Propionic Acid Promotes the Virulent Phenotype of Crohn’s Disease-Associated Adherent-Invasive Escherichia coli

Graphical Abstract

(1) The use of propionic acid in food and agriculture is increasing

(2) E. coli are becoming increasingly exposed to PA

   Adhesion
   Biofilm Formation
   Colonization
   Acid tolerance

(3) PA-exposed Adherent-invasive E. coli

   ‘Normal’

   Prior exposure to PA enables AIEC colonization of the gut where antimicrobial PA levels are naturally high

(4) ‘Abnormal’

Highlights

- PA induces virulence-associated changes in CD-associated AIEC
- PA-induced phenotype is reproducible in recently isolated clinical strains
- Phenotypic changes are transcriptional in nature and reversible
- Strains exposed to PA outcompete wild-type strains in a “humanized” murine model

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In Brief

The short chain fatty acid propionic acid is a bacterium-derived human intestinal antimicrobial and immune modulator used widely in Western food production and agriculture. Here, Ormsby et al. demonstrate that exposure to propionic acid induces virulence-associated phenotypic changes in Crohn’s disease-associated adherent-invasive Escherichia coli (AIEC).
Propionic Acid Promotes the Virulent Phenotype of Crohn’s Disease-Associated Adherent-Invasive Escherichia coli

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SUMMARY

Propionic acid (PA) is a bacterium-derived intestinal antimicrobial and immune modulator used widely in food production and agriculture. Passage of Crohn’s disease-associated adherent-invasive Escherichia coli (AIEC) through a murine model, in which intestinal PA levels are increased to mimic the human intestine, leads to the recovery of AIEC with significantly increased virulence. Similar phenotypic changes are observed outside the murine model when AIEC is grown in culture with PA as the sole carbon source; such PA exposure also results in AIEC that persists at 20-fold higher levels in vivo. RNA sequencing identifies an upregulation of genes involved in biofilm formation, stress response, metabolism, membrane integrity, and alternative carbon source utilization. PA exposure also increases virulence in a number of E. coli isolates from Crohn’s disease patients. Removal of PA is sufficient to reverse these phenotypic changes. Our data indicate that exposure to PA results in AIEC resistance and increased virulence in its presence.

INTRODUCTION

Short chain fatty acids (SCFAs) are naturally produced by gut bacteria through the breakdown of undigested carbohydrates and starches. This process results in the production of acetic acid (AA), butyric acid (BA), and propionic acid (PA), which together account for approximately 90% of intestinal SCFAs. PA has attracted significant interest due to its potent immuno-modulatory effects, with its supplementation shown to reduce the severity of colitis in murine models, suggesting its modulation has potential as a therapeutic intervention in inflammatory bowel disease (Tedelind et al., 2007; Smith et al., 2013). Crohn’s disease (CD) is a debilitating and incurable inflammatory disease of a multi-factorial etiology. The mechanisms underlying the disease are not fully understood; however, it is thought that defects in the immune response to the gut microbiota are a contributing factor (Hansen et al., 2010; Molodecky et al., 2011; Jostins et al., 2012; Mukhopadhya et al., 2012; Imhann et al., 2016; Keestra-Gounder et al., 2016). Sudden changes in diet have been shown to result in rapid changes in the gut microbiota (Turnbaugh et al., 2009; Martinez-Medina et al., 2014; Agus et al., 2016), whereas the inflammation associated with CD results in markedly decreased microbial diversity (Tamboli et al., 2004; Gophna et al., 2006; Frank et al., 2011). Levels of Enterobacteriaceae in particular are higher in intestinal samples from CD patients than in healthy controls (Willing et al., 2009; Morgan et al., 2012; Mukhopadhya et al., 2012; Honnefer et al., 2014). One group of Enterobacteriaceae that is of particular interest is the Escherichia coli pathotype adherent-invasive E. coli (AIEC). These bacteria are overrepresented in the ileal microbiota of CD patients, being present in 51.9% of mucosal samples from CD patients compared with 16.7% in healthy controls (Martinez-Medina et al., 2009). Key features of the AIEC pathotype that distinguish them from non-invasive commensal strains include adherence to and invasion of the intestinal epithelium, an increased ability to form biofilms, and the ability to survive and replicate within macrophages without inducing cell death (Martinez-Medina et al., 2009). Although AIEC strains are similar to extra-intestinal pathogenic E. coli (ExPEC) in terms of phylogenetic origin and genotype, they have few known virulence factors (Palmela et al., 2018). This apparent lack of virulence factors and the discovery of AIEC strains across all five major
diverse phylogroups of *E. coli* mean that an overarching explanation for the origin and virulence of AIEC has remained out of reach.

PA is manufactured on an industrial scale and is now commonly used in agriculture because, in addition to its anti-inflammatory effects, it is a potent antimicrobial. PA has demonstrated success in reducing pathogen numbers in poultry, particularly in reducing *Salmonella* and *Campylobacter* carriage (Hinton and Linton, 1988; Iba and Berchieri, 1995; Hung et al., 2013; González-Fandos et al., 2015). PA is also an effective antimicrobial agent used in Western food production and agriculture, reducing the need for antibiotic use amidst growing antibiotic resistance concerns (Deforêt et al., 2009; Haque et al., 2012; Khan and Iqbal, 2016). Inclusion in animal feed, grain, and food for human consumption accounted for almost 80% of PA consumption across the world in 2016, with Western Europe (40% of total use), North America (30%), and Asia (23%) as the main consumers (Bizzari and Blagoev, 2013). The success of SCFAs in reducing antibiotic dependence is now seeing their use spread to countries across Africa, the Middle East, and Central and South America (Bizzari and Blagoev, 2013). Although there is increasing evidence for antibiotic-driven enhanced genome-wide mutation rates and horizontal transmission of bacteria from food-producing animals to humans (Levy et al., 1976; Ojeniyi, 1989; Long et al., 2016; Ljubojevic et al., 2017), the role of alternative antimicrobials such as PA in such phenomena has yet to be addressed (Frana et al., 2013; Lazarus et al., 2015; Norizuki et al., 2017). Indeed, using animal models for human disease where SCFAs may play an important role has proven difficult at best. Murine models for human pathogens are limited by distinct differences in basal levels of SCFAs between the murine and human intestines, with murine levels significantly lower in the case of PA (Cummings et al., 1987). However, the significance of such differences in influencing the outcome or course of disease is not known but could be substantial.

