Differential impact of lipopolysaccharide defects caused by loss of RfaH in *Yersinia pseudotuberculosis* and *Yersinia pestis*

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RfaH enhances transcription of a select group of operons controlling bacterial surface features such as lipopolysaccharide (LPS). Previous studies have suggested that *rfaH* may be required for *Yersinia pseudotuberculosis* resistance to antimonial chemokines and survival during mouse infections. In order to further investigate the role of RfaH in LPS synthesis, resistance to host defense peptides, and virulence of *Yersinia*, we constructed Δ*rfaH* mutants of *Y. pseudotuberculosis* IP32953 and *Y. pestis* KIM6+. Loss of *rfaH* affected LPS synthesis in both species, resulting in a shorter core oligosaccharide. Susceptibility to polymyxin and the antimicrobial chemokine CCL28 was increased by loss of *rfaH* in *Y. pseudotuberculosis* but not in *Y. pestis*. Transcription of genes in the *ddhD-wzz* O-antigen gene cluster, but not core oligosaccharide genes, was reduced in Δ*rfaH* mutants. In addition, mutants with disruptions in specific *ddhD-wzz* O-antigen cluster genes produced LPS that was indistinguishable from the Δ*rfaH* mutant. This suggests that both *Y. pseudotuberculosis* and *Y. pestis* produce an oligosaccharide core with a single O-antigen unit attached in an RfaH-dependent fashion. Despite enhanced sensitivity to host defense peptides, the *Y. pseudotuberculosis* Δ*rfaH* strain was not attenuated in mice, suggesting that *rfaH* is not required for acute infection.

*Yersinia pestis* is a recently emerged clone of *Y. pseudotuberculosis*, and these two species provide a fascinating model for investigating the evolution of bacterial pathogens. *Y. pestis* is transmitted via an infected flea bite and is the causative agent of plague1. *Y. pseudotuberculosis* (*Yptb*) is a zoonotic pathogen, typically acquired by ingestion of contaminated food or water, that causes self-limiting gastroenteritis in humans. Both species share a tropism for growth in lymph nodes2.

*Yersinia* survival and replication within the small intestine, Peyer’s patches, liver, and spleen is enhanced by the carriage of a 70 kb virulence plasmid called pYV (or pCD1 in *Y. pestis*)3. This plasmid encodes a type III secretion system that injects *Yersinia* outer proteins (Yops)4. Yops have a wide variety of functions including counteracting pro-inflammatory cytokine production and preventing phagocytosis5, 6. However, *Yptb* P+ mutants lacking the pYV plasmid grow equally well in the mesenteric lymph nodes following oral infection2. In an effort to understand how P- strains survive in the absence of a type III secretion system, Crimmins et al. conducted a genome-wide screen to identify putative chromosomal virulence factors that enable survival in lymphoid tissues. Several mutants that appeared to have colonization defects had insertions in genes involved in lipopolysaccharide (LPS) synthesis, including *rfaH*7.

RfaH was originally identified as a component in the synthesis of LPS of *Salmonella enterica* serovar *typhimurium*8. Since this initial discovery, RfaH has been implicated in a wide array of processes in gammaproteobacteria, including F-plasmid conjugation9, hemolysin toxin production10, and expression of type II K15 capsule11. RfaH functions in a similar fashion to the essential NusG protein. It allows the RNA polymerase to bypass intrinsic terminator sites or DNA binding proteins in order to completely transcribe long operons12–14. The specificity of
Effect of RfaH on lipopolysaccharide synthesis in Y. pseudotuberculosis and Y. pestis. To determine the role of the rfaH gene in Yptb and Y. pestis, the entire coding region was first deleted from Y. pestis using lambda-red recombination. After the mutation was made in Y. pestis, the modified allele (rfaH upstream and downstream regions flanking a kanamycin resistance gene) was transferred to Yptb by allelic exchange. After verifying the genotypes of the resulting mutants (Supplementary Fig. S1), we determined whether RfaH influences the synthesis of LPS in these species.

