's t test.](image)
translocated intimin receptor (Tir), into the epithelial cells through the type III secretion system to facilitate its adherence and invasion.\textsuperscript{17,18}

The expression of most virulence genes in \textit{C. rodentium} is controlled by a regulator, Ler protein.\textsuperscript{44} It has been demonstrated that morphine treatment can activate the virulence factors of \textit{Pseudomonas} and induce gut-derived sepsis.\textsuperscript{7}

Increased bacterial virulence may promote \textit{C. rodentium} colonization in the gut and result in microbial dysbiosis. It has been shown in our study that morphine treatment increases the \textit{C. rodentium} growth in the gut and changes the diversity feature of gut microbiota. \textit{C. rodentium} infection and morphine treatment result in a distinct gut microbiome. In return, microbial dysbiosis enhances bacterial infections.\textsuperscript{45}

Mutual beneficial microbe–host interactions maintain gut homeostasis. The disruption of barrier function and increased growth of pathogenic bacteria are significant disturbances to gut homeostasis. This study showed that morphine treatment increases bacterial attachment to intestines in wild type mice in comparison with those who received the placebo treatment, suggesting that morphine can modulate the interaction of microbiota and intestinal surface. Our study shows that morphine treatment resulted in an increased expression of the virulence factors Ler and Tir.

The FISH visualization of \textit{C. rodentium} on intestinal cryostat sections has shown that morphine treatment increases the infiltration/adherence of bacteria to the gut epithelium. \textit{C. rodentium} infection alone is unable to damage tight junction

Figure 6. Morphine treatment alters the gut microbiome in the context of \textit{C. rodentium} infection. (a) Alpha diversity was assessed by using the chao1 index. (b) T-tests were conducted on the chao1 index. (c) Principal coordinates analysis (PCoA) of samples using the UniFrac metric at the OTU level. (d) UniFrac distance significant tests were performed using QIIME. ** indicates significantly different with a \textit{P} value<.01.
protein ZO-1 or to disrupt intestinal barrier function at day 5 post-infection indicating *C. rodentium* infection alone does not result in histological damage to the intestinal mucosal epithelial integrity or barrier function. Morphine treatment disrupts epithelial barrier function and results in impaired morphologic mucosal structure in the context of *C. rodentium* infection. The histological evidence reveals that the disruption of intestinal barrier function by morphine treatment may contribute to the systemic dissemination of *C. rodentium*.

Intestinal mucus is secreted by goblet cells in the epithelial lining of the mucosal surface and forms the first line of host defense against invading pathogens, preventing pathogen colonization.
and removing the adherent load from the mucosal surface.\textsuperscript{7,46} Commensal microbiota facilitates host barrier function through the upregulation of the mucus layer.\textsuperscript{47} \textit{C. rodentium} induces an increased number of goblet cells; however, morphine treatment decreases the amount of goblet cells in the villi of the mucosal epithelial surface in the context of \textit{C. rodentium} infection, indicating that mucus excreting goblet cells are attenuated. How morphine modulates goblet cells is still unclear.

Gut homeostasis is a balanced, mutually beneficial state that is composed of intestinal commensal microbiota and the host immune system.\textsuperscript{48} Innate barriers ensure a tolerant immune response to the microbiota.\textsuperscript{35} At the early phase of infection (4–7 days post-
infection), *C. rodentium* induces an IL-17 production. It is well demonstrated that robust IL-17A secretion is crucial for *C. rodentium* clearance. Furthermore, it has become clear that a rapid IL-17A-dependent innate immune response is necessary for maintaining gut homeostasis. Morphine-treated mice with *C. rodentium* infection exhibited low CD4+ T cell counts and decreased CD4+ T cell expression of the proinflammatory cytokine IL-17A, compared with those of the infected placebo group. This study demonstrates that