In this study, we show that PA exposure promotes increased virulence in AIEC. Using a murine model with increased intestinal PA concentrations, we have generated a more relevant model for human-gut-associated AIEC infection, resulting in increased AIEC virulence and persistence. This increased virulence is PA dependent and can be replicated in vitro by exposure...
was dramatically increased in LF82 recovered from mice fed PA relative to the WT strain, whereas there was no significant difference between LF82 recovered from control non-PA-fed mice and the WT strain (Figure 1C).

The Enhanced AIEC Phenotype Was Driven by PA

We hypothesized that the enhanced virulence of LF82 was driven by the increased murine intestinal PA concentration and was independent of other factors during in vivo infection. To examine this, LF82 was grown in minimal media with PA as the sole carbon source (20 mM). LF82 was able to grow in PA, whereas a human commensal E. coli strain included as a control, E. coli F-18 (Ormsby et al., 2016), replicated extremely poorly (Figure 2A). Subculturing the bacteria over five growth cycles in PA-supplemented minimal media generated a "PA-exposed" strain of LF82, termed LF82-PA. LF82-PA had a significantly increased growth rate with a doubling time of 3.98 h in PA compared to WT LF82 at 25.59 h (Figure 2A). This increased growth rate was specific to PA and was not observed in nutrient-rich broth (lysogeny broth [LB]; Figure 2B).

These results are surprising given the well-documented antimicrobial properties of PA (Hinton and Linton, 1988; Iba and Berchieri, 1995; Hung et al., 2013; González-Fandos et al., 2015).

Direct incorporation of PA into the membrane is a mechanism used by bacteria to minimize the toxic effects of excess PA in the environment (Jain et al., 2007; Lee et al., 2013; Si et al., 2016). Given that the phenotypic changes seen in AIEC, such as increased adhesion, were likely to be mediated by changes in the composition of the bacterial membrane following growth on PA, we investigated this further. Gas chromatography coupled to isotope ratio mass spectrometry (GC-IRMS) using 13C-labeled PA (1-13C sodium propionate) revealed that PA was not incorporated into odd chain long chain fatty acids (LCFAs). However, there was significant 13C-enrichment in 12 fatty acid methyl esters (FAMEs) that did not correspond to any of the 37 FAMEs in our reference standard. The proximity of these labeled peaks to known LCFAs is likely indicative of incorporation of PA into methylated or branched chain fatty acids (BCFAs), as described previously during Mycobacterium tuberculosis growth on, and detoxification of, PA (Lee et al., 2013). Therefore, this indicated that LF82 could both metabolize and detoxify an antimicrobial that exerts potent toxic effects on a number of other intestinal pathogens (Hinton and Linton, 1988; Iba and Berchieri, 1995; Hung et al., 2013; González-Fandos et al., 2015).
The observed changes in the bacterial membrane also rendered LF82-PA increasingly acid tolerant despite exposure in PA-supplemented minimal media being carried out at pH 7.4 (Figure 2F). A reduction in cell number was seen for both LF82 and LF82-PA at a pH of 3, but the LF82-PA strain survived in greater numbers for longer periods (at 20 min, LF82-PA was recovered in numbers >30.4-fold higher than LF82; at 40 min, LF82-PA was >22.7-fold higher). Taken collectively, these results indicate that the increased virulence observed after passage of LF82 through a PA-supplemented murine model can be replicated by exposure to PA in vitro.

The Enhanced Virulence Phenotype of LF82-PA Is Not Genome Encoded and Is Reversible

Genome sequencing of three biological replicates of LF82-PA, exposed independently in vitro to PA, revealed a number of single nucleotide polymorphisms (SNPs; Data S1, nucleotide analysis of PA-exposed LF82-PA, related to Figure 2). However, no SNPs were conserved across all isolates. Detailed analysis of the genes and pathways in which the SNPs were identified did not lead to the identification of any candidate pathways that may explain the changes in virulence observed. However, we cannot exclude the possibility that different combinations of small genomic changes may result in the same outcome at the transcriptional level. As the virulent phenotype persists over a number of generations and was not explained by genetic analysis, we hypothesized that the phenotype we see may be as a result of an epigenetic switch in LF82-PA. A long-term epigenetic memory switch with a role in controlling bacterial virulence modality was recently identified in enteropathogenic E. coli (EPEC) (Ronin et al., 2017). This “resettable phenotypic switch” results in populations of virulent and hypervirulent genetically identical subpopulations that are retained through generations. To further examine this possibility, LF82-PA was passaged through rich (LB) media with no PA-selective pressure. After five successive subcultures, this strain (LF82-PA-LB) had lost its increased growth rate in PA, and its virulence phenotype reverted to be more similar to that of WT LF82 (Figure 3). The epigenetic nature of this change was further confirmed through sequencing of the reverted LF82-PA-LB strains, which indicated that the SNPs present in the original LF82-PA strains were conserved and that the changes induced by PA were not due to SNPs or mutations (Data S1).

The PA-Driven Enhanced AIEC Phenotype Is Seen in Other Clinical AIEC Isolates

E. coli isolated from intestinal samples of pediatric patients with active CD was compared to LF82 for its ability to adhere to and invade an intestinal epithelial cell line before and after exposure to PA (Figure 3). All isolates exhibited an AIEC phenotype with an ability to adhere to and invade intestinal epithelial cells (Ormsby et al., 2019). Although there was an increase in the ability of all isolates to adhere to intestinal epithelial cells after PA exposure, this was only significant for clinical isolates B115 and B125 (Figure 3A). A similar increase was observed for invasion (Figure 3B). However, the phenotype was reversible in the same manner as previously for LF82 through removal of the PA pressure. Isolates were grown in rich media containing no PA for five successive growth cycles before re-examination of their ability to adhere to and invade intestinal epithelial cells, with all strains examined returning to WT levels of adhesion and invasion (Figure 3). These data indicated that PA-induced exacerbation of the AIEC phenotype occurred more widely in AIEC isolated from CD patients.

