Site-specific acylation of a bacterial virulence regulator attenuates infection

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Abstract

Microbiota generates millimolar concentrations of short-chain fatty acids (SCFAs) that can modulate host metabolism, immunity and susceptibility to infection. Butyrate in particular can function as a carbon source and anti-inflammatory metabolite, but the mechanism by which it inhibits pathogen virulence has been elusive. Using chemical proteomics, we discovered that several virulence factors encoded by Salmonella pathogenicity island-1 (SPI-1) are acylated by SCFAs. Notably, a transcriptional regulator of SPI-1, HilA, was acylated on several key lysine residues. Subsequent incorporation of stable butyryl-lysine analogs using CRISPR-Cas9 gene editing and unnatural amino acid mutagenesis revealed that site-specific modification of HilA impacts its genomic occupancy, expression of SPI-1 genes and attenuates Salmonella enterica serovar Typhimurium invasion of epithelial cells as well as dissemination in vivo. Moreover, a multiple-site HilA lysine-acylation mutant strain of S. Typhimurium was resistant to butyrate-mediated suppression in vivo. Our results suggest prominent microbiota-derived metabolites may directly acylate virulence factors to inhibit microbial pathogenesis in vivo.

Introduction

Short-chain fatty acids (SCFAs) generated by gut microbiota fermentation of dietary polysaccharides exhibit significant effects on host physiology and immunity1. SCFA concentrations in the mammalian gut range from 1 – 100 mM 2, depending on the location in the intestine, host diet and microbiota composition. Specific microbiota reconstitution studies have revealed that Clostridia cluster IV, XIVa, XVIII are key bacterial species involved in SCFA fermentation in the gut3. Amongst the SCFAs generated in vivo, butyrate has been reported to function as a nutrient source for colonocytes4, and as an agonist...
for G protein-coupled receptors (GPCRs: GPR41, GPR43, GPR109A) in multiple cell types to maintain intestinal barrier function. Moreover, butyrate has been suggested to function as a lysine deacetylase (KDAC) inhibitor to suppress inflammation in peripheral regulatory T (Treg) cells, neutrophils, macrophages and dendritic cells (DCs), as well as to prime an antimicrobial program in macrophages. Clostridia cluster IV, XIVa, XVIII production of butyrate is also implicated in peroxisome proliferator-activated receptor \( \gamma \) (PPAR\( \gamma \)) signaling to limit the lumen oxygen availability and prevent the expansion of \( S. \) Typhimurium in the intestinal lumen.

Beyond modulating host signaling pathways to fortify intestinal barrier integrity and suppress inflammation, microbiota-derived butyrate can also function as a nutrient source and directly attenuate the virulence of enteric pathogens. Depletion of microbiota with broad spectrum antibiotics or streptomycin alone increases host susceptibility to \( S. \) Typhimurium infection, which can be ameliorated by recolonization with butyrate-producing \( Clostridium \). Butyrate production by the microbiota has also been suggested to act as a carbon source for \( S. \) Typhimurium that expresses the \( ydiQRSTD \) operon. Notably, butyrate also inhibits the expression of virulence-associated genes in \( Salmonella \) pathogenicity island-1 (SPI-1) locus, which encodes transcriptional regulators, chaperones, secreted protein effectors and needle components of the type III secretion system (T3SS) required for invasion of epithelial cells and bacterial pathogenesis in vivo.

Nonetheless, the molecular mechanism by which butyrate directly inhibits the expression of SPI-1 virulence genes and \( S. \) Typhimurium infection is unclear.

To identify the protein targets of butyrate in \( S. \) Typhimurium, we employed a SCFA chemical reporter and label-free quantitative proteomics. We discovered that several virulence factors encoded within the SPI-1 locus could be acylated in \( S. \) Typhimurium. Amongst these acylated proteins, we focused on HilA, a key transcriptional regulator of SPI-1 gene expression and \( S. \) Typhimurium virulence. To address the potential site-specific functions of HilA acylation, we utilized CRISPR-Cas9 gene editing and genetic code expansion to install stable butyryl-lysine analogs at key lysine residues on chromosomally-expressed HilA. Our analysis of these engineered \( S. \) Typhimurium strains demonstrated that site-specifically butyrylation can inhibit and enhance HilA activity. Notably, acylation of lysine 90 impairs HilA occupancy and expression of SPI-1 genes as well as attenuates \( S. \) Typhimurium invasion of epithelial cells and pathogenesis in vivo. Moreover, a multi-site lysine-to-arginine and acylation-defective HilA mutant strain of \( S. \) Typhimurium was resistant to butyrate-mediated suppression of SPI-1 genes and slightly more virulent in microbiota-sufficient mice in vivo. Our results suggest that microbiota-derived SCFA acylation of key virulence factors may attenuate intestinal pathogen infection in vivo.

**Results**

**Proteomic analysis of acylated proteins in \( Salmonella \).**

Given that protein acylation is prevalent in bacteria, we explored whether microbiota-derived butyrate could acylate specific \( S. \) Typhimurium proteins involved in SPI-1 regulation using an alkyn-butyrate analog (alk-3, pentynoate) and chemical proteomics. Alk-3 behaved similar to butyrate and did not affect...
S. Typhimurium (strain 14028S) growth at 10 mM (Supplementary Fig. 1c), but inhibited expression of the SPI-1 gene sipA (Supplementary Fig. 1d) and S. Typhimurium invasion of HeLa cells (Supplementary Fig. 1e). To visualize acylated proteins, the total cell lysates from alk-3 treated S. Typhimurium cultures were reacted via bioorthogonal labeling with azide-rhodamine for in-gel fluorescence profiling (Supplementary Fig. 1b), which revealed many alk-3-modified proteins (Supplementary Fig. 1f). To identify these acylated proteins, alk-3-labeled total cell lysates were reacted with azide-biotin, enriched by streptavidin beads and analyzed by label-free quantitative (LFQ) proteomics. From this analysis, 56 proteins were selectively enriched more than 2-fold in alk-3 samples with P-values less than 0.05 (Fig. 1a and Supplementary Dataset). Bioinformatic analysis of the alk-3 hits recovered several proteins involved in metabolism as well as a few key effectors and regulators (SipD, SopB, SipA, SicA and Hila) of S. Typhimurium pathogenesis (Fig. 1a, Supplementary Dataset, and Supplementary Table 1). While the SPI-1 transcriptional regulator HilD has been suggested to modulate propionate activity in S. Typhimurium and could be regulated by acetylation, it was not labeled by alk-3 in our proteomic dataset (Supplementary Dataset) or when overexpressed (Supplementary Fig. 2a). Of note, Hila, a key transcriptional regulator of SPI-1 genes, was one of the most prominent acylated S. Typhimurium candidate proteins in the dataset (Fig. 1a and Supplementary Dataset).

Hila is a transcription regulator that regulates the expression of SPI-1 genes encoding T3SS components and substrates and also essential for S. Typhimurium infection \textit{in vitro} and \textit{in vivo}. Hila protein is predicted to contain an N-terminal DNA-binding domain (DBD) that is homologous to the OmpR/PhoB-family of transcription factors in bacteria, and a tetratricopeptide repeat (TPR) domain towards the C-terminus (Fig. 1b). To characterize Hila acylation, we expressed epitope-tagged Hila-HA-His6 in S. Typhimurium and validated labeling with alk-3 (Supplementary Fig. 2b), which was dose-dependent (Fig. 1c), resistant to hydroxylamine treatment (Supplementary Fig. 2c), and insensitive to the overexpression of S. Typhimurium acyltransferase YfiQ (Pat) or deacylase enzyme CobB, respectively (Supplementary Fig. 2d and e), suggesting Hila acylation occurs through posttranslational chemical acylation of lysine residues. In addition, deletion of other candidate protein acyltransferase genes (yfiQ, yiaC, or yjaB), homologous to \textit{E. coli} yiaC and yjaB, in S. Typhimurium did not affect alk-3 labeling of Hila (Supplementary Fig. 2f), further suggesting that millimolar levels of exogenous butyrate chemically acylates Hila through high energy acyl-CoA or phosphate intermediates in S. Typhimurium.