**Figure 9.** Morphine treatment disrupts the organization of ZO-1 tight junctions in the epithelium. Wild type mice were subcutaneously implanted with 25 mg morphine pellet and orally infected with *C. rodentium*. The small intestines and colons were excised and fixed. Images were analyzed by a Leica fluorescence microscope.
morphine treatment inhibits the C. rodentium-induced IL-17A immune response, thus suppressing gut mucosal immune protection against invading C. rodentium. A previous study in our laboratory showed that morphine treatment suppressed host defense against Streptococcus pneumoniae by disrupting IL-23/IL-17 immune axis. It is currently unclear whether the morphine-induced modulation of the host immune system results in gut microbial dysbiosis or whether morphine-induced dysbiosis contributes to an altered mucosal immunity. Morphine-induced gastrointestinal disorders such as constipation elicit the complex of the intestinal epithelial interface, host immune, and gut microbiota. These are areas of future studies that need to be systemically elucidated.

A proinflammatory response to a pathogen infection improves bacterial clearance and is part of the self-protective mechanism of host defense. However, inflammation may also lead to an over-reactive or dysregulated immune response that disrupts homeostasis and damages the host physiological function. Similarly, gut commensal microbiota, which are characterized by their adaptability and resilience against perturbations, play an important role in the maintenance of gut homeostasis, and they can also outcompete invading pathogens. However, highly virulent pathogens are capable of colonization of the gut, adherence to the mucosal epithelium, and translocation to systemic circulation. During this process, invading pathogens result in microbial dysbiosis, which therefore leads to a dysregulated immune response and intestinal barrier dysfunction. Thus, pathogenesis needs to be considered in the context of infection and disease and is highly dependent on host–microbe interactions. Hence, the overall

**Figure 10.** Morphine reduces the C. rodentium infection-induced proliferation of goblet cells in small intestine. Alcian blue staining of additional sections of the small intestines resulted in the bright blue staining of the cytoplasm of the goblet cells lining the intestinal epithelium. Goblet cells were evaluated by Alcian staining on jelly rolled intestinal sections. In each section, goblet cells were counted on 20 microvilli on the epithelial surface of large intestines. Significance tests were performed with Student’s t tests. *P ≤ 0.05, **P ≤ 0.01.
outcome depends on the resiliency and the stability of the host microbiome and the ability of the host to contain and eliminate the infection. We speculate that morphine treatment results in a decrease in the overall stability and resilience of the gut microbiome, which leads to a compromised mucosal immune system that allows for bacterial dissemination and colonization in systemic compartments. Thus, therapeutic strategies that are targeted at the gut microbiome may be a potentially noninvasive method for reducing the comorbidities that are associated with opioid use for pain management.

To summarize, we report that morphine treatment in a mouse model of *C. rodentium* infection results in 1) the promotion of *C. rodentium* systemic dissemination, 2) an increase in the expression of virulence factors and *C. rodentium* colonization in intestinal contents, 3) altered gut microbiome, 4) damaged integrity of gut epithelial barrier function, 5) goblet cell differentiation, and 6) a dysregulated IL-17A immune response. This is the first study to demonstrate that morphine promotes pathogen dissemination in the context of *C. rodentium* intestinal infection. This study demonstrates, further validates, and establishes a positive correlation between opioid drug use/abuse and an increased risk of infection. These results indicate that morphine modulates the virulence factor-mediated adhesion of pathogenic bacteria and induces the disruption of mucosal host defense during *C. rodentium* intestinal infection in mice, suggesting that the overprescription of opioids may increase the risk of the emergence of

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**Figure 11.** Morphine reduces the *C. rodentium* infection-induced proliferation of goblet cells in the colon. Alcian blue staining of additional sections of the large intestines resulted in bright blue staining of the cytoplasm of the goblet cells lining the intestinal epithelium. Goblet cells were evaluated with Alcian staining on jelly rolled intestinal sections. In each section, goblet cells were counted on 20 microvilli of the epithelial surface of large intestines. Significance tests were performed with Student’s t tests. *P*≤ 0.05, **P*≤ 0.01.
pathogenic strains, and opioids should therefore be used cautiously.