Enhanced LF82-PA Virulence Is Driven by Transcriptional Changes

Given that no definitive mutational basis for the observed increase in virulence was detected through genome analysis, we used a comparative RNA-seq approach to probe the global transcriptional profiles of LF82 and LF82-PA grown on PA. RNA-seq revealed 25 differentially expressed genes (DEGs; p ≤ 0.05) between LF82 and LF82-PA (Figure 4; Table S2); 24 were upregulated in the LF82-PA strain and 1 (mcbR; –20.85-fold) was downregulated. Of the 25 DEGs identified by RNA-seq, 21 including mcbR, were validated as significantly altered by PA using qRT-PCR (Figure S4). Functional grouping of these 21 DEGs revealed their roles in diverse processes, including biofilm formation, stress responses, metabolism, membrane integrity, and transport of alternative carbon sources (Figure 4; Table S1). Eight DEGs have well-described roles in biofilm formation, further adding to our in vitro findings indicating that PA was a driver of adhesion and biofilm formation (Figures 2C and 2E). Upregulation of another DEG, a regulator of membrane fatty acid composition yibT, adds further evidence for the potential detoxification of PA through membrane incorporation, as previously described (Lee et al., 2013; Si et al., 2016). Therefore RNA-seq analysis indicates...
that PA drives changes in virulence that are fundamental to the AIEC pathotype.

**Exposure to PA In Vitro Significantly Increases Persistence of LF82 In Vivo**

Given our findings of PA-driven changes in virulence, we determined the effect of PA exposure on long-term persistence of LF82-PA in vivo. Mice were again provided PA-supplemented (20 mM) water for 3 days prior to infection and for the 21-day duration of the infection. Mice given only sterile drinking water and infected with LF82 or LF82-PA or those treated with phosphate-buffered saline (PBS) were included as controls. In PA-fed mice, the colonization of LF82 was not significantly altered in either the ileum or colon compared to control mice (Figures 5A and 5B). However, LF82-PA was found to persist with a greater than 20-fold increase in the ileum and a greater than 18-fold increase in the colon in these mice compared to controls (Figures 5C and 5D). No significant difference in the persistence of either strain was observed in the caecum, irrespective of the presence of PA (Figure S5). These data indicate that PA-exposed strains retain virulence when transferred to a new model host, most likely through having an increased nutritional advantage as well as an increased ability to adhere to and invade intestinal cells and form biofilms.

**Exposure to PA In Vivo Gives LF82 a Competitive Advantage In Vivo**

As exposing LF82 to PA in vivo resulted in strains with an increased ability to adhere to and invade intestinal epithelial cells and form biofilms in vitro (Figures 1B and 1C), we examined the capacity of these strains to outcompete LF82 that had not been exposed to increased PA. LF82 and LF82lux were used to infect mice supplemented with either PA or sterile drinking water, before being recovered. A competition assay using equal mixtures of these re-isolated strains was then conducted in two subgroups of mice: one whose diet was supplemented with PA and a second whose diet was not. Our data indicated that LF82 recovered from a primary PA (PA\(^+\))-fed mouse out-competed bacteria from a primary water (W\(^-\))-fed mouse in subsequent infections of both PA and water-fed secondary mice (PA\(^2\) and W\(^2\), respectively) (Figure 6). PA\(^1\)-recovered bacteria outcompeted W\(^1\)-recovered bacteria in the ileum (competitive index [CI] = 2.91; p = 0.0078) and colon (CI = 1.91; p = 0.0156) of a PA\(^+\)-fed mouse. In a W\(^2\) mouse, PA\(^1\)-recovered bacteria outcompeted W\(^1\)-recovered bacteria in the ileum (CI = 2.07; p = 0.0938) and colon (CI = 2.75; p = 0.125).

**DISCUSSION**

SCFAs have significant effects on their hosts and are seen as key players in how the intestinal microbiome maintains health, modulates the immune system, controls invading pathogens, and even exerts effects on distal sites such as the brain (Smith et al., 2013; Jacobson et al., 2018; van de Wouw et al., 2018). Such positive effects have led to the suggested use of SCFAs as a therapeutic intervention strategy in inflammatory diseases, including inflammatory bowel disease (Tedelind et al., 2007). Here, however, we have shown a role for the SCFA PA in microbial infection, acting as a driver for virulence of the bacterial pathotype AIEC that is commonly isolated from the intestine of CD patients (Martinez-Medina et al., 2009). Exposure of AIEC to PA, in contrast to other pathogens where it is a negative regulator of virulence, stimulated adhesion and biofilm formation and induced an upregulation of an array of genes related to virulence.

Both the antimicrobial properties of PA and its negative regulation of bacterial virulence are the basis for its widespread use in agriculture as PA clears Salmonella and Campylobacter spp. rapidly post-treatment of poultry (Hinton and Linton, 1988; Ibá and Berchieri, 1995; Hung et al., 2013; González-Fandos et al., 2015). As well as its direct toxicity to these pathogens, this inhibitory effect is mediated by negative regulation of genes critical for intestinal colonization and is thought to be a response by these pathogens to the varying PA concentrations in the environment.
human intestine. In *Salmonella*, high concentrations of PA undermine the stability of virulence regulators, such as HilD, meaning *Salmonella* is less likely to colonize the lower intestine where PA levels are highest (Hung et al., 2013). In direct contrast, we have shown here that PA positively regulates virulence of the AIEC strain LF82. Increasing exposure to PA, and increasing concentrations in the murine intestine, results in a >20-fold increase in persistence as well as increasing the most notable phenotypic traits of AIEC, such as adhesion and invasion of the intestinal epithelium and biofilm formation. There are limited bacteria to draw comparisons to with regard to PA and virulence, as few respond positively to PA given its antimicrobial properties. Enterohemorrhagic *E. coli* (EHEC) and *Citrobacter* increase virulence by the type three secretion system (T3SS) in response to PA, as previously shown (Nakanishi et al., 2009; Connolly et al., 2018). However, *Mycobacteria* do become increasingly virulent in the presence of PA with overlapping strategies used by mycobacteria and AIEC as both metabolize and directly incorporate PA into their membrane lipids (Jain et al., 2007; Upton and McKinney, 2007; Lee et al., 2013; Si et al., 2016). This strategy of PA incorporation into the outer membrane of AIEC, as well as detoxifying PA, also increases resistance to pH and likely plays a role in the increased adhesion and invasion noted with LF82 after PA exposure (Figure 2).