To characterize Hila acylation, we expressed and purified Hila-HA-His6 from S. Typhimurium incubated with 10 mM butyrate to identify sites of acylation. Of the 34 lysine residues in Hila (Supplementary Fig. 3a), we identified 5 lysines (K90, K231, K324, K456, and K533) that were modified with acetate, propionate or butyrate by LC-MS/MS analysis of tryptic peptides (Supplementary Fig. 3b–d and Supplementary Table 2), consistent with previous Hila acetylation studies. Protein structure prediction of Hila by Robetta suggests that all 5 acylated-lysine residues are located at surface-exposed sites for potential chemical acylation and are in predicted DNA-binding (K90) or protein-protein (K231, K324, K456) interaction domains (Fig. 1b). Amongst the five SCFA-modified lysine residues, K90 was the most prominent acylation site when we compared the ratio of acylated and unmodified peptides (Fig. 1d and Supplementary Table 2).
Site-specific incorporation of acyl-lysine analogs into HilA.

To evaluate site-specific lysine-butyrylation of HilA in *S. Typhimurium* *in vivo*, we explored genetic code expansion with \( N^e \)-butyryl-lysine analogs (Supplementary Fig. 4a)\(^{37-39}\). Expression of wild-type *Methanosarcina mazei* pyrrolysine-tRNA synthetase (MmPylRS-WT) and tRNA\(^{Pyl}\)\(_{CUA}\) in *S. Typhimurium* showed poor incorporation of \( N^e \)-butyryl-lysine (buK) into overexpressed HilA-HA bearing an amber codon at K90 (HilA-K90-TAG-HA) (Supplementary Fig. 4b and c). We then evaluated other PylRS mutants with buK and other analogs for improved incorporation of potential butyryl-lysine mimics (Supplementary Fig. 4b). Analysis of HilA-HA protein levels revealed that \( N^e \)-pent-4-ynyl-oxy-carbonyl-lysine\(^{40}\) afforded near wild-type levels of HilA-K90-TAG-HA with *Methanosarcina barkeri* PylRS-ASF mutant (MbPylRS-ASF)\(^{40-42}\) (Supplementary Fig. 4b and c), which also cross-reacted with anti-butyryl-lysine antibody (Supplementary Fig. 4c) and was readily detected by bioorthogonal labeling (Supplementary Fig. 4c). We subsequently employed this compound as a lysine butyrylation-mimic (bmK) in *S. Typhimurium*.

To incorporate bmK into chromosomally-expressed HilA, we optimized CRISPR-Cas9 gene editing\(^43\) in *S. Typhimurium* using single strand DNA templates and liquid selection (Supplementary Fig. 5 and 6). Our optimized CRISPR-Cas9 gene editing protocol enabled chromosomal insertion of an HA-tag at the C-terminus of HilA as well as alanine, glutamine and TAG mutants at K90. The specificity of our CRISPR-Cas9 gene editing protocol was confirmed by whole-genome sequencing of HilA-HA and HilA-K90-TAG-HA *S. Typhimurium* strains (Supplementary Table 3). The activity of the HilA-HA, K90A and K90Q (an acetylation mimic) on SPI-1 gene expression was similar to the parent *S. Typhimurium* strain 14028S (Fig. 2a). However, HilA-K90-bmK-HA showed significant reduction of SPI-1 gene expression almost to \( \Delta \)hilA levels (Fig. 2a). These differences in SPI-1 gene expression were correlated with *S. Typhimurium* HilA-K90 mutant infection of HeLa cells (Fig. 2b) and not significant in RAW264.7 macrophages (Fig. 2c), which are less dependent on SPI-1 for invasion\(^44\). These results demonstrate that classical site-directed mutagenesis to canonical amino acids may not reveal how site-specific metabolite-modifications regulate protein function in cells.

Site-specific acylation of HilA affects *Salmonella* virulence.

We then generated additional *S. Typhimurium* strains with chromosomal amber codon mutants at K231, K324, K456, and K533 in HilA-HA to evaluate site-specific differences in activity. To control for non-specific effects of bmK incorporation into HilA, we also generated amber codon mutants at K57 and K527 that are not acylated based on our MS/MS analysis but could function as controls for protein expression and activity. Importantly, these engineered HilA-HA strains replicated at similar rates to the parent *S. Typhimurium* strain 14028S in liquid culture (Supplementary Fig. 7a) and efficiently incorporated bmK into the HilA-TAG-HA mutants that were readily detected by bioorthogonal labeling and anti-HA immunoblotting (Fig. 2d). To control for genetic code expansion, the HilA-HA *S. Typhimurium* strain (wt) was also transformed with plasmid encoding PylRS and tRNA\(^{CUA}\) and labeled with the same concentration of bmK for all subsequent experiments. A comparative analysis of HilA mutants showed that full-length HilA-HA expression in amber codon mutants depended on the presence of bmK (Fig. 2d, Supplementary Fig. 7c and
d). BmK-incorporation at K57, K90 and K527 yielded near wild-type levels of full length protein, whereas K231, K324, 456 and K533 amber codon mutants afforded slightly lower amounts of HilA-HA (Supplementary Fig. 7b and d). Nonetheless, the analysis of these S. Typhimurium strains demonstrated that HilA-bmK-HA protein levels are not correlated with the levels of SPI-1 secreted proteins (Fig. 2e), suggesting site-specific effects of lysine-butyrylation on HilA function.

We then evaluated the transcriptional activity of site-specifically acylated HilA mutants in S. Typhimurium. Quantitative RT-PCR analysis of HilA target genes showed that relative to wt HilA-HA, site-specific incorporation of bmK at K57, K90, K324 and K456 afforded reduced expression of prgH, invF, sipA, orgB and spaO located in different operons within the SPI-1 locus (Fig. 3a). While K57 is not acylated, it is predicted to be in the DNA-binding interface of HilA (Fig. 1c) and upon bmK incorporation showed significantly reduced expression of HilA target genes (Fig. 3a), serving as a positive control loss-of-function HilA mutant. Interestingly, acylated-K90 within the DNA-binding domain of HilA (Fig. 1c) also showed significantly reduced expression of HilA target genes with bmK incorporation (Fig. 3a). The incorporation of bmK into the TPR domain of HilA at K324 and K456 also impaired the expression of SPI-1 genes (Fig. 3a). Alternatively, bmK incorporation at K231 afforded slightly enhanced expression of HilA target genes compared to wt HilA-HA, bmK at K527 (non-acylated control for bmK incorporation) and K533 (Fig. 3a). While buK did not get incorporated into HilA-TAG-HA mutants as efficiently as bmK (Supplementary Fig. 4b), we observed similar site-specific effects on the expression of SPI-1 genes, albeit at lower levels (Supplementary Fig. 8a).

To investigate the significance of site-specific acylation of HilA on S. Typhimurium virulence, we analyzed the invasion of the HeLa epithelial cell line by S. Typhimurium. For these experiments, both wild-type and HilA mutant S. Typhimurium strains were transformed with PyrRS and tRNA^{CUA} expression plasmid, metabolically labeled with bmK, diluted with PBS buffer and used for infection. In comparison with the other lysine residues in HilA-HA, bmK incorporation at K57 (loss-of-function control), K90, K324 and K456 attenuated S. Typhimurium infection of HeLa cells, whereas K231 showed enhanced or similar levels of infection compared to wild-type HilA, K527 (neutral control) and K533 bmK-mutants (Fig. 3b). There was no significant differences in infectivity among the bmK-mutants in RAW264.7 macrophages (Fig. 3c), which is less dependent on SPI-1 invasion. These results demonstrate site-specific lysine-butyrylation of HilA modulates S. Typhimurium invasion of epithelial cells, consistent with its modulation of HilA-dependent SPI-1 gene expression (Fig. 3a) and secretion of SPI-1 substrates (Fig. 2d).

Since K90 is the most prominent butyrate modification site in HilA (Fig. 1e and Supplementary Table 2) and afforded significantly reduced expression of SPI-1 genes and S. Typhimurium infection with bmK incorporation, we focused on this site of HilA modification for additional functional studies. Indeed, HilA butyrylation levels were increased when S. Typhimurium was incubated with butyrate (Supplementary Fig. 8b), and K90 site-specific butyrylation was more than doubled (Supplementary Fig. 8c). To investigate site-specific effects of K90-butyrylation, we evaluated HilA occupancy of SPI-1 genes in S. Typhimurium. Crosslinking and HA-immunoprecipitation-sequencing
(x-IP-Seq) analysis of HilA-HA S. Typhimurium strains showed that HilA-K90-bmK-HA had decreased occupancy on the promoter regions of HilA-target genes (prgH and invF) compared to wt HilA-HA (Fig. 4a and Supplementary Fig. 9). Further analysis of HilA-HA variants by crosslinking and HA-immunoprecipitation-qPCR (x-IP-qPCR) revealed site-specific effects of bmK-incorporation on HilA-HA DNA binding (Fig. 4b). Indeed, bmK incorporation at K90 and K57 (loss-of-function control) showed markedly less occupancy of prgH and invF promoters compared to wt HilA and K527 (neutral control) (Fig. 4b). In contrast, bmK incorporation at K324 and K456 had similar HilA-HA occupancy of these SPI-1 locations (Fig. 4b), suggesting that their inhibitory effects may function not through DNA binding but through alternative mechanisms associated with the predicted TPR domain (Fig. 1c and Supplementary Fig. 10). Interestingly, bmK incorporation at K231 enhanced HilA-HA occupancy at invF and prgH promoter regions (Fig. 4b), consistent with elevated expression of SPI-1 genes (Fig. 3a) and invasion of HeLa cells (Fig. 3b), suggesting butyrylation of this site may promote HilA oligomerization, DNA-binding and/or transcriptional activity. These results demonstrate site-specific modification of acylated-lysine residues directly modulates the function of HilA in S. Typhimurium.