In Yptb and Y. pestis the composition of the sugars in LPS is known to change depending on growth temperature. We therefore extracted LPS from cultures grown at 21 °C and 37 °C from wild-type and mutant strains and analyzed these via polyacrylamide gel electrophoresis and staining of carbohydrates (Fig. 1). As expected, based on the ops element upstream of the O-antigen ddhD gene (Fig. 2), the Yptb ΔrfaH strain produced less high molecular weight O-antigen at 21 °C. Neither the wild-type or the Yptb ΔrfaH mutant strain produced...
Figure 2. Transcriptional changes in LPS genes caused by loss of rfaH. The ddhD-wzz O-antigen gene cluster (top) contains an ops regulatory sequence proximal to ddhD. The core oligosaccharide genes are found in 3 clusters as indicated (middle) and do not have ops sequences associated with them. The expression of individual LPS genes in the ΔrfaH mutant compared to the wild-type strains of Yptb (blue) or Y. pestis (green) were measured by qPCR. Data represent the mean fold changes in expression of specific genes, and statistically significant differences are indicated (*p < 0.05, **p < 0.01, ***p < 0.001). Similar results were obtained in two independent experiments, and data shown are from one representative experiment done in triplicate. The ddhD-wzz O-antigen genes are regulated by rfaH whereas the core oligosaccharide genes are not. The bottom image depicts the function of individual genes in producing Yptb/Y. pestis LPS.

O-antigen at 37 °C, which is consistent with known temperature-dependent O-antigen regulation in this strain. Restoration of O-antigen production in the Yptb ΔrfaH mutant was achieved by inserting the rfaH gene on plasmid pACYC184, which exists at about 15 copies per cell.

In contrast to the single O-antigen biosynthetic cluster, the core oligosaccharide genes exist in three separate clusters in Yptb and Y. pestis (Fig. 2). Cluster 1 contains the genes required for inner core synthesis including hldD, waaF and waaC. The outer core genes are contained in Clusters 2 (waaL and waaQ) and Cluster 3 (wabD and wabC). No ops-like sequences are apparent anywhere in Clusters 1–3, which suggests that RfaH likely does not play a role in transcriptional regulation of these operons. Nevertheless, we found that deleting rfaH altered the size of the Yptb oligosaccharide core at 21 and both species at 37 °C (Fig. 1). The rfaH+ plasmid successfully complemented O-antigen production in the Yptb ΔrfaH mutant, but did not affect size of the core oligosaccharide in the Y. pestis ΔrfaH mutant. As a size comparison, we included LPS from a Yptb hldD::Tn5 mutant strain, which fails to add L-α-heptose to the inner core. The ΔrfaH mutant core was significantly larger than that of hldD::Tn5 core, indicating that reduced transcription of the inner core was not likely responsible for the observed size change. We therefore considered that this size difference could reflect an alteration to the outer core or that conversely, Yptb (at 21 °C and 37 °C) and Y. pestis (at 37 °C) may produce a core with a small number of O-antigen oligosaccharide units (core-1OPS) in an RfaH-dependent process.

The LPS size changes we observed in the strains lacking rfaH (Fig. 1) suggested that expression of O-antigen and possibly outer core oligosaccharide biosynthesis genes may be regulated by RfaH. To test between these possibilities, RNA was isolated from wild-type and ΔrfaH mutant bacteria of both Yptb and Y. pestis. Transcription of several O-antigen and core oligosaccharide synthesis genes was measured using quantitative real-time PCR. As shown in Fig. 2, transcription of the ddhD gene cluster was significantly downregulated in the ΔrfaH mutant strains. Consistent with function of RfaH as an antiterminator, the downregulation of the more distal genes (wbyl and wbyk) was more pronounced than those closer to the promoter (ddhD and ddhA). Additionally, the effect of the ΔrfaH mutation was more pronounced in Yptb than in Y. pestis. Quantitative analysis of the transcription of core synthesis genes in clusters 1–3 showed that they were not significantly different in the ΔrfaH mutants, which is consistent with the absence of an ops sequence in these regions. These gene expression results suggested that the truncation we observed in the LPS of the ΔrfaH mutants was due to downregulation of the ddhD-wzz O-antigen cluster and not core oligosaccharide gene transcription.

