



---

All Theses and Dissertations

---

2016-06-01

# The Role of the Transcriptional Antiterminator RfaH in Lipopolysaccharide Synthesis, Resistance to Antimicrobial Peptides, and Virulence of *Yersinia pseudotuberculosis* and *Yersinia pestis*

Jared Michael Hoffman  
*Brigham Young University*

Follow this and additional works at: <https://scholarsarchive.byu.edu/etd>

 Part of the [Microbiology Commons](#)

---

## BYU ScholarsArchive Citation

Hoffman, Jared Michael, "The Role of the Transcriptional Antiterminator RfaH in Lipopolysaccharide Synthesis, Resistance to Antimicrobial Peptides, and Virulence of *Yersinia pseudotuberculosis* and *Yersinia pestis*" (2016). *All Theses and Dissertations*. 6401. <https://scholarsarchive.byu.edu/etd/6401>

This Thesis is brought to you for free and open access by BYU ScholarsArchive. It has been accepted for inclusion in All Theses and Dissertations by an authorized administrator of BYU ScholarsArchive. For more information, please contact [scholarsarchive@byu.edu](mailto:scholarsarchive@byu.edu), [ellen\\_amatangelo@byu.edu](mailto:ellen_amatangelo@byu.edu).

The Role of the Transcriptional Antiterminator RfaH in Lipopolysaccharide  
Synthesis, Resistance to Antimicrobial Peptides, and Virulence of  
*Yersinia pseudotuberculosis* and *Yersinia pestis*

Jared Michael Hoffman

A thesis submitted to the faculty of  
Brigham Young University  
in partial fulfillment of the requirements for the degree of  
Master of Science

David Erickson, Chair  
Eric Wilson  
William McCleary

Department of Microbiology and Molecular Biology  
Brigham Young University  
June 2016

Copyright © 2016 Jared Michael Hoffman

All Rights Reserved

## ABSTRACT

### The Role of the Transcriptional Antiterminator RfaH in Lipopolysaccharide Synthesis, Antimicrobial Peptide Resistance, and Virulence of *Yersinia pseudotuberculosis* and *Yersinia pestis*

Jared Michael Hoffman  
Department of Microbiology and Molecular Biology, BYU  
Master of Science

RfaH is a unique bacterial protein that enhances transcription of a select group of long operons in many Gram-negative bacteria. Operons regulated by RfaH possess an upstream operon polarity suppressor sequence, which recruits the RfaH protein to the RNA polymerase during transcription of genes, most of which are involved in the synthesis of cell-surface features. These include synthesis of the lipopolysaccharide (LPS) core and O-antigen in *Salmonella* and *Escherichia coli*, as well as F-plasmid conjugation pilus and capsule in *E. coli*. LPS is an important virulence factor in many Gram-negative bacteria, and protects *Y. pseudotuberculosis* against host antimicrobial chemokines. Recently published high-throughput transposon mutant screens have also suggested a role for RfaH in the ability of *Y. pseudotuberculosis* to colonize mice. However, the role of RfaH in *Y. pseudotuberculosis* and its descendent *Yersinia pestis* has not been carefully examined. In these studies we investigated the effect RfaH has on the structure of the LPS in both species at different temperatures. We also identified LPS-synthesis related genes that are regulated by RfaH. We determined the effect of RfaH on bacterial resistance to host defense peptides, and the ability of *Y. pseudotuberculosis* to colonize mice. We found that the loss of the *rfaH* gene had different effects in *Y. pseudotuberculosis* and *Y. pestis*. Loss of *rfaH* caused a truncation in the core region in *Y. pseudotuberculosis* strain IP32953 at both 21°C and 37°C, but only at 37°C in *Y. pestis* strain KIM6+. Similarly, we found that transcription of individual genes that are predicted to function in core or O-antigen synthesis were downregulated in the *rfaH* mutant strains in both species, but the impact of *rfaH* deletion was greater in *Y. pseudotuberculosis*. When tested for their ability to survive in the presence of antimicrobial peptides, the *Y. pseudotuberculosis rfaH* deficient bacteria were much more susceptible than wild-type to killing by polymyxin and by the antimicrobial chemokine CCL28. However, the *Y. pestis rfaH* mutant strain was equally susceptible to CCL28 as the wild-type strain. Infection of mice with *Y. pseudotuberculosis* show that *rfaH* deficient bacteria were able to survive as effectively as the wild-type following oral or intravenous inoculation, with or without the pYV virulence plasmid. Overall, our results show that while RfaH controls LPS gene expression in both *Y. pseudotuberculosis* and *Y. pestis*, its impact is much greater in *Y. pseudotuberculosis*. Furthermore, although loss of *rfaH* greatly reduces the ability of *Y. pseudotuberculosis* to resist antimicrobial peptides, it is not required for virulence in this species.