Site-specific acylation of HilA affects Salmonella dissemination.

Given that site-specific bmK incorporation into HilA yielded significant differences in S. Typhimurium infection in vitro, we explored the infectivity of K90 and K231 bmK-mutants in vivo. For these studies, we employed the streptomycin-treatment model of S. Typhimurium infection in mice, which has been shown to deplete butyrate-producing microbiota and provide a SCFA-deficient environment for evaluating site-specific modification of HilA mutants in vivo. Our initial analysis of bmK-labeled HilA-K90-TAG-HA and HilA-K231-TAG-HA S. Typhimurium strains showed similar levels of overall pathogenesis upon oral infection of streptomycin-treated mice compared to ΔhilA S. Typhimurium (Fig. 5a,b), likely due to depletion of bmK and inactivation of full-length HilA protein expression after multiple days in vivo. We thus focused on S. Typhimurium dissemination into mesenteric lymph nodes (mLN) and liver early (2 days) post-infection to evaluate acute SPI-1–dependent invasion in vivo. For these experiments, we orally infected streptomycin-treated mice with bmK-labeled wt and HilA mutant S. Typhimurium strains, harvested their mLN and liver and measured colony-forming units (CFUs) by Salmonella-selective agar plates. In comparison to wt S. Typhimurium, the bmK-labeled HilA-K90 mutant was defective in dissemination from the intestines, similar to ΔhilA, while the bmK-labeled HilA-K231 mutant was recovered from mLN and liver at similar levels to wt (Fig. 5c). These results are consistent with previous reports of ΔhilA in vivo, which is more attenuated than mutations in the SPI-1 needle complex alone since HilA also regulates motility and other environmental response elements in S. Typhimurium. Moreover, histological analysis of intestinal sections revealed that wt S. Typhimurium caused significant inflammation in the mice cecum, whereas the bmK-labeled HilA-K90 mutant had mild to no inflammation in the cecum (Fig. 5d, and Supplementary Fig. 11). These results demonstrate that site-specific butyrylation of K90 on HilA nearly phenocopies ΔhilA and is sufficient to attenuate S. Typhimurium invasion and dissemination in vivo.
Multi-site HilA mutant is resistant to butyrate inhibition.

Since site-specific HilA lysine butyrylation impaired *S. Typhimurium* virulence, we investigated whether these bacterial strains would be resistant to butyrate inhibition. We initially evaluated K90 to alanine and arginine mutants as potential acylation-deficient mutants, but both single-site mutants showed similar sensitivity to butyrate inhibition of invasion in HeLa cells (Supplementary Fig. 12a). Since K324 and K456 could also be acylated (Supplementary Fig. 3 and Supplementary Table 2) and were inhibited by site-specific bmK incorporation (Fig. 3a and b), we hypothesized that butyrylation of K324 and K456 could still inhibit HilA function and *S. Typhimurium* virulence. We thus tested overexpressed multi-site HilA mutants in which K90, K324, and K456 were all mutated to alanine (K90,324,456A) or arginine (K90,324,456R) (Supplementary Fig. 12b). While the HilA-K90,324,456A mutant showed defective SPI-1 secretion activity and was not useful for butyrate inhibition studies (Supplementary Fig. 12b), the HilA-K90,324,456R exhibited similar SPI-1 secretion activity to wt HilA (Supplementary Fig. 12b). We thus generated HilA-K90,324,456R chromosomal mutant strain, and found that it was resistant to butyrate inhibition of SPI-1 gene expression (Fig. 6a) as well as invasion of HeLa cells (Fig. 6b). We thus explored infectivity of HilA-K90,324,456R mutant strain in vivo. Infection of microbiota-sufficient mice containing higher levels of butyrate with the HilA-K90,324,456R mutant strain resulted in shorter survival time compared to wt *S. Typhimurium* (Fig. 6c), which was correlated with a more rapid expansion and shedding of *S. Typhimurium* (Fig. 6d). The difference between wt *S. Typhimurium* and the HilA-K90,324,456R mutant strain was dependent on the microbiota, as streptomycin-treated mice had similar survival time and *S. Typhimurium* fecal load when infected with wt or HilA-K90,324,456R mutant strain (Supplementary Fig. 12c and d). These results further demonstrate that acylation of key virulence factors such as HilA by exogenous butyrate may contribute to microbiota attenuation of *S. Typhimurium* infection in vivo.

Discussion

SCFAs are abundant metabolites generated by microbiota-fermentation of dietary polysaccharides, but the molecular mechanisms by which these prominent gut metabolites inhibit enteric pathogen virulence has been unclear. For example, propionate generated by Bacteroides is reported to mediate colonization resistance against *S. Typhimurium* and other Enterobacteriaceae by disrupting intracellular pH homeostasis as well as destabilizing HilD, another SPI-1 transcriptional regulator, suggesting specific mechanisms from different microbiota species and metabolites that may act cooperatively to prevent *S. Typhimurium* infection in vivo. Indeed, our analysis of butyrate, associated with other microbiota species, revealed direct acylation of other SPI-1 factors may be another mechanism to attenuate *S. Typhimurium* virulence. The most prominent target from our chemical proteomic studies was HilA, a key transcriptional regulator of SPI-1 genes required for *S. Typhimurium* invasion of intestinal epithelial cells and dissemination in vivo. To functionally evaluate site-specific HilA butyrylation, we installed butyryl-Lys analogs into chromosomally expressed HilA mutants using CRISPR-Cas9 gene editing and non-canonical amino acid mutagenesis. These constitutively butyryl-Lys-modified HilA mutants revealed site-specific effects on *S. Typhimurium* virulence gene expression, invasion
of epithelial cells and dissemination in vivo (Supplementary Fig. 13a). Abrogation of acylation on critical lysine residues on HilA results in resistance to butyrate inhibition in vitro and in vivo. While HilA acylation is substoichiometric, it can be increased by the millimolar levels of exogenous butyrate reported in the gut and may be further regulated by specific microbiota-butyrate producers in vivo. S. Typhimurium may therefore sense high concentrations of this SCFA through HilA-acylation to limit invasion of host cells and utilize butyrate as a nutrient source from the microbiota in the intestinal lumen (Supplementary Fig. 13b). Finally, our discovery of inhibitory acylation on specific proteins and amino acid residues suggests repurposing covalent protein modification agents may afford new anti-infectives for SCFA-sensitive pathogens.

Methods

Microbial Strains and Growth Conditions.

All strains used are listed in Supplementary Table 4. All Salmonella Typhimurium strains used were derivatives of S. Typhimurium 14028S\textsuperscript{25}. Salmonella strains were cultured at 37°C in liquid Miller Luria-Bertani (LB) medium [10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl] (Becton Dickinson, Difco\textsuperscript{TM}), SPI-1 inducing LB medium [10 g/L tryptone, 5 g/L yeast extract, 300 mM NaCl], or on Salmonella Shigella agar (Becton Dickinson). Cultures were grown at 37°C in Multitron shaking incubator (INFORS HT) at 220 rpm. When required, antibiotics were added to the medium as follows: carbenicillin 100 μg/mL, kanamycin 50 μg/mL, and chloramphenicol 10 μg/mL.

Animal Experiments.

C57BL/6J (000664) mice were purchased from the Jackson Laboratory and maintained at the Rockefeller University animal facilities under SPF conditions. Animal care and experimentation were consistent with the National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committee of the Rockefeller University.

Chemicals.

Sodium butyrate was purchased from Sigma-Aldrich (303410). Alk-3 (4-pentynoic acid) was purchased from Sigma-Aldrich (232211). Az-Rho was synthesized in the lab as previously described\textsuperscript{51}. Az-biotin was purchased from Sigma-Aldrich (762024). Butyryllysine was synthesized according to previously described\textsuperscript{38}. BmK was synthesized according to previously described\textsuperscript{40}.