We next hypothesized that if the altered LPS size in the ΔrfaH mutants were caused solely by reduced transcription of the ddhD-wzz cluster, then mutations in this cluster would also produce alterations in the LPS migration pattern resembling the ΔrfaH mutant strain. LPS from a series of Yptb Tn5 transposon mutants mapped to individual genes in this cluster supported this interpretation (Fig. 3A). The sizes of the LPS fractions for the mutants with insertions in ddhDABC, wzz, and wbyl genes were indistinguishable from the ΔrfaH mutant. The wbyH and wbyl (putative glycosyltransferase) Tn5 mutants produced LPS similar in size to the wild type strain, suggesting that these two genes are not essential for O-antigen synthesis. We also analyzed LPS from a Y. pestis ddhD::Tn5 mutant (Fig. 3B). Similar to the mutation in Yptb, disruption of the ddhD gene in Y. pestis
strongly reduced production of the larger putative core+1OPS oligosaccharide in comparison to the wild type strain (Fig. 3B).

RfaH is necessary for protection against CCL28 and polymyxin in Yptb but not Y. pestis. As LPS plays an important role in the defense against host antimicrobial peptides, Yptb and Y. pestis strains were examined to determine whether the loss of rfaH decreased resistance of the bacteria to the antimicrobial chemokine CCL28 and to polymyxin. We first measured the impact of rfaH deletion on binding to CCL28 using flow cytometry. As shown in Fig. 4A, deletion of rfaH from Yptb increased the proportion of cells that bind CCL28 from near zero in the wild type to approximately seventy percent in the mutant strain. Complementation of the mutant with the rfaH plasmid significantly reduced binding to CCL28 to near wild type levels. Interestingly, the level of CCL28 binding observed for the rfaH mutant was comparable to the Yptb hldD::Tn5 strain lacking 1-0 Heptose inner core residues, suggesting that there is a threshold at which increased truncation of LPS does not significantly change access of the antimicrobial peptide to the bacterial surface.

We also measured Yptb survival in the presence of CCL28 and polymyxin. Consistent with the binding results, the Yptb wild-type strain was largely unaffected by CCL28 whereas the rfaH mutant strain exhibited enhanced sensitivity (Fig. 4B). Complementation with the rfaH+ plasmid restored the survival rates to wild-type levels. Susceptibility to polymyxin was also affected by rfaH mutation. Yptb is relatively resistant to polymyxin, but as shown in Fig. 4C, the rfaH mutant strain shows a dramatic decrease in survival when exposed to polymyxin as compared to the wild-type. Consistent with the CCL28 survival results, the complemented strain and the wild type strain showed similar resistance to polymyxin.

In contrast to Yptb, CCL28 binding did not detectably change as a result of rfaH mutation in Y. pestis (Fig. 4A). We also found that rfaH mutation did not affect bacterial survival in the presence of CCL28, with the wild type and mutant strains exhibiting an 80% percent survival rate similar to the Yptb wild type strain (Fig. 4B). Interestingly, although Y. pestis appears similarly susceptible to the chemokine CCL28 as Yptb (independently of rfaH), Y. pestis is far more sensitive to polymyxin than Yptb at 37 °C (Fig. 4C). This sensitivity was slightly enhanced by loss of rfaH in Y. pestis, but the difference was not statistically significant under these conditions. These results suggest that addition of unpolimerized O-antigen to the Y. pestis outer core does not significantly affect susceptibility to these antimicrobial peptides. Conversely, the putative core+1OPS contributes to the polymyxin and CCL28 resistance of Yptb.