Although PA has significant immunomodulatory properties in the intestine, disease context is highly important. Although PA supplementation in certain murine models reduces disease through signaling to specific immune cells, here, PA supplementation in the presence of AIEC resulted in significant overgrowth of the bacteria due to PA-driven phenotypic switches that occurred (Smith et al., 2013). The dramatic increase in colonization with increasing PA in the murine intestine also highlights a significant problem with murine models of intestinal disease. SCFAs and other antimicrobial molecules by the intestinal microbiota (Jacobson et al., 2018). Interestingly, in this regard, the caecum where the majority of SCFAs are produced in the murine intestine was distinct from the ileum and colon during infection, as no significant increase in colonization by AIEC was detected here. It is possible that the levels and types of antimicrobials being produced in the caecum still proved refractory to increased AIEC colonization despite its PA adaptation.

Our ability to recapitulate in *vitro* the effects of PA on AIEC indicates that PA in isolation exerts a significant effect on bacterial virulence. This was further demonstrated using clinical isolates of *E. coli* derived from CD patients that were exposed to PA and tested for their ability to adhere to and invade human colonic epithelial cells. Although the clinical isolates were from the intestine of CD patients, it is unknown if they are true AIECs given the confusion over what constitutes the AIEC pathotype (Elhenawy et al., 2018). However, our phenotypic examination of these isolates suggests that they are likely to be AIEC (Figure 3). Exposure to PA induced significant increases in virulence in all clinical strains. In comparison, the human commensal isolate F-18 was not able to adapt to PA. These data suggest that certain *E. coli* isolates recovered from the human CD intestine are readily adaptable to PA and that contrary to its effects on other pathogens, PA is actually a driver for AIEC virulence and does not exert antimicrobial effects. Further analysis of the PA effect on a large range of *E. coli* pathobionts is necessary to definitively determine if this effect is AIEC specific.

Here, we have shown through our work using *in vivo* models that LF82 isolated post-PA supplementation in a murine model is more virulent than without such treatment. This indicates that rather than being directly inhibited by the antimicrobial
effects of PA, these strains instead show potential to be readily adaptable to the naturally higher concentrations of PA in the human intestine. Given the wide use of PA environmentally and agriculturally, it is not inconceivable that bacteria such as AIEC come into contact with such concentrations of PA, as those in animal water, feed, and silage are reported to be 20 mM and higher (Hinton and Linton, 1988; European Food Safety Authority, 2013; Hung et al., 2013; González-Fandos et al., 2015) Such exposure would likely make the PA concentrations in the human intestine, which increases from 1.5 mM in the ileum to 27 mM in the colon, easily tolerable to E. coli strains as we have indicated here (Cummings et al., 1987; Hung et al., 2013). Horizontal transmission of strains from poultry to humans as previously seen, driven by antibiotics, would therefore seem highly possible. Additionally, recent evidence has suggested the food additive trehalose is a contributory factor in the emergence and hypervirulence of two epidemic lineages of Clostridioides difficile (Collins et al., 2018). Therefore, although the focus rightly remains on antibiotic resistance, more work is needed to determine the long-term effects of alternative antimicrobials in generating more resistance and more virulent bacteria capable of horizontal transmission. Although, in addressing one resistance problem, we must be careful that another is not inadvertently created.

Most encouragingly, we were able to show that the in vitro and ex vivo phenotypes that we observed were directly relatable to increased in vivo virulence (Figure 6). Our competitive assay between isolates passaged through WT mice and mice with a PA-supplemented diet revealed that those isolates that were exposed to PA in vivo were able to outcompete those that were not, in a PA-fed secondary mouse. Although in a water-fed secondary mouse this out-competition was not significant, there was a still an observed trend toward those isolates initially recovered from a PA-fed mouse. However, this recapitulates the in vitro observations made previously, in that the PA-phenotype is reversible with the removal of PA selective pressure.

Our findings here are not without precedent. Dietary additives and a mock Western diet have been demonstrated to contribute to increased colonization of AIEC in murine models (Martinez-Medina et al., 2014; Agus et al., 2016). This work explores the finding of PA as a paradoxical pro-virulence factor in AIEC, which is at odds with its perceived role as an antimicrobial. The growing use of PA in the Western diet, coupled with the rapid expansion of CD incidence in recent years, highlights the importance of this work in suggesting a potential mechanism for diet as a key driver of selection in the gut, which would favor the carriage and transformation of an emerging pathogenic E. coli variant.

**STAR METHODS**

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Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2020.01.078.

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AUTHOR CONTRIBUTIONS

M.J.O. designed and performed the experiments, analyzed the data, and prepared the manuscript. S.A.J. assisted in experimental design, animal experiments, and RT-PCR. N.C. and D.W. assisted in the design and performance of animal experiments. L.M.M., R.J.G., and D.G.E.S. assisted in experimental design. A.M. provided technical assistance throughout the experiments. J.P.R.C. and A.J.R. advised on and assisted in RNA-seq analysis. C.C.M. was awarded the funding, developed the initial concept, designed experiments, and RT-PCR. N.C. and D.W. assisted in the design and performance of SCFA experiments. D.M. conducted GC-IRMS experiments. G.L.H. and R.H. provided BISCUIT isolates and relative clinical information. D.M.W. was awarded the funding, developed the initial concept, designed experiments, and prepared the manuscript. All authors contributed to editing the manuscript for publication.