Keywords: *rfaH*, lipopolysaccharide, CCL28, *Yersinia pseudotuberculosis*, *Yersinia pestis*

## ACKNOWLEDGMENTS

I have many people to thank who helped to contribute to this thesis. First, my committee members: Dr. Erickson, who continually provided guidance and support. Dr. Wilson was instrumental in helping with the immunology and mouse portions of this thesis. Dr. McCleary is extremely knowledgeable in all things bacteria.

I would like to thank the other members in the Erickson lab who helped to complete various experiments and gave ideas and support in the tough times: Shea Sullivan, Erin Wu, Lauren Johnson, Michael Olson, and Brett Schacterle.

I am grateful for my family who was with me every step of the way, and for BYU who gave me the opportunity to continue growing bacteria.

## TABLE OF CONTENTS

Title Page .....	i
Abstract .....	ii
Acknowledgments .....	iii
Table of Contents .....	iv
List of Tables .....	v
List of Figures .....	vi
Introduction.....	1
Materials and Methods.....	14
Results.....	20
Discussion.....	29
Tables and Figures .....	37
References.....	47

## LIST OF TABLES

Table 1. Gene deletion and complementation primers. ....	37
Table 2. qPCR primers.....	37
Table 3. Lipopolysaccharide synthesis genes .....	38
Table 4. qPCR gene fold expression changes. ....	38
Table 5. qPCR temperature related gene fold expression differences.....	39

## LIST OF FIGURES

Figure 1. <i>Yersinia</i> lipopolysacchride structure. ....	40
Figure 2. <i>ddhD</i> gene cluster. ....	40
Figure 3. <i>rfaH</i> gene deletion strategy. ....	41
Figure 4. Lipopolysaccharide gel analysis.....	42
Figure 5. CCL28 binding assays. ....	43
Figure 6. CCL28 killing assays.....	44
Figure 7. Polymyxin killing assays.....	45
Figure 8. <i>In vivo</i> mouse infections.....	46

## INTRODUCTION

### *Yersinia*

The genus *Yersinia* is a member of the *Enterobacteriaceae* family. There are eleven species, three of which are pathogenic to humans: *Yersinia pestis*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica* [1]. These studies focus on *Y. pestis* and *Y. pseudotuberculosis*, which are extremely closely related and are a fascinating model system for investigating the evolution of bacterial pathogens. *Y. pestis* is a Gram-negative, facultatively anaerobic, nonmotile coccobacillus. It is the causative agent of plague which has been responsible for three pandemics and the death of two hundred million in recorded history [1]. The strains of *Y. pestis* are subdivided into four groups based on their ability to ferment sugars and reduce nitrate: Antiqua, Medievalis, Pestoides, and Orientalis [2]. *Y. pestis* uses rodents as its reservoir and can be transmitted to humans through a flea bite, which can result in bubonic or septicemic plague [3]. *Y. pestis* can also be spread through inhalation and cause pneumonic plague [4].