RfaH does not affect Yptb acute virulence following oral and intravenous mouse infections. The truncated LPS and increased susceptibility to antimicrobial peptides in the Yptb ΔrfaH strain suggested that this strain would exhibit a survival defect during in vivo mouse infections. To test this hypothesis, we first compared the ability of Yptb wild-type and ΔrfaH strains that carry the pYV virulence plasmid (P+) to colonize after oral infection. Three days after infection, the mesenteric lymph nodes, Peyer’s patches, spleen, and liver were collected and the bacterial loads determined. As shown in Fig. 5 there was no significant difference in survival of wild-type P+ and ΔrfaH P+ bacteria at this time point. Mice infected with the wild type strain or the ΔrfaH mutant appeared equally sick, with ruffled fur and lethargy prior to being euthanized. Given this unexpected result, we next determined whether rfaH would affect in vivo Yptb survival in strains lacking the pYV virulence plasmid (P-). As observed with the P+ infections, there were no significant differences in bacterial numbers in any organ between the P- ΔrfaH and wild-type strains (Fig. 5B). These results clearly indicate that rfaH is not required for survival and dissemination of Yptb strain IP32953 after oral infection in mice three days post infection.

Previous studies have also suggested that transposon insertion mutations in rfaH may result in reduced ability to colonize via intravenous infection. To investigate this possibility, we infected groups of mice via intravenous retro-orbital infections with P- Yptb wild-type ΔrfaH mutant bacteria. The bacterial loads in the liver and spleens of these mice were measured three days following infection (Fig. 5C). Similar to the oral infection results, survival of the ΔrfaH mutant was not decreased in comparison to wild-type bacteria in any of the organs. These data
demonstrate that although rfaH deletion alters LPS structure and increases susceptibility to antimicrobial peptides in vitro, these changes do not significantly affect bacterial survival during mouse infection.

**Discussion**

As has been seen in other bacterial species, we found that RfaH regulates the synthesis of the LPS in both *Yptb* and *Y. pestis*. Loss of rfaH eliminated high molecular weight O-antigen production in *Yptb* and caused truncation of a portion of the LPS that, because of its size, we initially suspected was within the core oligosaccharide (Fig. 1). Gene expression analysis indicated that core oligosaccharide genes were not affected; however, several genes within the dhhD-wzz O-antigen gene cluster were downregulated in the ΔrfaH strains (Fig. 2). In agreement with these gene expression results, LPS from several mutants with disruptions in the dhhD-wzz O-antigen locus had the same electrophoretic mobility as LPS from the ΔrfaH mutant (Fig. 3). This demonstrates that the dhhD-wzz cluster is the relevant target of RfaH responsible for the LPS truncation. Semi-rough LPS consisting of lipid A plus core oligosaccharide with a single O-antigen unit has been observed in *Yptb* O3, O4, and O8 strains 23, 24. Other *Y. enterocolitica* O:3 and O:9 strains also have a gene cluster required for synthesis of a single O unit that is not polymerized but which is attached to the inner core of the LPS 25, 26. However, no reports of *Y. pestis* strains producing semi-rough LPS have been published previously. Our results suggest that both *Yptb* IP32953 (O:1b) and *Y. pestis* KIM6+ can produce this form at 37°C and *Yptb* at both 21 and 37°C.

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**Figure 4.** *Yptb* rfaH affects antimicrobial peptide susceptibility but *Y. pestis* rfaH does not. (A) Binding of CCL28 to *Yptb* but not *Y. pestis* is significantly enhanced by loss of rfaH, expressed as a percentage of cells that stain positive by flow cytometry. (B and C) Relative survival of bacteria (expressed as a percentage of the number of live cells counted in the unexposed control of the same strain) in the presence CCL28 (B) or polymyxin (C). Asterisks denote that the result obtained was significantly different from the wild type strain at the given concentration by Two-way ANOVA (**p < 0.0001, *p < 0.05**). Similar results were obtained in three independent experiments, and data shown are from one representative experiment done in triplicate.