DECLARATION OF INTERESTS

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# STAR METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and Virus Strains** | | |
| LF82: Wild-type | Prof. Daniel Walker, Uni. of Glasgow | LF82 |
| LF82-PA.1 (LF82 exposed to 20 mM PA; rep 1) | This study | LF82-PA.1 |
| LF82-PA.2 (LF82 exposed to 20 mM PA; rep 2) | This study | LF82-PA.2 |
| LF82-PA.3 (LF82 exposed to 20 mM PA; rep 3) | This study | LF82-PA.3 |
| LF82ΔeutR: LF82 with eutR knocked out | This study | LF82ΔeutR |
| LF82-PAΔeutR: LF82-PA.1 with eutR knocked out | This study | LF82-PAΔeutR |
| F-18 | Ormsby et al., 2018 | F-18 |
| B94 | UK Clinical Research Network (9633) | B94 |
| B115 | UK Clinical Research Network (9633) | B115 |
| B122 | UK Clinical Research Network (9633) | B122 |
| B125 | UK Clinical Research Network (9633) | B125 |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| Lysogeny Broth (LB) media | LabM | NCM0088A |
| Bacto Agar | Formedium | A0A02 |
| Sodium chloride | Merck | N/A |
| Ammonium chloride | Fisher Scientific | 12125-02-9 |
| Potassium hydrogen phosphate | Merck | 7758-11-4 |
| Trace metal solution | Cold Spring harbor protocols | N/A |
| Magnesium sulfate | VWR Chemicals | 7487-88-9 |
| Calcium chloride | Fisher Scientific | 22189-08-8 |
| Thiamine hydrochloride | Fisher Scientific | 67-03-8 |
| Iron chloride | Fisher Scientific | 10025-77-1 |
| Ethylenediaminetetraacetic Acid (EDTA) | Fisher Scientific | 60-00-4 |
| Taurocholic acid | Fisher Scientific | 345909-26-4 |
| D-glucose | Sigma | 50-99-7 |
| Sodium propionate | Sigma | 137-40-6 |
| 1,2-Propanediol | Sigma | 57-55-6 |
| Ethanolamine | Sigma | 141-43-5 |
| RPMI-1640 | Thermofisher | 31870025 |
| Foetal calf serum | Fisher Scientific | 11573397 |
| L-glutamine | Fisher Scientific | 15430614 |
| No-Carbon-E (NCE media) | Cheng et al., 2011 | N/A |
| Cyano-cobalamin | Fisher Scientific | 68-19-9 |
| RNAProtect | QIAGEN | 76526 |
| RNAlater | Thermofisher | 10391085 |
| Penicillin/streptomycin | Sigma | P4333 |
| Triton X-100 | Merck | T9284 |
| Acetic Acid | VWR Chemicals | 20104-334 |
| Crystal Violet | Merck | C0775 |
| M9 Minimal Salts, 5x | Merck | M6030 |
| Dulbeccos Modified Eagle Medium (DMEM) | Sigma | D5671 |
| Foetal Bovine Serum, Heat Inactivated | Invitrogen | 10500064 |
| Phosphate Buffered Saline, PBS | Invitrogen | 14190086 |

(Continued on next page)
LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr Daniel M. Wall (Donal.Wall@glasgow.ac.uk). All unique reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

The Caco-2 human intestinal epithelial cell (IEC) line obtained from the American Type Culture Collection (ATCC) was maintained in Dulbecco’s Modified Eagle Medium (DMEM) medium (Sigma) supplemented with 10% Heat-inactivated FBS (Sigma), L-glutamine and penicillin/streptomycin (Sigma).

RAW 264.7 murine macrophages
RAW 264.7 macrophages were obtained from the ATCC and maintained in Roswell-Park Memorial Institute (RPMI) media supplemented with 10% Fetal bovine serum (FBS), L-glutamine and penicillin/streptomycin (Sigma). Cells were maintained at 37 °C and 5% CO₂ with regular media changes.

Animal experiments
All animal procedures were approved by an internal University of Glasgow ethics committee and were carried out in accordance with the relevant guidelines and regulations as outlined by the UK Home Office (PPL 70/8584). Male C57BL/6J mice aged between eight

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### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Streptomycin Sulfate Salt | Sigma | S9137 |
| Erythromycin | Sigma | E5389 |
| Ampicillin | Sigma | A1593 |
| Gentamicin | Merck | G1264 |
| LPS from Salmonella Typhimurium | Merck | L7770 |
| Hydrochloric Acid | Merck | 258148 |
| Turbo DNase | Thermofisher | AM2238 |

Critical Commercial Assays

| Affinity Script cDNA multi-temp Synthesis Kit | Agilent | 26000-50 |
| PerfeCTa SYBR Green FastMix | Quanta Biosciences - VWR | 200436 |
| MicrobeExpress mRNA Kit | Invitrogen | AM1905 |
| RNEasy Mini Kit | QIAGEN | 74104 |

Deposited Data

| RNA-seq and Genomic sequence data | This paper | ENA:PRJEB36206 |

Experimental Models: Cell Lines

| Caco-2 Human Intestinal Epithelial cells | American Type Culture Collection (ATCC) | ATCC HTB-37 |
| RAW264.7 murine macrophage cell line | American Type Culture Collection (ATCC) | ATCC TIB71 |

Experimental Models: Organisms/Strains

| Mouse C57BL/6J (Male; 6-8 Weeks old) | Envigo | N/A |

Oligonucleotides (5'-3')

| Oligonucleotides can be found in Table S1 | Sigma | N/A |

Software and Algorithms

| GraphPad Prism v7.0c | https://www.graphpad.com | N/A |
| Mascot search engine v2.6.2 | http://www.matrixscience.com | N/A |
| MAUVE v2.4.0 | http://darlinglab.org/mauve/mauve.html | N/A |
| CLC Genomics Workbench v7.0.1 | https://digitalinsights.qiagen.com/ | N/A |
| ExPASy | https://www.expasy.org | N/A |
| EMBOSS Needle | https://www.ebi.ac.uk/Tools/psa/emboss_needle | N/A |

Other

| Breathe-Easy Sealing Membrane | Merck | 2380059-1PAK |
and ten weeks were obtained from The Jackson Laboratory (Envigo). Twenty millimolar sodium propionate was administered to C57BL/6 mice in drinking water three days prior to infection. Control mice were given only sterile water. Twenty-four hours prior to infection, mice were treated with an oral dose of 20 mg streptomycin before oral infection with 0.1 mL PBS (mock-infected) or with approx. $1 \times 10^9$ colony forming units (CFU) of LF82 or LF82lux. After 7 days of infection, mice were euthanized 3 days after infection for colonization experiments and 21 days after infection for persistence experiments, with caecal contents collected for SCFA analysis. Ileal, caecal and colonic tissue were weighed and homogenized for enumeration of bacterial numbers. Bacterial numbers were determined by plating tenfold serial dilutions onto LB agar containing the appropriate antibiotic. After 24 h of incubation at 37 °C, colonies were counted and expressed as CFU per gram of tissue.