*Y. pseudotuberculosis* is a food borne pathogen that causes self-limiting gastroenteritis [5]. *Yersinia pseudotuberculosis* was first discovered in 1883 from guinea pigs that had tuberculosis like lesions [6]. It has diverse animal reservoirs such as in brown hares and sheep located in many different countries. Transmission to humans is uncommon and can occur sporadically or in outbreaks of contaminated food [6, 7]. In Russia, *Y. pseudotuberculosis* has been linked to outbreaks which cause Far-East scarlet-like fever which is characterized by severe systemic inflammatory symptoms [8]. There are 21 different serotype variants of *Y. pseudotuberculosis* that are characterized by the structure of the lipopolysaccharide O-antigen [9].



As an enteric pathogen, *Yersinia pseudotuberculosis* enters the human body through contaminated food or water. Once it passes through the stomach, *Y. pseudotuberculosis* travels to the ileum of the small intestine and translocates through lymphoid associated tissues known as Peyer's patches [10]. The Peyer's patches are specialized intestinal areas that contain specialized M cells which bind and transport luminal antigens in order to commence an adaptive immune response [11]. Normally M cells phagocytose antigens and pass them to local antigen-presenting cells such as macrophages and dendritic cells. These cells present them to local lymphocytes or bring them to the mesenteric lymph nodes [5, 12]. *Y. pseudotuberculosis* uses a chromosomally-encoded protein called invasin to bind to  $\beta_1$ -integrins on mammalian cells to be taken up by the M cells [10]. Approximately 24 hours after an oral infection, *Y. pseudotuberculosis* can be found within the mesenteric lymph nodes and after 48 - 72 hours it appears in the spleen and liver [5]. Colonization of the local lymphatic tissue can lead to dissemination of *Y. pseudotuberculosis* into the blood stream via lymph drainage in the thoracic duct [5].

The three pathogenic strains of *Yersinia* in humans contain a 70 kb virulence plasmid called pYV (or pCD1 in *Y. pestis*) [13]. This virulence plasmid encodes a type III secretion system which *Yersinia* uses to inject *Yersinia* outer proteins (Yops) to survive and replicate within lymphoid tissue [14]. The effector Yops proteins have a wide variety of functions including counteracting proinflammatory cytokine production and preventing phagocytosis [15, 16]. A *Y. pseudotuberculosis* mutant strain lacking several of the Yops proteins was unable to colonize the gastrointestinal tract and Peyer's patches in an oral infection [17]. However, it was subsequently discovered that *Y. pseudotuberculosis* lacking the plasmid grow just as well in the mesenteric lymph nodes as those that had the plasmid following oral infection [18]. The pYV plasmid-negative ( $P^-$ ) *Y. pseudotuberculosis* reside extracellularly in B and T cells in the cortex

and paracortex of the mesenteric lymph nodes. These areas offer protection against phagocytic cells. The P<sup>-</sup> strains also caused a milder inflammatory response [19]. In order to find chromosomal virulence factors that allow the bacteria to survive in internal organs in the absence of the pYV virulence factors, a collection of transposon mutants of P<sup>-</sup> strains of *Y.*

*pseudotuberculosis* was passaged through mice. During oral infection, only a few bacteria among the thousands that enter the intestine are able to pass through the intestinal barrier to colonize deeper tissues. These few founding colonizers then go on to replicate to great numbers. In order to bypass the severe bottleneck presented by the intestinal mucosal tissue, the researchers infected mice intravenously with pools of mutants and then isolated surviving bacteria from the liver. This allowed them to find chromosomal virulence factors based on the inability to detect many P<sup>-</sup> transposon mutants in these organs. Many of the mutants that failed to survive in liver had insertions in genes involved in lipopolysaccharide synthesis, including the transcriptional antiterminator RfaH [18] Mutants with insertion in *rfaH* were estimated to have a 100,000- fold decrease in their ability to survive in the liver.