**Figure 5.** Loss of rfaH in *Yptb* does not affect bacterial replication and dissemination. Mice were infected with 10⁷ CFU of IP32953 wild type or ΔrfaH mutant containing the pYV virulence plasmid (P⁺) or 10⁶ CFU of the strains without the plasmid (P⁻). (A) Scatterplot of bacterial burden in each organ following oral infection with P⁺ strain. (B) Scatterplot of bacterial burden following oral infection with P⁻ strain. (C) Scatterplot of bacterial burden in spleen and liver following intravenous infection with P⁻ strain. Each dot represents 1 mouse, with the mean indicated. Data represent 3 independent experiments (n = 1–4 per experiment). No significant differences were detected when bacterial burdens for the wild type or ΔrfaH mutants in any of the organs were compared (Mann-Whitney test).
It is interesting that although Y. pestis strains are believed to be rough, all of them retain the ddhD-wzz gene cluster. Early sequencing of Y. pestis strains CO92 and EV76 indicated that several O-antigen genes (eg. ddhB, wbyl, gnda, and fch) were likely inactivated early in the emergence of Y. pestis from its ancestral strain. However, examination of more recently added genomes from diverse strains predicts that several carry functional versions of at least some of these genes. It has also been suggested that some of the mutations may be phase-variable since they occur in repetitive sequences prone to mismatch repair. We have shown here that a transposon disruption within ddhD changes the LPS electrophoretic mobility in Y. pestis KIM6+ (Fig. 3B), showing that this locus does have a function in Y. pestis. This ddhD::Tn5 mutant was identified based on altered colony phenotype on Congo-red agar plates and it exhibits increased clumping in liquid media, phenotypes that are consistent with altered cell envelope properties. It is unlikely that any Y. pestis strains produce high molecular weight polymerized O-antigen as it is known to interfere with the function of the plasminogen activator protease, which is essential for plague pathogenesis. Our studies are the first to our knowledge to suggest the production of oligosaccharide with a single O-unit in Y. pestis. It is also interesting that this form only appeared during growth at 37°C. The structure of the core oligosaccharides of some Y. pestis strains has been determined via high-resolution analyses including NMR and electrospray ionization mass spectrometry (ESI MS). These investigations did not detect the O-antigen sugars parasite, fucose, or mannose. However, they did find temperature-dependent differences in the oligosaccharide composition. Differences in serologic specificities of antibodies to LPS from Y. pestis have been suggested, thought to be primarily due to temperature-dependent variations in the structural properties of lipooligosaccharides. Similar detailed structural analysis of LPS from additional Y. pestis strains may be warranted to verify the effect of RfaH that we propose in these studies.

Changes in LPS can significantly affect resistance of Gram-negative bacteria to complement and antimicrobial peptides. Loss of rfaH altered LPS structure in Yptb, dramatically increasing susceptibility to polymyxin B (Fig. 4). We observed similar trends with binding and killing by the antimicrobial chemokine CCL28 in the ΔrfaH mutant. However, in Y. pestis we saw no significant difference in polymyxin or CCL28 susceptibility between the wild type and the ΔrfaH strains. Additionally, even though Y. pestis is much more sensitive than Yptb to polymyxin, it is equally susceptible to CCL28. This may indicate polymyxin and CCL28 have different targets, or that some features of Y. pestis not found in Yptb, such as capsule or the Pla protease (carried on plasmids pPCP1 and pMT1), could limit the access of CCL28 (but not polymyxin) to the bacterial surface.

Plasmid complementation with the rfaH gene mostly restored the wild-type phenotypes in the Yptb ΔrfaH strain in the LPS analyses and antimicrobial assays. We attempted to complement the Y. pestis ΔrfaH mutant using the same plasmid with the rfaH gene from Yptb, but surprisingly this plasmid failed to restore the wild-type phenotypes. Since the Y. pestis rfaH sequence differs from the Yptb sequence by one nucleotide (causing a single glycine-valine difference at position 75, see Supplementary Fig. 1), we also created a plasmid containing the Y. pestis version of this gene. This plasmid also failed to restore the wild-type phenotypes (data not shown).