Generation of HilA-K90bu site-specific antibody.

HilA-K90bu peptide (CRI LSED(butyrylK)EHRYIE) and HilA-K90 peptide (CRI LSED KEHR YIE) was synthesized in The Rockefeller University Proteomics Resource Center. HilA-K90bu peptide was sent to Thermo Fisher for custom antibody production with a 70-day rabbit immunization protocol. Serum was collected on Day 35, 56 and 58 and measured for titer with ELISA. Site-specific polyclonal antibodies were affinity purified and counter-selected with HilA-K90 peptide.
**Salmonella growth curve.**

Overnight cultures of *Salmonella* strains in Miller LB were diluted 1:100 to 5 mL fresh SPI-1 inducing LB medium in Falcon round-bottom 15 mL tubes. Cultures were taken out aliquots of 750 μL from 0 hour to 5 hours at 1-hour interval. Aliquots were added to 10 mm polystyrene cuvettes (Sarstedt) and OD₆₀₀ was measured with Biophotometer plus (Eppendorf).

**Preparation of *Salmonella* bacterial total cell lysates.**

One to fifty dilutions of overnight Miller LB cultures of *Salmonella* Typhimurium strain 14028s WT overnight culture were grown in 4 mL SPI-1 inducing LB for 4 h at 37°C with 220 rpm shaking. For alk-3 labeling experiments, cultures were incubated with or without 10 mM fresh alk-3 (in dH₂O). For incorporation of ncAA into overexpressed HilA, cultures were added with 1 mM ncAA and 0.2% arabinose. For incorporation of ncAA into endogenous HilA, cultures were added with 100 uM ncAA and 0.01% arabinose. *S. Typhimurium* cells were pelleted at 15000 g for 1 min, and pellets were lysed with 200 μL lysis buffer (phosphate-buffered saline (PBS) containing 0.5% Nonidet P-40, 1X EDTA-free protease inhibitor cocktail (Roche), 0.5 mg/mL lysozyme (in dH₂O) (Sigma), and 1:1,000 dilution of Benzonase (Millipore)). After re-suspension, pellets were sonicated for 10 sec for 3 times, then were incubated on ice for 30 min. Cell lysates were centrifuged at 15000 g for 1 min to remove cell debris and supernatants were collected. Protein concentration was estimated by BCA assay with BCA Protein Assay Kit (Thermo).

**Salmonella protein immunoprecipitation and immunoblotting.**

From *Salmonella* total cell lysates prepared as described above, protein samples were boiled with 1X Laemmli buffer 95°C for 5 min. 20 μL of each sample was loaded onto a 4–20% Tris-HCl gel (Bio-Rad) for SDS-PAGE. Proteins were transferred onto 0.45 μm nitrocellulose membrane (Bio-Rad) with Trans-Blot Turbo Transfer System (Bio-Rad) at 25 V for 30 min. Membrane was blocked with 5% non-fat milk in PBS with 0.1% Tween-20 (PBS-T) for 30 min, and primary antibody was added to solution before incubating membrane at 4°C overnight. Dilution of primary antibodies were as follows: for HA-tagged proteins, 1:2,000 anti-HA rabbit antibody H6908 (Sigma); for FLAG-tagged proteins, 1:2000 anti-FLAG rabbit antibody F7425 (Sigma); for HilA-K90Bu antigen, 1:200 anti-HilAK90Bu rabbit custom antibody (Thermo Fisher) or 1:200 anti-HilAK90 rabbit custom antibody (Thermo Fisher). Membrane was washed with PBS-T 3 times, and incubated with 1:10,000 goat polyclonal anti-rabbit HRP ab97051 (Abcam) in PBS-T with 5% non-fat milk at room temperature for 1 hour. Membrane was washed with PBS-T 3 times, and imaged with Clarity Western ECL substrate (Bio-Rad) and ChemiDoc XRS+ System (Bio-Rad).

For *Salmonella* protein immunoprecipitation, 250 μg of each total cell lysates were incubated with 20 μL PBS-T-washed EZview™ Red Anti-HA Affinity Gel (Sigma) at 4°C for 1 hour with end-to-end rotation. Samples were washed with 200 μL PBS-T for 3 times, before being boiled with 1X Laemmli buffer 95°C for 5 min. 20 μL of each sample was loaded onto a 4–20% Tris-HCl gel (Bio-Rad) for SDS-PAGE and further immunoblotting.
In-gel fluorescence analysis of alk-3 labeling.

For in-gel fluorescence analysis of alk-3 labeled Salmonella proteome, from the alk-3-treated or control total cell lysates prepared as described above, 45 μL of each total cell lysates (~50 μg) was added with 5 μL of click chemistry reagents as a 10X master mix (az-Rho: 0.1 mM, 10 mM stock solution in DMSO; tris(2-carboxyethyl)phosphine hydrochloride (TCEP): 1 mM, 50 mM freshly prepared stock solution in dH2O; tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA): (0.1 mM, 2 mM stock in 4:1 t-butanol: DMSO); CuSO4 (1 mM, 50 mM freshly prepared stock in dH2O). Samples were mixed well and incubated at room temperature for 1 h. After incubation, samples were mixed with 200 μL cold methanol, 150 μL cold water, and 75 μL cold chloroform. Sample proteins were precipitated at 18000 g for 1 min at 4 °C. After gently removing the aqueous layer, protein pellets were washed with 200 μL cold methanol, spinning down at 18000 g for 1 min at 4 °C, and liquid was gently decanted. After washing twice, pellets were allowed air-dried before boiling with 1X Laemmli buffer.

For in-gel fluorescence analysis of alk-3 labeled HilA, from the alk-3-treated or control total cell lysates prepared as described above, 250 μg of each total cell lysates were immunoprecipitated with 20 μL PBS-T-washed EZview™ Red Anti-HA Affinity Gel (Sigma). After samples were washed with 200 μL PBS-T for 3 times, 36 μL of PBS was added to each sample. 4 μL of click chemistry reagents as a 10X master mix mentioned above were added to each sample. Samples were mixed well and incubated at room temperature for 1 h. After incubation, samples were washed with 200 μL PBS-T for 3 times.

Samples were boiled with 1X Laemmli buffer 95°C for 5 min before being loaded onto a 4–20% Tris-HCl gel (Bio-Rad) for SDS-PAGE. In-gel fluorescence scanning was performed using a Typhoon 9400 imager (Amersham Biosciences).

Alk-3 labeling Label-Free Quantitative proteomics.

One to fifty dilutions of overnight Miller LB cultures of Salmonella Typhimurium strain 14028s WT overnight culture were grown in 20 mL SPI-1 inducing LB for 4 h at 37°C with 220rpm shaking, each sample growing in 4 mL aliquots. Cultures were incubated with or without 10 mM fresh alk-3 (in dH2O). Cultures were pooled back to 20 mL per sample in Falcon tubes, and lysed in lysis buffer described above. After re-suspension in 1 mL lysis buffer, bacteria were sonicated for 15 sec with Sonic Dismembrator Model 500 (Fisher Scientific) with 5 sec on and 10 sec off per cycle. Cell lysates were centrifuged at 15000 g for 1 min to remove cell debris and supernatants were collected. Each total cell lysates (~2 mg) was added with 100 μL of click chemistry reagents as a 10X master mix (az-Biotin: 0.1 mM, 10 mM stock solution in DMSO; tris(2-carboxyethyl)phosphine hydrochloride (TCEP): 1 mM, 50 mM freshly prepared stock solution in dH2O; tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA): (0.1 mM, 2 mM stock in 4:1 t-butanol: DMSO); CuSO4 (1 mM, 50 mM freshly prepared stock in dH2O). Samples were mixed well and incubated at room temperature for 1 h. After incubation, samples were mixed with 4 mL cold methanol and incubated at −20°C overnight. Protein pellets were centrifuged at 5000 g for 30 min at 4°C, and were washed with 1 mL cold methanol 3 times. After last wash, pellets were let
air dried before being re-solubilized in 250 μL 4% SDS PBS with bath sonication. Solutions were diluted with 750 μL PBS, and incubated with 60 μL PBS-T-washed High Capacity NeutrAvidin agarose (Pierce) at room temperature for 1 h with end-to-end rotation. Agarose were washed with 500 μL 1% SDS PBS 3 times, 500 μL 2M Urea PBS 3 times, and 500 μL PBS 3 times. Agarose were then reduced with 100 μL 10 mM DTT (Sigma) in PBS for 30 min at 37°C, and alkylated with 100 μL 50 mM iodoacetamide (Sigma) in PBS for 20 min in dark. On-bead proteins were digested with 400 ng Trypsin/Lys-C mix (Promega) at 37°C overnight with shaking. Digested peptides were collected and lyophilized before being desalted with custom-made stage-tip containing Empore SPE Extraction Disk (3M). Peptides were eluted with 2% acetonitrile, 2% formic acid in dH₂O.