**In vivo competition assay**

Male C57BL/6 mice aged between eight and ten weeks were obtained from Envigo. Twenty millimolar sodium propionate was administered to C57BL/6 mice in drinking water three days prior to infection. Control mice were given only sterile water. Twenty-four hours prior to infection, PA-treated and control mice were given an oral dose of 20 mg streptomycin before oral infection with 0.1 mL PBS (mock-infected) or with approx. $1 \times 10^9$ colony forming units (CFU) of LF82 or LF82lux. Mice were euthanized 3 days after infection for colonization experiments and 21 days after infection for persistence experiments, with caecal contents collected for SCFA analysis. Ileal, caecal and colonic tissue were weighed and homogenized for enumeration of bacterial numbers. Bacterial numbers were determined by plating on LB plates supplemented with ampicillin or erythromycin. Mice were challenged as previously bacteria recovered through plating of ileum and colon homogenates on LB agar supplemented with ampicillin or erythromycin. Competitive indices were determined by normalization to the initial inoculum ratios.

**METHOD DETAILS**

**Bacterial strains and growth conditions**

Pathogenic AIEC strain LF82 and intestinal commensal *E. coli* strain F-18 were used in this study and were cultivated on Lysogeny broth or agar. M9 minimal medium supplemented with 20 mM PA (M9-PA [20% M9 salts (32 g Na2HPO4·2H2O (Merck), 12.5 g NaCl (Merck), 2.5 g NH4Cl (Fisher scientific), 7.5 g KH2PO4 (Merck) and 400 mL H2O), 0.1% Trace metal solution, 0.2 mM MgSO4 [VWR chemicals], 0.02 mM CaCl2 [Fisher scientific], 1 mM Thiamine, 0.01% 5 g/L FeCl3, 0.01% 6.5 g/L EDTA, 0.1% taurocholic acid, 20 mM Sodium propionate and dH2O]) was used for growth. Strains were grown in 100 mL of M9-PA at 37°C at 180 rpm, unless stated. Bacterial growth was measured at optical density 600nm (OD600nm). To obtain adapted cells, upon reaching stationary phase, cultures were back-diluted into fresh M9-PA. Strains for infection were back-diluted after overnight growth into 10 mL cultures of RPMI-1640 (Sigma) supplemented with 3% fetal calf serum (FCS) and L-glutamine. These were then grown at 37 °C in a shaking incubator at 180 rpm to an OD600nm of 0.6 before further dilution to give final multiplicities of infection (MOI) of 10 or 100. Real-time PCR was conducted using bacteria grown in No-Carbon-E (NCE) media (Davis et al., 1980). Twenty millimolar sodium propionate (Sigma), 1,2-propanediol (Fisher Scientific) or D-glucose (Sigma) were added with 200 nM cyano-cobalamin (Sigma) to act as an electron acceptor (Price-Carter et al., 2001). Cultures were grown overnight in LB, washed three times in NCE media with no carbon source added, and inoculated 1:100 into 10 mL NCE media containing each respective carbon source. Cultures were grown until mid-log phase (OD600 of 0.6) and used for RNA-extraction.

Clinical isolates (B94, B115, B122 and B125) were from the “Bacteria in Inflammatory bowel disease in Scottish Children Undergoing Investigation before Treatment” (BISCUIT) study (Hansen et al., 2013). Isolates B94, B115, 122 and 125 were recovered from patients with Crohn’s disease. The median (range) age was 13.7 (11.2 to 15.2), height z-score was $-0.7 (-3.4$ to $-0.1)$, BMI z-score was $-1.3 (-4.0$ to $-0.4$). Symptom duration prior to diagnosis was median 7.5 months (5 to 12). 50% had granulomas present on initial histology. Phenotypes by Paris criteria (Levine et al., 2011) at diagnosis were: B94- colonic, non-stricturing/non-penetrating (L2, B1); B115- colonic, non-stricturing/non-penetrating (L2, B1); B122- ileocolonic, strictureing (L3, B2); B125- ileocolonic, non-stricturing/non-penetrating (L3, B1). This study is publically registered on the United Kingdom Clinical Research Network Portfolio (9633).

**Biofilm assays**

Crystal violet static biofilm assays were performed essentially as described previously (O'Toole, 2011). Briefly, bacteria were grown in RPMI to an OD600nm of 0.6 at 37 °C with shaking at 180 rpm. Cultures were further diluted 1:5 before 100 µl was loaded into a 96-well plate, in technical triplicate. The outer wells of the 96-well plate were filled with PBS, only. Plates were sealed with clear plastic seals (Sigma) and placed in a humid chamber. Where indicated, PA was added to a final concentration of 20 mM. Anaerobic culture conditions were achieved using a microaerobic cabinet. Biofilms were enumerated using the crystal violet method after 5 days of incubation (O'Toole, 2011). All experiments were conducted in biological triplicate.
Measurement of 1-\(^{13}\)C-PA incorporation by gas chromatography coupled to isotope ratio mass spectrometry (GC-C-IRMS)