Materials and methods

Experimental animals

Pathogen-free C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). All mice were female and 8–10 weeks old. A maximum of four mice were housed per cage. Food and tap water were available ad libitum. The animal housing facilities were maintained on a 12-h light/dark cycle with a constant temperature (72 ± 1°F) and 50% humidity. All animals were maintained in specific-pathogen-free facilities, and all procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee (IACUC). The IACUC protocol number was 1203A11091. All procedures were

Figure 12. Morphine disrupts the infection-induced IL-17A immune response. Flow cytometry analysis of cells isolated from mesenteric lymph nodes (mLN). WT mice were subcutaneously treated with placebo or 25 mg morphine pellet for 24 hr prior to infection. Mice were sacrificed at day 5 post-infection. (N ≥ 4), P < .05, Student’s t test.
conducted in line with the guidelines set forth by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Bacterial strains**

The *C. rodentium* strain (DBS 100) was obtained from American Type Culture Collection (ATCC; Manassas, VA). Nalidixic acid resistant *C. rodentium* was selectively screened on LB agar plates with 20 μg/ml nalidixic acid. A spontaneous mutant of *C. rodentium* DBS100 that was resistant to nalidixic acid (20 μg/ml) was obtained by growing DBS100 at 37°C in LB medium supplemented with nalidixic acid at 5 μg/ml for 7 h before spreading the liquid culture onto LB agar plates that were supplemented with nalidixic acid at 20 μg/ml. The selective LB agar plates and broth were made by adding up to 20 μg/ml nalidixic acid to selectively culture *C. rodentium* in the future study. The nalidixic acid solution was made with Mili-Q water in fume hood, and the stock solution was sterilized through a 0.22 μm filter using a 10-ml syringe in the sterile hood. Kanamycin-resistant *C. rodentium* was obtained from the laboratory of Dr. Bruce A. Vallance at the University of British Columbia, Vancouver, Canada. Bioluminescent strains of *C. rodentium* were constructed by introducing the plasmid pT7, which carried the entire lux operon from *Photorhabdus luminescens*. For selective culture of *C. rodentium*, kanamycin (SKU 60615, Sigma) was added into the Luria-Bertani broth or agar plates at a concentration of 30 μg/ml.

**Animal treatment and bacterial oral infection**

Mice received morphine through the pellet implantation method, as previously described. Using this method, plasma levels of morphine were maintained in the 0.6–2.0 μg/ml range (range observed in opioid abusers and patients on opioids for moderate to severe pain). Furthermore, this model is commonly used in studies of opiate dependence and addiction. Briefly, placebo or 25 mg morphine pellets (National Institutes of Health [NIH]/National Institute on Drug Abuse [NIDA], Bethesda, MD) were inserted in a small pocket on the dorsal side of animals that was created by a small skin incision; incisions were closed using surgical wound clips (Stoelting, 9 mm stainless steel, Wooddale, IL). Mice were subcutaneously treated with placebo or 25 mg morphine pellet for 24 h prior to infection. Mice were infected with 1x10⁹ (CFU value) *C. rodentium* in 200 μl medium via oral gavage. Mice were sacrificed for study at day 5 post-infection with *C. rodentium*.

**Colonial forming units**

Bacteria were cultured on blood agar plates without antibiotics or on LB agar plates with 20 μg/ml nalidixic acid or 30 μg/ml kanamycin overnight at 37°C.

**Histology**

Tissues were harvested and preserved in 10% buffered formalin and embedded in paraffin. Paraffin specimens were cut into 5 μm sections and mounted on microslides. Hematoxylin and eosin (H&E) and Alcian blue staining were performed by the Comparative Pathology Shared Resource (CPSR and Bionet) at the University of Minnesota, and the slides were imaged using a Leica DM5500 B microscope (Leica biosystems, Buffalo Grove, IL). Representative images are shown.

**Fluorescence in situ hybridization (FISH)**

The detection of commensal bacteria was performed with Alexa-Fluor 488 (FITC)-labeled Eub338 (5’-GCT GCC TCC CGT AGG AGT-3’), which is a universal probe that is complementary to the 16s RNA of virtually all bacteria, and the infiltration of bacteria into the colonic epithelium was determined. This allows the direct visualization of bacteria within the colonic mucosa. The blue color indicates nuclei staining with DAPI; the green color indicates bacteria staining with FITC.