Cases where phenotypes are unable to be complemented in mutant strains can be due to additional compensatory mutations in non-target genes or disruptions to flanking genes during mutagenesis. PCR reactions with primers within genes flanking rfaH (hemB and udiD) gave the expected size products (Supplementary Fig. 1), suggesting that the recombination had not disrupted nearby genes. The Y. pestis ΔrfaH mutant was remade using the same allelic exchange plasmid which was used to generate the Yptb mutant strain. The same mutant phenotypes were observed in this new strain, but again the mutation was not able to be complemented via transformation with either the rfaHP or rfaHcas plasmids. This result suggests that secondary, non-target mutations may arise extremely quickly in the Y. pestis ΔrfaH strains that prevent restoration of RfaH function. Alternatively, we considered the possibility that in Y. pestis multiple copies of the rfaH gene carried on plasmids could result in incorrect expression levels or other effects that prevent proper function. Therefore, a separate complementation strategy was attempted using a Tn7 transposon to insert a single copy of rfaH into the chromosome in the ΔrfaH strains. However, despite successful insertion of rfaH at the Tn7 site, the LPS remained truncated in this strain (data not shown). After multiple attempts via different methods it remains unclear why complementation of the rfaH gene in Y. pestis has not been successful. Because of the absence of complementation, at this time we cannot rule out the possibility that expression changes observed in the Y. pestis ΔrfaH mutant are influenced by other mutations.

The importance of rfaH to virulence of E. coli and Salmonella is well established, and rfaH mutants are sufficiently attenuated to make them potential live vaccine candidates. Given its potential role in host immune evasion, RfaH could be an attractive target for the development of new anti-virulence treatments against these species. Recent studies have also suggested a possible role for rfaH in Yersinia pathogenesis. For instance, a Y. enterocolitica ΔrfaH mutant was shown to have greater sensitivity to polymyxin, but more resistance to serum complement. We also previously identified rfaH in a screen for Yptb IP32953 mutants with increased antimicrobial chemokine binding suggesting a role for RfaH in bacterial colonization. Other groups have also found that mutants with transposon insertions in rfaH in a Yptb YPII P- strain background were less competitive for growth in liver and spleen following intravenous infection in BALB/c mice. Mutation of O-antigen genes, which we show here are regulated by RfaH (Fig. 2), reduced survival in competitive Yptb genome-wide transposon mutagenesis studies following orogastric, intraperitoneal, or intravenous infection of mice. These high-throughput screens involving competition between thousands of mutants strongly implicate RfaH and genes regulated by RfaH in virulence. In previous studies, fitness defects observed in Yptb rfaH mutants were calculated to be up to 100,000-fold. In addition, during the course of our studies Green et al. demonstrated that in a 1:1 competition with the wild-type strain, Yptb strain IP26666 rfaH mutants have an approximately 10-fold growth defect in mouse livers and spleens after intravenous injection. They also found that the fitness defect of this mutant was even further enhanced when mice were first depleted of neutrophils.

In this study, single strain infections were performed comparing the virulence of the wild type and an ΔrfaH mutant IP32953 serotype O:1b strain. Unexpectedly, we found that the ΔrfaH mutant did not appear to be...
attenuated, regardless of whether the plasmid encoding the type III secretion system was present, or whether the mice were infected orally or intravenously (Fig. 5). In addition to the strain and serotype differences between the strains used in our study and those published previously, it is likely that competition assays would give a more sensitive measure of any defects caused by rfaH mutation. It is also possible that measuring bacterial colonization at earlier or later time points could reveal subtle differences between the wild type and mutant strains used here. However, our results suggest that rfaH mutation by itself may not be universally sufficient for Yptb attenuation, and may lessen the attractiveness of RfaH as an antibacterial target for Yersinia.

Materials and Methods

Bacterial strains and growth conditions. Y. pestis KIM6+ and Yptb serotype O:1b IP32953 were routinely grown in Terrific Broth (TB) at either 21 °C or 37 °C. Kanamycin (30 µg/mL) and chloramphenicol (10 µg/mL) were included when necessary. Escherichia coli strain MFDpir was grown in Luria Broth (LB) at 37 °C. Yptb mutants with Tn5 transposon insertion in hldD and in genes within the dhdD-wzz locus were previously described18. A Y. pestis mutant with an insertion in dhdD was obtained using the same transposon delivery method and selection strategy.