Peptide LC-MS analysis was performed as described previously. Briefly, samples were analyzed with a Dionex 3000 nano-HPLC coupled to an Orbitrap XL mass spectrometer (Thermo Fisher). Peptide samples were pressure-loaded onto a home-made C18 reverse-phase column (75 μm diameter, 15 cm length). A 180-minute gradient from 95% buffer A (HPLC grade water with 0.1% formic acid) and 5% buffer B (HPLC grade acetonitrile with 0.1% formic acid), to 25% buffer A and 75% buffer B in 133 minutes was used at 200 nL/min. The Orbitrap XL was operated in top-8-CID-mode with MS spectra measured at a resolution of 60,000@m/z 400. One full MS scan (300–2000 MW) was followed by three data-dependent scans of the nth most intense ions with dynamic exclusion enabled. Peptides fulfilling a Percolator-calculated 1% false discovery rate (FDR) threshold were reported.

Label-free quantification of alk-3 labeled proteins was performed with the label-free MaxLFQ algorithm in MaxQuant software as described. The search results from MaxQuant were analyzed by Perseus (http://www.perseusframework.org/). Briefly, the control replicates and alk-3 labeled sample replicates were grouped correspondingly. The results were cleaned to filter off reverse hits and contaminants. Only proteins that were identified in all alk-3 labeled sample replicates and with more than two unique peptides were subjected to subsequent statistical analysis. LFQ intensities were used for measuring protein abundance and logarithmized. Signals that were originally zero were imputed with random numbers from a normal distribution, whose mean and standard deviation were chosen to best simulate low abundance values below the noise level (Replace missing values by normal distribution with Width = 0.3 and Shift = 2.2). Significant proteins enriched in alk-3 labeled sample group versus control group were determined by a threshold strategy, in which ratio larger than or equals to 2 and P-value smaller than 0.05 were categorized as hits. The resulting table was exported as Supplementary Dataset.

MS/MS detection of protein PTM.

One to fifty dilutions of overnight Miller LB cultures of Salmonella Typhimurium strain 14028s ΔhilA pBAD-HilA-HA-His overnight culture were grown in 500 mL SPI-1 inducing LB with 10 mM sodium propionate or 10 mM sodium butyrate for 2 h at 37°C with 220rpm shaking, before 0.2% arabinose was added to induce HilA-HA-His expression for 3 h. Bacteria was harvested with 5000 g for 10 min at 4°C, and lysed in 25 mL lysis buffer described above with 1 mM EDTA and 50 mM nicotinamide (NAM). bacteria were sonicated for 5 min with Sonic Dismembrator Model 500 (Fisher Scientific) with 5 sec
on and 10 sec off per cycle. Lysates were centrifuged at 5000 g for 10 min at 4°C, and supernatants were filtered with 0.22 um filter before being loaded onto HisTrap FF 5mL column (GE Healthcare). HilA-HA-His protein was purified with wash with 3 Column Volume (CV) 100% buffer A (PBS-T, 50 mM NAM), wash with 5 CV 10% buffer B (PBS-T, 50 mM NAM, 300 mM imidazole), and 5 CV elution with gradient from 10% to 100% buffer B. Fractions containing HilA was pooled and dialyzed to PBS-T with 3 spin-dilution cycles in 10,000 MWCO filter (Amicon). Purified HilA-HA-His was immunoprecipitated with anti-butyryllysine antibody (PTM Biolab)- or anti-propionyllysine antibody (PTM Biolab)-conjugated protein A/G magnetic beads (Pierce), and samples were boiled and run on SDS-PAGE. Coomassie Blue-stained bands corresponding to HilA was cut out, reduced with 10 mM DTT (Sigma) in fresh 100 mM ammonium bicarbonate (ABC) for 30 min at 37°C, and alkylated with 50 mM iodoacetamide (Sigma) in fresh 100 mM ABC for 20 min in dark. Gel pieces were digested with 200 ng Trypsin/Lys-C mix (Promega) at 37°C overnight with shaking.

**Salmonella CRISPR-Cas9 genome editing.**

To make electrocompetent Salmonella Typhimurium, overnight culture of Salmonella Typhimurium strains were diluted 1:50 to 100 mL fresh LB, and were grown at 37°C in shaking incubator at 220 rpm for 2 hours, until OD_{600} reached 0.5 to 0.7. Cells were pelleted at 5000 g for 10 min at 4°C, and washed with 50 mL ice-cold 10% glycerol twice. Cell pellets were resuspended in 500 μL 10% glycerol, and aliquoted 50 μL per tube. Electrocompetent parent Salmonella Typhimurium strains were transformed with pKD46 via electroporation with Gene Pulser II (Bio-Rad) at 2.5 kV and 25 μF in 2 mm cuvette, and selected on Ampicillin agar plates at 30 °C overnight. The resulting Salmonella Typhimurium pKD46 strains were made into electrocompetent cells after grown at 30°C with 0.2% arabinose and ampicillin. Salmonella Typhimurium pKD46 electrocompetent cells were transformed with 2 μL pWJ297-sgRNA (∼100 ng) and 10 μL 10 uM ssDNA editing template and selected on Chloramphenicol agar plates at 37 °C overnight. All colonies on the plate were collected with cell scraper and resuspended in 4 mL LB with Chloramphenicol. The bacterial suspension were diluted 1:50 to 4 mL fresh LB with Chloramphenicol, and were grown at 37°C in Multitron shaking incubator (INFORS HT) at 220 rpm for 2 hours. Culture was streaked onto Chloramphenicol agar plates, and colonies from the plates were randomly picked for colony PCR to confirm successful editing. Successfully edited colonies were streaked onto plain agar plates to cure pWJ297-sgRNA, and curing was confirmed by streaking on Chloramphenicol agar plates.

Optimization of Salmonella CRISPR-Cas9 genome editing are described as follows. The protocol for CRISPR-Cas9 genome editing in *E. coli* resulted in very high false-positive rate and an estimated editing efficiency of less than 5% (data not shown). To increase editing efficiency, we introduced synonymous co-mutation of nearest PAM together with targeted sequence, and used gRNA specific for that PAM to guide Cas9 (Supplementary Fig. 5a). As an example, we set out to mutate Lys90 to Ala in HilA. Synonymous PAM co-mutation (c243g) increased editing efficiency and resulted in successful editing (Supplementary Fig. 5b). Colonies selected for pWJ297 (pCas9) and pCRISPR were mostly false-positive, meaning that CRISPR-Cas9 system did not impose an adequate selection pressure. We
surmised that *Salmonella* has an intrinsic suppressive mechanism to inactivate exogenous CRISPR-Cas9 system. Therefore, we collected all colonies from the plate of first-round selection, suspended them in liquid medium LB with corresponding antibiotics, and grew them to early stationary phase. The resulting culture was plated again on selection agar plate. This liquid selection protocol drastically increased frequency of successfully edited clones (Supplementary Fig. 5c). The plasmid pCRISPR is very resistant to curing, taking up to one month of serial purification (data not shown), which is presumably due to its small size and high copy number. Therefore, we incorporated sgRNA sequence directly at pWJ297 BsaI cloning site, sparing the usage of pCRISPR. The editing efficiency of pWJ297-sgRNA is similar to pWJ297 + pCRISPR combination (data not shown), while the former was cured in about 1 day. SsDNA recombination efficiency is much higher than dsDNA in *E. coli* because ssDNA has higher transformation efficiency\(^{54}\). Therefore, we decided to use ssDNA template when editing site-specific mutation of single codons. CRISPR-Cas9 genome editing with ssDNA provided higher editing efficiency compared to that with long dsDNA template that has the same mutation site (Supplementary Fig. 5d). We observed no significant difference of editing efficiency among ssDNA templates that matches either the same strand or the complementary strand of sgRNA, nor between templates matching either the leading or the lagging strand of the genome (Supplementary Fig. 5e). To summarize, we have optimized the scarless CRISPR-Cas9 genome editing protocol to facilitate the editing process in *Salmonella* that only takes 3–4 days (Supplementary Fig. 6).