Detection of *C. rodentium*: Paraffin-embedded sections were deparaffinized and rehydrated as described above. Sections were incubated overnight at 37°C in the dark with a Texas red-conjugated EUB338 general bacterial probe (5’-GCT GCC TCC CGT AGG AGT-3’) and an AlexaFluor 488
conjugated GAM42a probe (5′-GCC TTC CCA CAT CGT TT-3′) that recognizes bacteria that belong to the γ-Proteobacter class; both probes were diluted to a final concentration of 2.5 ng/μl each in the hybridization solution (0.9 M NaCl, 0.1 M Tris pH 7.2, 30% formamide, 0.1% SDS). Sections were then washed once in the dark with the hybridization solution for 15 min with gentle shaking. This step was repeated once with wash buffer (0.9 M NaCl, 0.1 M TRIS pH 7.2), and sections were placed in nuclease-free H2O and then mounted using ProLong Gold Antifade reagent with DAPI (Molecular Probes) and imaged using a Leica DM5500B Microscope.

**Fecal sample collection and DNA extraction**

Stool samples were collected in 1.7 ml RNase/DNase-free tubes (Catalog #: C-2170, Denville Scientific, Holliston, MA, USA) at various time points. The fecal samples were immediately frozen on dry ice and then stored at −80°C; DNA extractions from the fecal matter were carried out using the PowerSoil DNA isolation kit (Catalog #: 12888–100, MO BIO Laboratories, Carlsbad, CA, USA). All extracted DNA samples were stored at −80°C until amplification.

**Quantitative real-time PCR amplification for detection of virulence genes**

Fecal DNA samples (25 ng) were used as templates for PCR amplification of the virulence genes-specific primers. All samples were run in triplicate, and relative mRNA expression levels were determined after normalizing all values to 16S rRNA. 16S rRNA gene universal primers used were as follows: rrsA (16SRNA); 5′-AGG CCT TCG GTG TGT AAA GT-3′ and 5′-ATT CCG ATT AAC GCT TGC AC-3′. Ler F 5′-AAT ATA CCT GAT GGT CTT CCT CAT TCA ATA ATG CTT CTT-3′; Tir F 5′-TAC ACA TTC GGT TAT TCA GCA G-3′; R 5′-GAC ATC CAA CCT TCA GCA TA-3′. The qPCR program was as follows: (i) initial denaturation at 95°C (10 min) and (ii) 45 cycles of 95°C (15 sec), 60°C (45 sec followed by fluorescence plate read). Quantitative real-time polymerase chain reaction (PCR) was performed on an Applied Biosystems 7500 Real-time PCR Detection system.

**Quantitative real-time PCR amplification for illumina sequencing**

The 16S sequencing procedure was performed at the University of Minnesota Genomic Center. Method optimizations and the protocol have been recently published.55,56 Fecal DNA samples (25 ng) were used as templates for PCR amplification of the V4 region of the 16S rRNA gene. Degenerate primer sets were designed with Illumina index sequences on the 5′ end of the reverse primer, which were specific to each fecal DNA sample and allowed for multiplex sequencing. Primers also contained Illumina PCR primer sequences (reverse primer) and Illumina TruSeq Universal Adapter sequences (forward primers) for library creation. The primer sequences (16S-specific portion in bold) used were Meta_V4_515F (TCGTCGAGCGGTAGTGTATGATGTAAGATACAGGTGTGACAGCAGTCTACTACAG) and Meta_V4_806R (GTCTCGTGGGCTCGGAAGATGTGTATAAGAGACCAGCTACTACHVGGGTWTCTAAT). The indexing primers are as follows: This step adds both the index and the flow cell adapters. [i5] and [i7] refer to the index sequence codes used by Illumina. The p5 and p7 flow cell adapters are in bold. Forward indexing primer: ATGATCGGCACCCGAGATCTACAC [i5]TCGTCGAGCGGTAGTGTATG; Reverse indexing primer: CAAGCAGAAGACGGCATACGAT [i7] GTCTCGTGGGCTCGG. PCR reactions were performed using KAPA HiFidelity Hot Start Polymerase. PCR 1 (using the Meta_V4_515F/Meta_V4_806R primer pair): 95°C 5 min, 20 cycles (98°C 20 s, 55°C 15 s, 72°C 1 min), followed by holding at 4°C. After the first round of amplification, PCR 1 products were diluted 1:100 and 5 μl of 1:100 PCR 1 was used in the second PCR reaction. PCR 2 (using different combinations of forward and reverse indexing primers): 95°C 5 min, 10 cycles (98°C 20 s, 55°C 15 s, 72°C 1 min), followed by holding at 4°C.