Gene Deletions and Complementation. The rfaH gene was deleted from Y. pestis via lambda-red recombination40, 41. Primers (Supplementary Table S1) were designed to amplify three individual segments with complementary overhangs, representing 500 bp upstream and downstream segments flanking the rfaH gene, and the kanamycin resistance gene from plasmid pKD13. These three PCR products were combined using overlap-extension PCR. This DNA was then electroporated into Y. pestis KIM6+ expressing recombinase via plasmid pKOBEG-sacB. After growth on kanamycin plates, several colonies were tested for the correct ΔrfaH mutation by PCR (Supplementary Fig. S1). An allelic exchange plasmid pREI1226 was used to create the mutation in Yptb. The rfaH upstream and downstream region from the Y. pestis ΔrfaH mutant was amplified by PCR and ligated into pREI122 using the Sacl and Kpnl restriction sites, and transformed into chemically competent E. coli MFDpir43. The resulting suicide plasmid was transferred in bi-parental matings with Yptb, and transconjugants were selected by plating on media containing kanamycin and 10% sucrose. The desired mutation was verified by PCR (Supplementary Fig. S1).

To complement the mutant strains, the rfaH gene and its native promoter from either Yptb or Y. pestis were inserted into the SalI and XbaI sites of plasmid pACYC18444. The resulting plasmids (rfaH+) were then electroporated into the ΔrfaH mutant strains. A complemented strain was also constructed using transposon Tn7. The rfaH gene was inserted into the pGRG36 plasmid45, and electroporated into the Yptb ΔrfaH and Ypestis ΔrfaH strains. The Tn7 transposon inserts transgenes into a defined neutral site in the chromosome (attTn7). The insertions were verified using attTn7 site primers.

Lipopolysaccharide isolation and analysis. Bacteria were grown overnight at 21 °C or 37 °C in TB and adjusted to an A600nm of 1.0. LPS was then extracted as described previously18, 46. Briefly, 1.5 mL cultures were pelleted and suspended in 200 µL of SDS sample buffer. The lysed cells were boiled for 15 min, cooled, and treated with proteinase K at 59 °C overnight. The samples were then extracted with Tris-saturated phenol at 65 °C for 15 minutes, and then with diethyl ether at room temperature. Following centrifugation, the bottom blue layer was collected which contained the isolated LPS. The extracted LPS samples were separated on 4–20% polyacrylamide gradient gels. The gels were stained using the Pro-Q Emerald 300 Staining kit (Invitrogen) following the manufacturer’s protocol.

RNA Isolation and Gene Expression Analysis. RNA was isolated from cultures (n = 3 for wild type or ΔrfaH mutant strains) grown in TB at 37 °C for 6 hours using the rBAC RNA Isolation Kit (IBI Scientific) according to the manufacturer’s instructions. Residual DNA contamination was removed using the Ambion Turbo DNAse Free Kit (ThermoFisher Scientific) and the integrity of the isolated RNA was checked by agarose gel electrophoresis. The concentration of RNA was measured using a Nanodrop spectrophotometer, and cDNA was made from the RNA using a ProtoScript II First Strand Synthesis Kit (New England Biolabs). The cDNA samples were diluted to 70 ng/µL and used as template in quantitative PCR reactions (qPCR).

Primers specific for each gene were designed to give 100–150 bp products (Supplementary Table S1). Reactions consisted of qPCR 2 × SybrGreen Master Mix, High ROX (Genesee Scientific) with 3 µM each of forward and reverse primers and were run on a StepOne Real-Time PCR System. The cycling conditions were the following: 95 °C for 15 min followed by 40 cycles of 95 °C for 15 seconds then 60 °C for 1 min. A melt curve analysis was then performed to confirm the specificity of the PCR amplification. The resulting Ct values were normalized to the stably-expressed gene dnaE47, 48. Comparative ΔΔCt values were used to calculate the fold changes49. A one-sample T-test using GraphPad Prism software was performed to determine if the mean fold change for each gene was significantly different from a hypothetical value of 1.0 (no change).