LF82-PA was grown as before in M9 minimal medium here supplemented with 20 mM 1-\(^{13}\)C-PA. Cultures were harvested at OD 0.6, pelleted and washed with PBS. Air-dried cells were treated using a saponification and methylation procedure to produce fatty acid methyl esters (FAMES) of all cell fatty acids. Briefly, to air-dried cells was added 1 mL of methanol:heptane:toluene:2,2-dimethoxypropane:conc H\(_2\)SO\(_4\) (39:34:20:5:2 by vol) and samples vortexed and then heated at 80 °C for 30 mins. Upon cooling, 100 \(\mu\)L of the upper heptane phase containing FAMES was extracted to a clean vial ready for analysis. Samples were analyzed using gas chromatography coupled to isotope ratio mass spectrometry through a combustion interface (GC-C-IRMS). FAMES separated by GC (Agilent 6890, ZB-FFAP column (30 m x 0.25 mm x 0.25 \(\mu\)m), He carrier (2ml/min), temperature program of 80 °C start followed by 7.5 °C / min to 150 °C, 2 °C / min to 225 °C and finally 5 min dwell at 225 °C) and eluting FAMES were oxidized to CO\(_2\) over hot copper oxide (GVI Isochrome, Manchester, UK) in a He flow to the IRMS. An open split design allowed a portion of the eluting CO2 in He to enter the IRMS where ions at mass to charge (m/z) 44, 45 and 46 were analyzed continuously and identified peaks were integrated against a reference CO\(_2\) peak to yield the background and Craig corrected \(^{13}\)C/\(^{12}\)C ratio expressed in the normal units versus the internationally accepted scale for \(^{13}\)C/\(^{12}\)C measurements, VPDB. Samples were bracketed by a certified reference FAME mix (Supelco® 37 Component FAME Mix, Sigma-Aldrich, UK; containing Butyate, Hexanoate, Octanoate, decanoate, Undecanoate, Laurate, tridecanoate, tetradecanoate, Myristoleic, Pentadecanoate, cis-10-pentadecanoic, Palmitate, palmitoleic, heptadecanoic, cis-10-Heptadecenoic, octadecanoic, trans-9-Elaidic, cis-9-Oleic, Linolealidic, linoleate, Arachidate, gamma-Linolenic, cis-11-eicosenoate, Linolenate, heneicosanoate, cis-11,14-Eicosadienoic, docosanoate, cis-8,11,14-Eicosatrienoic, Erucate, cis-11,14,17-Eicosatrienoic, tricosanoate, cis-5,8,11,14-Eicosatetraenoic, cis-13-16-Docosadienoic, lignocerate, cis-5,8,11,14,17-Eicosapentaenoate, Nervonate, cis-4,7,10,13,16,19-Docosahexaenoate) to retention time lock for 37 odd and even chain FAMES.

Adherence and invasion assays

Caco-2 IECs were washed once before infection and bacterial suspensions were added at an MOI of 10. Plates were centrifuged after the initial inoculation (700 x g, 15 min), before the infection was allowed to proceed for 2 h at 37 °C in 5% CO\(_2\) atmosphere. Non-adhered bacteria were washed away and the infected cells were lysed with 1% Triton X-100 for 5 min. Bacteria were serially pelleted and washed with PBS. Air-dried cells were treated using a saponification and methylation procedure to produce fatty acid methyl esters (FAMES) of all cell fatty acids. Briefly, to air-dried cells was added 1 mL of methanol:heptane:toluene:2,2-dimethoxypropane:conc H\(_2\)SO\(_4\) (39:34:20:5:2 by vol) and samples vortexed and then heated at 80 °C for 30 mins. Upon cooling, 100 \(\mu\)L of the upper heptane phase containing FAMES was extracted to a clean vial ready for analysis. Samples were analyzed using gas chromatography coupled to isotope ratio mass spectrometry through a combustion interface (GC-C-IRMS). FAMES separated by GC (Agilent 6890, ZB-FFAP column (30 m x 0.25 mm x 0.25 \(\mu\)m), He carrier (2ml/min), temperature program of 80 °C start followed by 7.5 °C / min to 150 °C, 2 °C / min to 225 °C and finally 5 min dwell at 225 °C) and eluting FAMES were oxidized to CO\(_2\) over hot copper oxide (GVI Isochrome, Manchester, UK) in a He flow to the IRMS. An open split design allowed a portion of the eluting CO2 in He to enter the IRMS where ions at mass to charge (m/z) 44, 45 and 46 were analyzed continuously and identified peaks were integrated against a reference CO\(_2\) peak to yield the background and Craig corrected \(^{13}\)C/\(^{12}\)C ratio expressed in the normal units versus the internationally accepted scale for \(^{13}\)C/\(^{12}\)C measurements, VPDB. Samples were bracketed by a certified reference FAME mix (Supelco® 37 Component FAME Mix, Sigma-Aldrich, UK; containing Butyate, Hexanoate, Octanoate, decanoate, Undecanoate, Laurate, tridecanoate, tetradecanoate, Myristoleic, Pentadecanoate, cis-10-pentadecanoic, Palmitate, palmitoleic, heptadecanoic, cis-10-Heptadecenoic, octadecanoic, trans-9-Elaidic, cis-9-Oleic, Linolealidic, linoleate, Arachidate, gamma-Linolenic, cis-11-eicosenoate, Linolenate, heneicosanoate, cis-11,14-Eicosadienoic, docosanoate, cis-8,11,14-Eicosatrienoic, Erucate, cis-11,14,17-Eicosatrienoic, tricosanoate, cis-5,8,11,14-Eicosatetraenoic, cis-13-16-Docosadienoic, lignocerate, cis-5,8,11,14,17-Eicosapentaenoate, Nervonate, cis-4,7,10,13,16,19-Docosahexaenoate) to retention time lock for 37 odd and even chain FAMES.

Gentamicin protection assay

Intracellular replication was analyzed through a gentamicin protection assay over a time course of infection. Bacteria were added at an MOI of 10 to LPS-activated RAW 264.7 cells and the infection allowed to proceed for 1 h. After 1 h, non-internalised bacteria were removed by washing three times in media containing gentamicin (50 \(\mu\)g/ml). Cells were then held in gentamicin containing media for 2 h. After 2 h (time point = 0h), cells were washed three times in PBS, before being lysed with 1% Triton X-100. Bacteria were enumerated via serial dilution. A further time point at 4 h post the 0 h time point was analyzed.