**DNA sequencing**

Genomic DNA sequencing was performed using Illumina MiSeq at the University of Minnesota Genomic Center (UMGC). Pooled, size-selected samples were denatured with NaOH, diluted to
8 μM in Illumina’s HT1 buffer, spiked with 15% PhiX, and heat-denatured at 96°C for 2 min immediately prior to loading. The MiSeq 600 cycle v3 kit was used to sequence the sample. Nextera adapter sequences for post-run trimming were as follows:

Read 1: CTGTCTCTTATACACATCTCCGAGCCACGAGACNNNNNNATCTCGTATGCCG-TCTCTGCTTG

Read 2: CTGTCTCTTATACACATCTGACGCTGCC-GACGANNNNNNGTGTAGATCTCGGTGG-TCGCCGTATCATT

The raw data files for 16s rDNA sequencing have been deposited with ArrayExpress with the accession numbers E-MTAB-7503.

**Sequence processing and analysis**

Microbial operational taxonomic units (OTUs) and their taxonomic assignments were obtained using default settings in QIIME version 1.8.0 by reference-mapping at 97% similarity against representative sequences of 97% OTU in Greengenes (release GG_13_8), following which chimeric sequences were removed from subsequent analyses. Sequences showing 97% or greater similarity were clustered into operational taxonomic units (OTUs) using the USEARCH method, and representative sequences were assigned taxonomies using the RDP classifier.

**Statistical analysis**

For the microbiome analysis, QIIME 1.8 was used to calculate the α diversity (alpha_rarefaction.py) and to summarize taxa (summarize_taxa_through_plots.py). Principal coordinates analysis (PCoA = multidimensional scaling, MDS) is a method that is used to explore and to visualize interobject similarity/dissimilarity in a low-dimensional, Euclidean space. Principal coordinate analysis (PCoA) of unweighted UniFrac phylogenetic distances between microbial communities were performed using this program with observation ID level. The input for PCoA is the OTU table containing the number of sequences that were observed in each OTU (rows) for each sample (columns). OTU tables were rarefied to the sample containing the lowest number of sequences in each analysis. An estimate of the false discovery rate (q-value < 0.10) was calculated to take into account the multiple comparisons that occur in microbiome analyses. The P-value in the UniFrac distance comparison was Bonferroni-corrected. The tests of significance were performed using a two-sided Student’s two-sample t-test.

For the real-time PCR and histology studies, data analyses were performed using GraphPad Prism software version 5.0 (GraphPad Software Inc.). The differences between two groups were evaluated using a Student’s t test (parametric) or Mann–Whitney U test (nonparametric). For the multiple comparisons, statistical analyses were performed using one-way ANOVA (parametric), the Kruskal–Wallis test (nonparametric), and the Bonferroni test for parametric samples. Differences at P < .05 were considered significant.

**Author Contributions**

SR conceptualized the project. FW and SR designed the experiments. FW, JM, and LZ performed the experiments and data analysis. FW and SR wrote the manuscript.

**Data availability**

The microbiome datasets supporting the conclusions of this article are available in the ArrayExpress repository. The accession numbers are E-MTAB-7503. The hyperlink to datasets is [https://www.ebi.ac.uk/arrayexpress/](https://www.ebi.ac.uk/arrayexpress/).

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**Disclosure of Potential Conflicts of Interest**

The authors declare that they have no competing interests.

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