Antimicrobial chemokine binding assay. Bacterial binding to the antimicrobial protein CCL28 was measured as previously described18. Briefly, bacteria grown to mid-logarithmic phase at 37 °C were diluted in filtered PBS supplemented with bovine serum albumin (BSA). The bacteria were incubated with 250 nM Human CCL28, washed in PBS, and then incubated with biotin- conjugated anti-chemokine antibody. The percentage of bacteria with detectable CCL28 bound to the surface was then measured with fluorescent streptavidin conjugates using a BD Accuri C6 Flow Cytometer and analysed using FACSDiva software (BD Biosciences). The statistical significance of specific comparisons was assessed via two-way ANOVA with Dunnett’s correction using GraphPad Prism software.
Antimicrobial peptide susceptibility assays. The ability of CCL28 or polymyxin to kill bacteria was tested as previously described\(^5\). Bacteria were diluted into 0.1\(\mu\)M potassium phosphate buffer (PPB) and incubated with 250 nM CCL28 or 10\(\mu\)g/\(\mu\)l polymyxin B diluted in 0.01 mg/ml BSA, or in BSA alone for 2 hours. Samples of the bacteria were removed and mixed with freshly prepared Polystyrene 15 \(\mu\)m Microsphere counting beads and Propidium Iodide (PI). The numbers of bacteria per 30,000 beads were then determined by flow cytometry as described above for the binding assay, and percent survival was calculated by dividing bacteria in the treated sample by the bacteria in the BSA control sample. The statistical significance of specific comparisons was assessed via two-way ANOVA with Dunnett’s correction using GraphPad Prism software.

Mouse Infections and Ethics Statement. Mouse infections were carried out in a biosafety level 2 laboratory in accordance with standard operating procedures approved by the Brigham Young University Institutional Animal Care and Use Committee. Carriage of the pYY virulence plasmid in Yptb strain IP32953 colonies was assessed by growth on media containing Congo-red\(^20\) and verified by PCR targeting the yopM and lcrV genes. Wild-type and \Delta yopHYptb bacteria were grown overnight at 28 °C in TB and then subcultured until they reached an A\(_{600nm}\) of 1.0. After centrifugation and resuspension in PBS, 100 \(\mu\)l of bacterial suspension was inoculated orally using a tube and ball syringe to BALB/c mice approximately 3 months of age. The numbers of bacteria in the inoculum was measured by serial dilutions and plating onto Yersinia Selective Agar (YSA) plates (which contain bile salts, crystal violet, and irgasan). For the P\(^+\) strains, mice were infected with 2 \(\times\) 10\(^5\) CFU, and 2 \(\times\) 10\(^8\) CFU were used for the P\(^-\) strains. The mice were fasted for 16 hours before infection and were then given food 2 hours following infection. After 3 days, the mice were sacrificed and the mesenteric lymph nodes, Peyer’s patches, spleens, and livers were collected. These organs were weighed, homogenized in PBS, and serial dilutions were plated onto YSA. Colonies were counted after 24 h of growth and the CFU/g of each organ was calculated. For intravenous infections, mice were infected retroorally under anesthesia with 100 \(\mu\)l of bacterial suspension containing 1 \(\times\) 10\(^8\) CFU of P\(^+\) Yptb. After 3 days, the mice were sacrificed and the liver and spleen were harvested, homogenized, and then plated on YSA.

Data Availability. Most of the data generated or analyzed during this study are included in this published article (and its Supplementary Information file). Other data are available from the corresponding author on reasonable request.

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Author Contributions

J.H., S.S. and E.Wu., conducted experiments, interpreted the data, and helped prepare figures; E. Wilson and D.E. designed and performed experiments, interpreted the data, and prepared and edited the manuscript.

Additional Information

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