Acid survival assays

Cultures of bacteria were grown overnight at 37 °C in LB. The pH of these cultures was lowered to pH 3 using 1 M HCl. Samples were taken every 20 min for 1 h and serially diluted in LB. Dilutions were plated in triplicate onto LB agar and incubated overnight at 37 °C. To determine bacterial invasion, cells were infected for 2 h, extracellular bacteria were then washed away and 50 \(\mu\)g/ml gentamycin sulfate was added for 1 h to kill any remaining cell-associated bacteria before Triton X-100 treatment.

Total RNA extraction and mRNA enrichment

Cultures were grown overnight in LB, washed three times in NCE media with no carbon source added, and inoculated 1:100 into 10 mL NCE media containing each respective carbon source. Cultures were grown until mid-log phase (OD600 of 0.6) and mixed with two volumes of RNAprotect reagent (QIAGEN, Valencia, CA, USA), before incubating for 5 min at room temperature. Total RNA was extracted, genomic DNA removed and samples enriched for mRNA as described previously by Connolly et al. (2016). Samples for RNA-sequencing (RNA-seq) analysis were QC tested for integrity and rRNA depletion using an Agilent Bioanalyzer 2100 (University of Glasgow, Polyomics Facility).

Genomic analysis and SNP identification

A bacterial lawn generated from single overnight colonies of LF82 and three independent cultures of LF82-PA were resuspended in a microbank bead tube, inverted four times and incubated at room temperature for 2 min. The cryopreservative was removed and the samples sent to MicrobesNG (Birmingham University, UK) for sequencing. Genomic DNA was extracted using a Illumina Nextera XT DNA sample kit as per manufacturer’s protocol (Illumina, San Diego, USA). Samples were sequenced on the Illumina MiSeq using a 2x250 paired-end protocol, De novo assembled using SPAdes version 3.5, aligned to the reference genome using BWA-MEM 0.7.5. Variants were called using samtools 1.2 and VarScan 2.3.9 and annotated using snpEFF 4.2. Subsequent genomic analysis
was performed using a combination of MAUVE, CLC genomics (Version 7.0.1), ExPASY and EMBOSS Needle. The sequence reads in this paper have been deposited in the European Nucleotide Archive under accession number PRJEB36206.

**RNA-seq transcriptome generation and data analysis**
cDNA synthesis and sequencing was performed at the University of Glasgow Polyomics Facility, essentially as described by Connolly et al. (2016). Briefly, sequencing was performed using an Illumina NextSeq 500 platform obtaining 75 bp single end reads. Samples were prepared and sequenced in triplicate. Raw reads were QC checked using FastQC (Babraham Bioinformatics, Cambridge, UK) and trimmed accordingly using CLC Genomics Workbench (CLC Bio, Aarhus, Denmark). Trimmed reads were mapped to the LF82 reference genome (NCBI accession number: CU651637) allowing for 3 mismatches per read. Analysis of differential expression was performed using the Empirical analysis of DGE tool, which implements the EdgeR Bioconductor tool (Robinson et al., 2010). Differentially expressed genes were identified by absolute fold change (cutoffs log2) and a P value of ≤ 0.05. Volcano plots were generated in CLC Genomics Workbench. The sequence reads in this paper have been deposited in the European Nucleotide Archive (PRJEB36206).

**Quantitative real-Time PCR (qRT-PCR)**
cDNA was generated from total RNA using an Affinity Script cDNA Synthesis Kit (Agilent) following the manufacturer’s instructions. Levels of transcription were analyzed by qRT-PCR using PerfeCTa® SYBR® Green FastMix® (Quanta Biosciences). Individual reactions were performed in triplicate within each of three biological replicates. The 16S rRNA and rpoS genes were used to normalize the results. RT-PCR reactions were carried out using the ECO Real-Time PCR System (Illumina, San Diego, CA, USA) according to the manufacturer’s specifications and the data were analyzed according to the 2^ΔΔCT method (Livak and Schmittgen, 2001). All primers used are listed in Table S1.

**Construction of p16Slux**
LF82 and LF82-PA lux integrated strains containing the erythromycin cassette were generated using the protocol of Riedel et al. (2007). The bioluminescent properties of these strains allowed visualization of the establishment of infection but despite bacteria being recovered it was noted that bioluminescent signal was lost. However, upon plating the murine microbiome onto LB agar containing ampicillin (100 μg/ml), it was observed that several members of the microbiota also harbored ampicillin resistance. LB supplemented with erythromycin (500 μg/ml) did not support the growth of any microbiota species; therefore utilizing the erythromycin cassette inserted as part of the lux integration allowed for the selection of LF82 and LF82-PA, and was used in subsequent animal experiments. Strains containing the lux cassette were only used during in vivo infections and subsequent in vitro experiments when these strains were re-isolated from the murine intestine and tested for virulence.

**SCFA analysis by gas chromatography**
Faecal contents of murine caeca were isolated from PBS treated mice three dpi. The concentrations of acetate, propionate and butyrate per gram of dry weight were measured by gas chromatography as previously described (Laurentin and Edwards, 2004) and expressed as a ratio for comparison to the known human acetate:propionate:butyrate SCFA ratio (Cummings et al., 1987). The effect of PA supplementation in drinking water on PA levels was measured by extraction of caecal contents from PA treated and untreated mice and the levels of SCFAs again calculated per gram of dry weight.

**QUANTIFICATION AND STATISTICAL ANALYSIS**
Values are represented as means and standard deviation. All statistical tests were performed with GraphPad Prism software, version 7.0c. All replicates in this study were biological; that is, repeat experiments were performed with freshly grown bacterial cultures, immortalized cells and additional mice, as appropriate. Technical replicates of individual biological replicates were also conducted, and averaged. Significance was determined as indicated in the figure legends. RT-PCR data was log-transformed before statistical analysis. Values were considered statistically significant when p-values were *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

**DATA AND CODE AVAILABILITY**

The sequence reads in this paper have been deposited in the European Nucleotide Archive (ENA:PRJEB36206).