#### Evolutionary relationship between *Y. pestis* and *Y. pseudotuberculosis*

Comparative genomic analysis of genes in *Y. pestis* show that it is a clone that evolved from *Y. pseudotuberculosis* 1,500-20,000 years ago [20]. *Y. enterocolitica* represents a distinct evolutionary lineage [1]. Phylogenetic analysis using single nucleotide polymorphisms (SNPs) from 133 strains of *Y. pestis* has shown that the *Y. pseudotuberculosis* genomes share the same SNPs as *Y. pestis* and from this sequential mutations in the evolution of *Y. pestis* can be defined [21]. Recent studies sequenced hundreds of *Y. pestis* genomes and found that *Y. pestis* evolved from *Y. pseudotuberculosis* in China and spread to West Asia and Africa [22]. The extremely

close genetic relationship and established ancestry between these species of bacteria provide an excellent model for the study of pathogen evolution.

While the genomes of *Y. pestis* and *Y. pseudotuberculosis* are very similar, there are many changes that occurred in the evolution of *Y. pestis* that allowed it to take advantage of a completely different route of infection. *Y. pestis* has 32 chromosomal genes that are not present in *Y. pseudotuberculosis*, as well as two *Y. pestis* specific plasmids. One of the important genes found on the *Y. pestis* specific plasmids is *ymt*, which encodes the phospholipase D enzyme that allows *Y. pestis* to infect the midgut of fleas [23]. *Y. pestis* has 317 pseudogenes that have been inactivated by frameshift mutations or insertion sequences, but which are functional in *Y. pseudotuberculosis* [24]. One such gene that has been inactivated in *Y. pestis* is the *ureE* gene. This gene encodes urease activity which while useful to an enteric pathogen is orally toxic to fleas [25]. Five inactivated genes are located in the O-antigen gene cluster in *Y. pestis*. The inactivation of these genes prevent the synthesis of the O-antigen in *Y. pestis* which is necessary for the proper function of the Pla protease. The Pla protease is another plasmid-encoded protein necessary for activation of human plasminogen and is not found in *Y. pseudotuberculosis* [26]. *Y. pseudotuberculosis* has a fully functional Rcs regulatory system, which negatively regulates extracellular polysaccharide and biofilm formation, but this system has been inactivated in *Y. pestis* [27]. The loss of these genes was essential for enhanced biofilm formation and efficient transmission of *Y. pestis* from fleas to mammals. Thus, mutations in regulatory pathways affecting cell envelope structures including LPS and capsule polysaccharides has contributed to vastly different lifestyles and pathogenic potential among these two closely related species.

*Yersinia* lipopolysaccharide

*Y. pestis* and *Y. pseudotuberculosis* are Gram-negative bacteria and as such they possess lipopolysaccharide (LPS) structures in the outer leaflet of their outer membranes. LPS serves as a virulence factor as it provides resistance from antibiotics, complement, and other host defenses [28]. The LPS is composed of three major parts: the lipid A, core oligosaccharide, and O-antigen.

Lipid A, also known as endotoxin, causes an excessive release of pro-inflammatory cytokines by host macrophages [28]. Lipid A is absolutely required for growth of the bacteria [29]. It is composed of biphosphorylated glucosamine residues that have four or six acyl chains attached which allow it to embed in the outer leaflet of the outer membrane [30]. In *Yersinia*, the number of acyl chains changes depending on the growth temperature of the bacteria. At 21°C *Y. pestis* and *Y. pseudotuberculosis* produce hexaacylated lipid A and tetraacylated lipid A at 37°C [31]. Reducing the number of acyl chains at 37°C results in a less immunostimulatory LPS and likely helps *Y. pestis* to avoid activation of the immune system [31].

The core oligosaccharide is attached to the lipid A and consists of the inner and outer core. The inner core is made up of five conserved saccharide moieties: 2 3-Deoxy-D-manno-oct-2-ulosonic acid (Kdo) and 3 L-glycero-D-manno-Heptoses (L-D-Heptose) residues [28]. *Y. pestis* also has an additional glucose residue bound to one of the heptose residues (Figure 1). As with the lipid A portion, the inner core of *Y. pestis* changes in response to a decrease in temperature. At 21°C, one of the Kdo residues is replaced with a D-glycero-D-talo-oct-ulosonic acid (Ko) residue. *Y. pestis* also lacks the typical outer core found in other Gram-negative bacteria, which includes an additional four residues of glucose and galactose, and possess an additional D-D-Heptose residue or D-galactose residue (at 21°C) [28].