**Salmonella** whole-genome sequencing.

One mL of *Salmonella* cultures were processed with Quick-DNA Fungal/Bacterial kit (Zymo Research) per manufacturer’s manual. Purified *Salmonella* genomes were sent to Rockefeller University Genomics Center for processing with Nextera XT gDNA library preparation and sequencing with MiSeq 75 Pair-End sequencing. Sequencing results were analyzed with Geneious software.

**Salmonella** Quantitative Reverse-Transcription PCR.

Five hundred μL of *Salmonella* cultures were processed with RNeasy Mini Kit (Qiagen) per manufacturer’s manual. Concentrations of purified RNA were normalized to 100 ng/μL with RNase-free water. Quantitative Reverse-Transcription PCR (qRT-PCR) were performed with Power SYBR Green RNA-to-C\(_T\) 1-Step Kit (Applied Biosystems) per manufacturer’s manual and primers listed in Supplementary Table 5.

**Salmonella** HilA crosslink-IP-qPCR and crosslink-IP-seq.

Four mL *Salmonella* cultures were crosslinked with 1% methanol-free PFA (Thermo) for 20 min at room temperature. Crosslinking were quenched with 125 mM Glycine (Fisher). Bacteria were centrifuged 16000 g at 4°C for 1 min and washed with 1 mL PBS twice. Pellets were resuspended in 500 μL ChIP Lysis Buffer (10 mM Tris-HCl, pH 8.0, 20% sucrose, 50 mM NaCl, 10 mM EDTA, 10 mg/mL lysozyme), and incubated at 37°C for 30 min. Lysates were added with 500 μL 2X RIPA buffer (100 mM Tris-HCl, pH 8.0, 300 mM NaCl, 2% Nonidet P-40 (NP-40), 1% sodium deoxycholate, 0.2% SDS) and sonicated with Sonic Dismembrator Model 500 (Fisher Scientific) for 10 sec. Resulting solutions were centrifuged at 16000 g for 1 min at room temperature. 100 μL supernatants were saved as
total inputs. 750 μL of the remaining supernatants of each were incubated with 2 μL anti-HA ChIP-grade polyclonal antibody (ab9110, Abcam) at 4°C for 1 h with end-to-end rotation. The solutions were then added to 30 μL PBS-T-washed protein A/G magnetic beads (Pierce) and incubated at 4°C for 1 h with end-to-end rotation. Beads were washed with 500 μL 1X RIPA buffer twice, 500 μL LiCl Wash buffer (10 mM Tris-HCl, pH 8.0, 250 mM LiCl, 1 mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate) twice, and 500 μL Tris-EDTA buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) once. Samples were eluted with 100 μL SDS Elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS) at 65°C for 10 min. Each total input sample and ChIP sample were added with 5 μL 20 mg/mL proteinase K (Qiagen) and de-crosslinked at 65°C overnight. All de-crosslinked samples were purified with E.Z.N.A. Cycle Pure kit (Omega Bio-tek) and eluted with 100 μL elution buffer in the kit. ChIP-qPCR were performed with PowerUp SYBR Green Master Mix (Applied Biosystems) per manufacturer’s manual and primers listed in Supplementary Table 5. Same samples were sent to Rockefeller University Genomics Center for library preparation and sequenced with NextSeq High Output 75 Single-Read sequencing.

**In vitro invasion assay and intracellular survival assay.**

HeLa cells were cultured in 12-well tissue culture plates at 80–90% confluency. Wells were added with *Salmonella* cells at an MOI = 10:1 and centrifuged at 1000 g for 5 min. Cells were incubated at 37°C with 5% CO$_2$ for 30 min to allow invasion. The media was then replaced with medium containing 100 μg/mL gentamicin and incubated for an additional hour to kill extracellular *Salmonella*. Wells were then washed 3 times with PBS, and cells were lysed with 500 μL 1% Triton X-100 PBS. Lysates were serially diluted and drip-dropped on *Salmonella Shigella* agar plates (BD 211597) to determine the number of invaded bacteria.

For intracellular survival assay of *Salmonella*, after incubation with medium containing 100 μg/mL gentamicin for 1 h, media was replaced with medium containing 10 μg/mL gentamicin and incubated for additional 4.5 hours at 37°C, 5% CO$_2$. Intracellular bacterial counts were obtained by lysing cells and drip-dropping serial dilutions on *Salmonella Shigella* agar plates.

**S. Typhimurium infection of mice.**

For streptomycin treatment experiments, C57BL/6J SPF mice were gavaged with a single dose of 20 mg of streptomycin 24 hours before infection. Bacterial cultures of different *S. Typhimurium* strains were washed and re-suspended in sterile phosphate-buffered saline (PBS) at 10$^7$ CFU/mL. Mice were gavaged with 100 μL of the bacterial suspension (10$^6$ CFU). Leftover inocula were serially diluted and plated to confirm the number of CFU administered.

For 48 h infection experiments, mice were euthanized 48 hours after *S. Typhimurium* gavage. Colony-forming units (CFU) in the livers and mesenteric lymph nodes (mLN) were determined by plating five serial dilutions of livers or mLN suspended in sterile 0.1% Triton X-100 PBS on *Salmonella Shigella* agar (BD 211597). Resulting quantities were normalized to liver or mLN weight.
For *S. Typhimurium* infection survival assay, mice weight loss was monitored starting just before infection, and mice were euthanized when they reached 80% baseline weight, appeared hunched or moribund, or exhibited a visibly distended abdomen (indicative of peritoneal effusion), whichever occurred first. Death was not used as an end point.

**Quantification and Statistical Analysis.**

Comparisons and statistical tests were performed as indicated in each figure legend. Briefly, pairwise comparisons were generated with two-tailed t tests. For comparisons of multiple groups over time or with two variables, a two-way analysis of variance (ANOVA) was used with an appropriate Bonferroni posttest comparing all groups to each other, all groups to a control, or selected groups to each other. Survival data were analyzed using a log-rank (Mantel-Cox) test with a Bonferroni correction for the degrees of freedom based on the number of comparisons made. To compare two groups with non-normal distribution or low sample size, the medians of the two groups were compared using a Mann-Whitney test, unless one data set contained only zero values. In these cases, a Mann-Whitney test could not be performed because all values in the group were identical; a Wilcoxon test was performed instead. For comparisons of multiple groups with only one variable, a one-way ANOVA or Kruskal-Wallis test was performed for data with underlying normal or non-normal distribution, respectively, with Bonferroni, Dunnett’s, or Dunn’s posttests where appropriate. Statistical analyses were performed in GraphPad Prism software. A *P* value of less than 0.05 was considered significant, denoted as *P* ≤ 0.05, **P** ≤ 0.01, and ***P** ≤ 0.001 for all analyses.

**Data and materials availability**

The data and materials that support the findings of this study are available from the corresponding author upon reasonable request.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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References:

1. Rooks MG & Garrett WS Gut microbiota, metabolites and host immunity. Nature Reviews Immunology 16, 341–352 (2016).

2. Cummings JH, Pomare EW, Branch WJ, Naylor CP & Macfarlane GT Short chain fatty acids in human large intestine, portal, hepatic and venous blood. Gut 28, 1221–1227 (1987). [PubMed: 3678950]

3. Atarashi Ket al. Induction of colonic regulatory T cells by indigenous Clostridium species. Science (80-. ). 331, 337–41 (2011).

4. Donohoe D Ret al. The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon. Cell Metab. 13, 517–526 (2011). [PubMed: 21531334]

5. Elinav Eet al. NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. Cell145, 745–757 (2011). [PubMed: 21565393]

6. Voltolini Cet al. A novel antiinflammatory role for the short-chain fatty acids in human labor. Endocrinology153, 395–403 (2012). [PubMed: 22186417]

7. Trompette Aet al. Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. Nat. Med20, 159–166 (2014). [PubMed: 24390308]

8. Singh Net al. Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. Immunity40, 128–139 (2014). [PubMed: 24412617]

9. Macia Let al. Metabolite-sensing receptors GPR43 and GPR109A facilitate dietary fibre-induced gut homeostasis through regulation of the inflammasome. Nat. Commun6, 6734 (2015). [PubMed: 25828455]

10. Furusawa Yet al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature504, 446–50 (2013). [PubMed: 24226770]

11. Arpaia Net al. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. Nature504, 451–455 (2013). [PubMed: 24226773]

12. Smith PMet al. The Microbial Metabolites, Short-Chain Fatty Acids, Regulate Colonic Treg Cell Homeostasis. Science (80-. ). 341, 569–573 (2013).