The O-antigen is a repeating polysaccharide chain that extends out from the core oligosaccharide [32]. *Y. pestis* does not produce O-antigen due to frameshift mutations in the *ddhB*, *wbyI*, *wzy*, *gmd*, and *fcl* genes which are located in the *ddhD-wzz* gene cluster [9]. *Y. pseudotuberculosis* produces diverse O-antigens, which forms the basis for serotyping of *Y. pseudotuberculosis* strains [33]. There are 21 known serotypes of *Y. pseudotuberculosis*. Based on phylogenetic analysis of the O-antigen gene cluster, *Y. pestis* is thought to have evolved from a serotype O:1b ancestor [9].

Each portion of the LPS plays a specific role in the defense of the bacteria against host defense peptides. It has been shown previously that mutations in core LPS genes which result in truncation of the LPS in *Burkholderia cenocepacia* decreased resistance to polymyxin B and melittin [34]. When genes related to the synthesis of the O-antigen were knocked out in *B. cenocepacia*, it did not increase susceptibility to polymyxin B [34]. Therefore, the core region conferred resistance to antimicrobial peptides.

#### Lipopolysaccharide synthesis

Synthesis of LPS proceeds in distinct stages and locations in order to deliver the final product in the outer membrane of the bacterial cell. The lipid A and Kdo regions are synthesized in the cytoplasmic side of the inner membrane [29]. The first step in this process is the attachment of fatty acids to oxygen and nitrogen residues within a UDP-GlcNAc (N-acetylglucosamine) molecule [35]. The enzyme WaaA then adds on the Kdo residues and following this additional fatty acid chains can be added on to the oxygens of the carboxylic acid groups [36].

The core region is also synthesized in the cytoplasm by a series of enzymes as shown in Figure 1. The first sugar L,D-Heptose I is added to the Kdo residue by the enzyme WaaC. [37]. This sugar is synthesized from sedoheptulose-7-P in a series of steps requiring the HldD enzyme (also known as GmhA) [38]. The genes *waaE*, *waaF*, *waaL*, *waaQ*, and *wabC/D* encode glycosyltransferases. These enzymes add glucose, L,D-Heptose II, GlcNAc, L,D-Heptose III, and D-D Heptose or galactose (WabC/WabD depending on the temperature) to assemble the core. Once the core is completed, The MsbA flippase protein transports the completed lipid A and core structure from the cytoplasmic to the periplasmic side of the inner membrane [39].

The O-antigen is synthesized by genes that are usually arranged in clusters or operons, and in *Yersinia* they are found between the *hemH* and *gsk* genes [40]. Glycosyltransferase enzymes encoded in this region synthesize heteropolymeric O-units in the cytoplasm, which are transported to the periplasm via a Wzy-dependent process [37]. The O-antigen in *Yersinia pseudotuberculosis* contains rare sugars synthesized from glucose-1-phosphate [40]. These are known as 3,6-dideoxyhexose sugars and their synthesis requires the *ddh* genes. DdhA and DdhB catalyze the reaction of glucose-1-phosphate to CDP-6-deoxy-D-xylo-4-hexulose. DdhC and DdhD then interact to perform C-3 deoxygenation of CDP-6-deoxy-D-xylo-4-hexulose into CDP-3,6-dideoxyhexoses [40]. The O-units are transported to the periplasmic space and are assembled by Wzy and Wzz. Wzy is the O-antigen polymerase and Wzz determines the O-antigen chain length [41]. Once completed, these are added to the lipid A + core on the periplasmic side of the inner membrane by the ligase protein WaaL [37]. The Lpt (LptA to LptG) proteins are responsible for transporting the completed LPS molecule from the inner membrane to the outer membrane [42]. The LptB<sub>2</sub>CFG protein complex extracts the LPS from the inner membrane. LptA, LptC, and LptD form a bridge to transport the LPS molecule from the inner membrane to

the outer membrane [43]. The LptDE outer membrane translocon has a barrel shape and inserts the LPS molecule in the outer leaflet of the outer membrane [44].

### Antimicrobial peptides

Antimicrobial peptides are an important part of the innate immune system. They are a first line of defense against bacteria as they provide broad-spectrum antimicrobial activity [45]. Examples of antimicrobial peptides include defensins, cathelicidins, and histatins. Their primary mechanism of action is to disrupt bacterial membranes [46]. Many antimicrobial peptides have a cationic charge which gives them an affinity to negatively charged bacterial membranes [45]. Some peptides interact with the acyl chains of the lipid membrane and oligomerize to form an aqueous pore. Some bacteria resist the activity of cationic antimicrobial peptides by modifying their lipopolysaccharide in order to change the ionic charge of the membrane [47].

### Antimicrobial chemokines

Chemokines are a class of small (8-12 kDa) chemotactic cytokines. The primary role of these chemokines is to recruit immune cells to sites of infection. Recently it was discovered that some of these chemokines may have antimicrobial effects upon bacteria, similar to the activity of antimicrobial peptides such as defensins [48]. For example, CCL28, also known as mucosae-associated epithelial chemokine, is an antimicrobial chemokine that binds to the chemokine receptors CCR10 and CCR3 [49]. CCL28 is expressed abundantly in the mucosal in the trachea, colon, salivary, and mammary glands. It is constitutively expressed in the epithelial cells of the salivary glands and found in high concentrations in the saliva and milk. It exhibits broad-spectrum antimicrobial activity against gram negative and gram positive bacteria [50]. CCL28 has been shown to have potent *in vitro* antimicrobial activity against organisms that cause

mastitis in dairy cattle, including *Staphylococcus aureus* and *E. coli* [51]. Although the exact mechanism of killing is unknown, it is believed to function like the  $\beta$ -defensin antimicrobial peptides by binding through electrostatic interactions and forming membrane pores. The antimicrobial activity of CCL28, like many antimicrobial peptides, requires low osmolarity solutions to exert its effects [52].

It has been shown that the LPS provides resistance to antimicrobial chemokines. Modifications to the lipid A moiety by adding 4-amino-4-deoxy-L-arabinose can help to mask the negative charge of the bacterial membrane and can provide resistance to CCL28 [53]. When the LPS was truncated in response to a transposon insertion in the *hldD* gene it increased susceptibility to CCL28 [54]. A previous study used transposon mutagenesis in *Y. pseudotuberculosis* to identify genes that function in providing resistance to antimicrobial chemokines CCL25 and CCL28 [55]. One of the genes that was identified in this screen which decreased the resistance of *Y. pseudotuberculosis* to CCL28 was *rfaH*. A separate study found that when the *rfaH* gene was deleted from *Y. enterocolitica*, it caused the bacteria became more susceptible to polymyxin and detergent due to changes in the LPS [56].

#### Transcriptional antiterminator, RfaH

In bacteria, groups of functionally related genes are often organized into operons that are regulated at the level of transcription [57]. Typically this involves repressors or activators that bind to promoters and controls access of RNA polymerase (RNAP). Another method of transcriptional regulation and modification of genes is through RNAP associated proteins that function in transcriptional elongation. During transcription of long operons, the RNAP might experience random or programmed pause events and without help, it may prematurely stop



transcription of long operons [58]. To counteract pausing, the essential NusG protein may bind to the RNAP after the  $\sigma$  factor disassociates. It functions to increase transcriptional processivity by suppressing RNAP pausing, ensuring that all genes in an operon are transcribed [59].