13. Vinolo MARet al. Suppressive effect of short-chain fatty acids on production of proinflammatory mediators by neutrophils. J. Nutr. Biochem22, 849–855 (2011). [PubMed: 21167700]

14. Chang PV, Hao L, Offermanns S & Medzhitov R The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. Proc. Natl. Acad. Sci 111, 2247–2252 (2014). [PubMed: 24390544]

15. Schulthess Jet al. The Short Chain Fatty Acid Butyrate Imprints an Antimicrobial Program in Macrophages. Immunity50, 432–445.e7 (2019). [PubMed: 30683619]

16. Byndloss MXet al. Microbiota-activated PPAR-γ signaling inhibits dysbiotic Enterobacteriaceae expansion. Science (80-. ). 357, 570–575 (2017).

17. Rivera-Chávez Fet al. Depletion of Butyrate-Producing Clostridia from the Gut Microbiota Drives an Aerobic Luminal Expansion of Salmonella. Cell Host Microbe19, 443–454 (2016). [PubMed: 27078066]

18. Bronner DNet al. Genetic Ablation of Butyrate Utilization Attenuates Gastrointestinal Salmonella Disease. Cell Host Microbe23, 266–273.e4 (2018). [PubMed: 29447698]

19. Gantois Iet al. Butyrate specifically down-regulates Salmonella pathogenicity island 1 gene expression. Appl. Environ. Microbiol72, 946–949 (2006). [PubMed: 16391141]

20. Lawhon SD, Maurer R, Suyemoto M & Altier C Intestinal short-chain fatty acids alter Salmonella typhimurium invasion gene expression and virulence through BarA/SirA. Mol. Microbiol 46, 1451–1464 (2002). [PubMed: 12453229]

21. Groisman E. a & Ochman H Cognate gene clusters govern invasion of host epithelial cells by Salmonella typhimurium and Shigella flexneri. EMBO J. 12, 3779–3787 (1993). [PubMed: 8404849]
22. Hentchel KL & Escalante-Semerena JC Acylation of Biomolecules in Prokaryotes: a Widespread Strategy for the Control of Biological Function and Metabolic Stress. Microbiol. Mol. Biol. Rev 79, 321–346 (2015). [PubMed: 26179745]

23. Yang Y-Y, Ascano JM & Hang HC Bioorthogonal chemical reporters for monitoring protein acetylation. J. Am. Chem. Soc 132, 3640–3641 (2010). [PubMed: 20192265]

24. Grammel M & Hang HC Chemical reporters for biological discovery. Nature Chemical Biology 9, 475–84 (2013). [PubMed: 23868317]

25. Hung C-CC et al. The intestinal fatty acid propionate inhibits Salmonella invasion through the post-translational control of HilD. Mol. Microbiol87, 1045–1060 (2013). [PubMed: 23289537]

26. Sang Yet al. Acetylation Regulating Protein Stability and DNA-Binding Ability of HilD, thus Modulating Salmonella Typhimurium Virulence. J. Infect. Dis 216, 1018–1026 (2017). [PubMed: 28329249]

27. Lostroh CP, Bajaj V & Lee CA The cis requirements for transcriptional activation by HilA, a virulence determinant encoded on SPI-1. Mol. Microbiol 37, 300–315 (2000). [PubMed: 10931326]

28. Bajaj V, Lucas RL, Hwang C & Lee CA Co-ordinate regulation of Salmonella typhimurium invasion genes by environmental and regulatory factors is mediated by control of hilA expression. Mol. Microbiol 22, 703–714 (1996). [PubMed: 8951817]

29. Ellermeier JR & Slauh JM Adaptation to the host environment: regulation of the SPI1 type III secretion system in Salmonella enterica serovar Typhimurium. Curr. Opin. Microbiol 10, 24–9 (2007). [PubMed: 17208038]

30. Bajaj V, Hwang C & Lee CA hilA is a novel ompR/toxR family member that activates the expression of Salmonella typhimurium invasion genes. Mol. Microbiol 18, 715–727 (1995). [PubMed: 8817493]

31. Lostroh CP & Lee CA The HilA box and sequences outside it determine the magnitude of HilA-dependent activation of P(prgH) from Salmonella pathogenicity island 1. J. Bacteriol 183, 4876–85 (2001). [PubMed: 11466291]

32. Eichelberg K & Galan JE Differential Regulation of Salmonella typhimurium Type III Secreted Proteins by Pathogenicity Island 1 (SPI-1)-Encoded Transcriptional Activators InvF and HilA. Infect. Immun 67, 4099–4105 (1999). [PubMed: 10417179]

33. Boddicker JD, Knosp BM & Jones BD Transcription of the Salmonella Invasion Gene Activator, hilA, Requires HilD Activation in the Absence of Negative Regulators. J. Bacteriol 185, 525–533 (2003). [PubMed: 12511499]

34. Christensen DG et al. Identification of Novel Protein Lysine Acetyltransferases in Escherichia coli. MBio9, e01905–18 (2018).

35. Sturm AB, Rist of ttss-1 genes in Salmonella Typhimurium. (Diss., Eidgenössische Technische Hochschule ETH Zürich, Nr. 19635, 2011, 2011). doi:10.3929/ethz-a-006715280

36. Kim DE, Chivian D & Baker D Protein structure prediction and analysis using the Robetta server. Nucleic Acids Res. 32, W526–W531 (2004). [PubMed: 15215442]

37. Chin JW, Expanding and reprogramming the genetic code. Nature550, 53–60 (2017). [PubMed: 28980641]

38. Gattner MJ, Vrabel M & Carell T Synthesis of ε-N-propionyl-, ε-N-butyryl-, and ε-N-crotonyllysine containing histone H3 using the pyrrolysine system. Chem. Commun. (Camb) 49, 379–81 (2013). [PubMed: 23192406]

39. Neumann H, Peak-Chew SY & Chin JW Genetically encoding N(epsilon)-acetyllysine in recombinant proteins. Nat. Chem. Biol 4, 232–4 (2008). [PubMed: 18278036]

40. Li Jet al. Ligand-free palladium-mediated site-specific protein labeling inside gram-negative bacterial pathogens. J. Am. Chem. Soc 135, 7330–7338 (2013). [PubMed: 23641876]

41. Zhang Met al. A genetically incorporated crosslinker reveals chaperone cooperation in acid resistance. Nat. Chem. Biol17, 671–677 (2011). [PubMed: 21892184]

42. Lin Set al. Site-specific incorporation of photo-cross-linker and bioorthogonal amino acids into enteric bacterial pathogens. J. Am. Chem. Soc 133, 20581–7 (2011). [PubMed: 22084898]

43. Jiang W, Bikard D, Cox D, Zhang F & Marraffini L. a. RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Nat. Biotechnol 31, 233–9 (2013). [PubMed: 23360965]
44. Drecktrah D, Knodler LA, Ireland R & Steele-Mortimer O The mechanism of Salmonella entry determines the vacuolar environment and intracellular gene expression. Traffic 7, 39–51 (2006). [PubMed: 16445685]

45. Barthel Met al. Pretreatment of mice with streptomycin provides a Salmonella enterica serovar Typhimurium colitis model that allows analysis of both pathogen and host. Infect. Immun 71, 2839–2858 (2003). [PubMed: 12704158]

46. Ellermeier CD, Ellermeier JR & Slauch JM HilD, HilC and RtsA constitute a feed forward loop that controls expression of the SPI1 type three secretion system regulator hilA in Salmonella enterica serovar Typhimurium. Mol. Microbiol 57, 691–705 (2005). [PubMed: 16045614]

47. Hapfelmeier Set al. The Salmonella Pathogenicity Island (SPI)-2 and SPI-1 Type III Secretion Systems Allow Salmonella Serovar typhimurium to Trigger Colitis via MyD88-Dependent and MyD88-Independent Mechanisms. J. Immunol 174, 1675–1685 (2005). [PubMed: 15661931]

48. Thijs IMVet al. Delineation of the Salmonella enterica serovar Typhimurium HilA regulon through genome-wide location and transcript analysis. J. Bacteriol 189, 4587–4596 (2007). [PubMed: 17483226]

49. Jacobson Aet al. A Gut Commensal-Produced Metabolite Mediates Colonization Resistance to Salmonella Infection. Cell Host Microbe 24, 296–307.e7 (2018). [PubMed: 30057174]

50. Sorbara MTet al. Inhibiting antibiotic-resistant Enterobacteriaceae by microbiota-mediated intracellular acidification. J. Exp. Med 216, 84–98 (2019). [PubMed: 30563917]

References

51. Rangan KJ, Yang Y-Y , Charron G & Hang HC Rapid visualization and large-scale profiling of bacterial lipoproteins with chemical reporters. J. Am. Chem. Soc 132, 10628–10629 (2010). [PubMed: 20230003]

52. Peng T & Hang HC Bifunctional fatty acid chemical reporter for analyzing S-palmitoylated membrane protein-protein interactions in mammalian cells. J. Am. Chem. Soc 137, 556–9 (2015). [PubMed: 25575299]

53. Cox Jet al. Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. Mol. Cell. Proteomics 13, 2513–26 (2014). [PubMed: 24942700]

54. Court DL, Sawitzke J. a & Thomason LC Genetic engineering using homologous recombination. Annu. Rev. Genet 36, 361–88 (2002). [PubMed: 12429697]
Figure 1. Proteomic analysis of acylated proteins in S. Typhimurium.

(a) LFQ proteomic analysis of alk-3-labeled proteins in S. Typhimurium (n = 4). S. Typhimurium cell lysates were reacted with az-biotin for the enrichment of alk-3-labeled proteins with streptavidin beads and identification by LC-MS/MS. Proteins that were enriched by alk-3 (top right corner) were colored according to their annotated biological function. Blue, metabolic enzymes. Red, SPI-1 proteins. Black, other proteins. (b) Domain architecture (top) and predicted structure of HilA (bottom). Domain architecture was assigned by NCBI Conserved Domain Search. Structure prediction was performed with Robetta. DBD, DNA-binding domain, yellow. TPR, tetra-tricopeptide repeat, blue. Lysine residues detected to be acylated (K90, K231, K324, K456, K533) and two additional lysine residues that serve as controls (K57, K527) are highlighted in red. K324 is buried in the back (see Supplementary Fig. 9). (c) Alk-3 dose-dependent labeling on HilA. S. Typhimurium ΔhilA overexpressing HilA-HA-His were grown with different concentrations of alk-3 before cell lysates were reacted with az-Rho, followed by anti-HA immunoprecipitation, SDS-PAGE in-gel fluorescence scanning (top), and anti-HA immunoblotting (bottom). Also see Supplementary Fig. 14. (d) MS/MS spectrum of HilA K90 butyrylated peptide detected from HilA purified from S. Typhimurium grown with 10 mM butyrate.
Figure 2. Site-specific mutation of HilA affects *S. Typhimurium* virulence.

(a) Relative expression level of SPI-1 gene *sipA* in *S. Typhimurium* WT, HilA-HA strain, ΔhilA strain, HilAK90A-HA and HilAK90Q-HA mutants measured by qRT-PCR (*n* = 7–8). All mutants were compared to WT with one-way ANOVA and Dunnett post-test. Error bar, SD. **, *P*-value < 0.01; ***, *P*-value < 0.001. Comparisons with no asterisk had *P*-value > 0.05 and were not considered significant. (b and c) Gentamicin protection assay of *S. Typhimurium* WT, HilA-HA strain, ΔhilA strain, HilAK90A-HA, HilAK90Q-HA, and HilAK90bmK-HA mutants infecting HeLa cells (b) or RAW264.7 macrophages (c) at MOI = 10 (*n* = 3–6). All mutants were compared to HilA-HA with one-way ANOVA and Dunnett post-test. Centerline, average. **, *P*-value < 0.01. Comparisons with no asterisk had *P*-value > 0.05 and were not considered significant. (d) Endogenous expression of HilA-bmK-HA mutants in *S. Typhimurium*. *S. Typhimurium* HilA-HA strain (wt) and amber codon mutants (HilA-TAG-HA) with amber codon suppression system were grown with or without 100 μM bmK. Anti-HA immunoprecipitated samples were analyzed with SDS-PAGE in-gel fluorescence scanning (top) and anti-HA immunoblotting. Also see Supplementary Fig. 14. (e) Secreted proteins of *S. Typhimurium* endogenous HilA-bmK-HA mutants. *S. Typhimurium* HilA-HA strain (wt) and amber codon mutants (HilA-TAG-HA) with amber codon suppression system were grown with or without 100 μM bmK. Supernatants of the cultures were precipitated with 10% trichloroacetic acid (TCA) and analyzed with SDS-PAGE Coomassie blue staining.
Figure 3. Site-specific acylation of HilA affects S. Typhimurium transcriptional activity and invasion in vitro.

(a) Relative expression level of SPI-1 genes in several operons (prgH, invF, sipA, orgB, and spaO) in S. Typhimurium HilA-HA strains and HilA-bmK-HA mutants measured by qRT-PCR ($n = 6–8$). All mutants were compared to HilA-HA with one-way ANOVA and Dunnett post-test. Centerline, average. Error bar, SD. *, $P$-value < 0.05; **, $P$-value < 0.01; ***, $P$-value < 0.001. Comparisons with no asterisk had $P$-value > 0.05 and were not considered significant. (b and c) Gentamicin protection assay of S. Typhimurium endogenous HilA-bmK-HA mutants infecting HeLa cells (b) or RAW264.7 macrophages (c) at MOI = 10 ($n = 4–8$). All mutants were compared to HilA-HA with one-way ANOVA and Dunnett post-test. Centerline, average. *, $P$-value < 0.05; ***, $P$-value < 0.001. Comparisons with no asterisk had $P$-value > 0.05 and were not considered significant.
Figure 4. HilAK90 acylation affects its occupancy at target genes.
(a) Crosslink-IP-Seq result on S. Typhimurium HilA-HA and S. Typhimurium HilA-K90bmK-HA in SPI-1 region. Yellow shade, prgH promoter region (left) and invF promoter region (right). RPKM, Reads Per Kilobase Million. (b) Crosslink-IP-qPCR of prgH promoter region (left) or invF promoter region (right) of S. Typhimurium endogenous HilA-bmK-HA mutants (n = 3). All mutants were compared to HilA-HA with one-way ANOVA and Dunnett post-test. Error bar, SD. *, P-value < 0.05; **, P-value < 0.01. Comparisons with no asterisk had P-value > 0.05 and were not considered significant.
Figure 5. HilAK90 acylation affects *S*. Typhimurium infection and intestinal inflammation in *vivo*.

(a and b) C57BL/6 mice were orally infected with $10^6$ CFU *S*. Typhimurium HilA-HA strain, ΔhilA strain, HilAK90bmK-HA or HilAK231bmK-HA mutant ($n = 3$). (a) survival and (b) weight loss are shown. Weights of all mutants at day 4 were compared to HilA-HA with one-way ANOVA and Dunnett post-test. Error bar, SD. *, $P$-value $< 0.05$; **, $P$-value $< 0.01$. (c) *S*. Typhimurium bacterial CFU counted from mesenteric lymph nodes (mLN, left) or liver (right) of mice orally infected with *S*. Typhimurium HilA-HA, ΔhilA, HilA-K90bmK-HA or HilA-K231bmK-HA at 48h post-infection ($n = 12$). HilA-K90bmK-HA and HilA-K231bmK-HA mutants were compared to HilA-HA or ΔhilA with Kruskal-Wallis test and Dunns post-test. Centerline, median. *, $P$-value $< 0.05$; **, $P$-value $< 0.01$. ns, not significant. Dashed line, detection limit. (d) Representative images of cecum tissue stained with H&E, harvested 48h post-infection from mice infected with *S*. Typhimurium HilA-HA or HilA-K90bmK-HA. Scale bar, 100 μm.
Figure 6. HilA-K90,324,456R mutant resists butyrate inhibition in vitro and in vivo.

(a) Relative expression level of SPI-1 gene sipA in S. Typhimurium HilA-HA strains and HilA-K90,324,456R-HA mutant in the presence or absence of 10 mM butyrate measured by qRT-PCR (n = 6). Mutants were compared with one-way ANOVA and Bonferroni post-test. Centerline, average. Error bar, SD. ***, P-value < 0.001. ns, not significant. (b) Gentamicin protection assay of S. HilA-HA strains and HilA-K90,324,456R-HA mutant in the presence or absence of 10 mM butyrate infecting HeLa cells at MOI = 10 (n = 8). Mutants were compared with one-way ANOVA and Bonferroni post-test. Centerline, average. Error bar, SD. ***, P-value < 0.001. ns, not significant. (c) Survival curve of C57BL/6 mice orally infected with 10^6 CFU S. Typhimurium HilA-HA or K90,324,456R mutant (n = 13). Groups were compared with Gehan-Breslow-Wilcoxon test. *, P-value < 0.05. (d) S. Typhimurium fecal load of mice as in (c). Error bar, SD. Dashed line, detection limit.