The RfaH protein also functions as a transcriptional antiterminator and shares significant structural similarity with NusG, but it acts as a different set of operons in the genome [57]. RfaH was originally discovered as a component in the synthesis of the lipopolysaccharide in *Salmonella enterica* serovar *typhimurium* [60]. Since this initial discovery, RfaH has been shown to function in a wide array of cellular functions including: F-plasmid conjugation pilus [61], haemolysis toxin [62], and the *E. coli* type II K15 capsule [63]. In *E. coli rfaH* mutants, it has been shown that there is a minor decrease in transcription of genes proximal to the promoter of RfaH-regulated operons, but the transcription of more distal genes was more severely reduced resulting in transcriptional polarity [64]. Similar to NusG, the RfaH protein allows the RNAP to bypass intrinsic terminator sites, DNA binding proteins, or DNA lesions and transcribe the entire operon completely [65, 66]. Functional complementation studies also indicate that despite disparate primary amino acid sequences, the function of RfaH is conserved throughout the gamma-proteobacteria [67]. In addition to its role in transcriptional regulation, RfaH also has a role in coupling transcription to translation, which is an important feature in bacteria. RfaH can undergo a conformational change from a closed state to an open state while bound to the RNAP. This open configuration facilitates translation by binding to the ribosome and is important for translation activation of the operons regulated by RfaH [68].

The specificity of RfaH to certain genes depends upon a regulatory site called the operon polarity suppressor (ops) [69]. The ops sequence is typically found within the 5'-proximal transcribed sequence of operons under control by RfaH and they display high sequence

conservation among bacterial species with homologous operons (Figure 2) [67]. The common 8-bp ops sequence motif is 5' –GGCGGTAG – 3' (reported as a longer 12-bp motif in some studies: 5'-RGGCGGTAGYnT-3') and when this sequence is deleted it results in reduced expression of genes close to the promoter while distal gene expression is almost abolished [62, 70]. During transcription, the ops sequence causes the RNAP to pause briefly. The exposed non-template strand with the ops sequence interacts with the RNAP structure and recruits the RfaH protein [71]. The RfaH protein then binds to  $\beta'$  subunit clamp in the RNAP, which is the same binding site as the  $\sigma$  initiation factor [72]. In *rfaH* mutant bacteria, the poor transcription of distal genes can be restored by purified RfaH protein. When the ops is present in *in vitro* experiments, the amount of RfaH required to restore the transcription of these genes is reduced [57]. The requirement of the ops sequence has been abolished *in vivo* if the RfaH protein is overexpressed [57].

#### Effects of RfaH in Gram-negative pathogens

RfaH has been found to function in the synthesis of many components of Gram-negative bacteria, some of which have direct consequences in infection. For instance, the *chuA* gene in uropathogenic *E. coli* strain 563 is regulated by RfaH. The ChuA protein is an outer membrane protein that functions in hemin utilization and is important for iron acquisition in this pathogen [73]. Without RfaH, mutants of this strain are less virulent in mice [74]. In a different strain of extraintestinal pathogenic *E. coli*, *rfaH* has been shown to be required for fitness in the intestinal niche. These researchers found that the fitness defect of *rfaH* mutants was comparable to deep-rough LPS mutants [75].

Similar attenuation was also observed in a *rfaH* mutant strain of pathogenic *Salmonella enterica* serovar Typhimurium strain SL1344 in orogastric and intraperitoneal mouse infections. One possible explanation for the defect is that the loss of *rfaH* increases susceptibility to antimicrobial peptides, which is a key defense against *Salmonella* [76]. As a consequence of the attenuation, the researchers also found that by infecting the mice first with the *rfaH* mutant they could immunize and protect the mice from a second infection with the wild-type pathogenic strain [77].

While the effect of RfaH on *in vivo* virulence in *Yersinia* has not been investigated, a recent study showed that the loss of RfaH in *Y. enterocolitica* O:3 reduces expression of O-antigen and outer core gene clusters and causes truncation of the LPS [56]. These changes cause the bacteria to become more susceptible to polymyxin and detergent, but also more resistant to serum complement attack. The researchers found *Y. enterocolitica* RfaH has greater specificity for binding because there were >10 candidate ops sequences adjacent to genes with other functions but their activity was not regulated by RfaH. In *Y. pseudotuberculosis* serotype O3 strain YPIII, transposon insertions in *rfaH* reduced the ability of the bacteria to compete with other mutants in the livers and spleens of mice following intravenous injection. The researchers speculated that loss of O-antigen production in the *rfaH* mutants could be the reason for their reduced fitness, since many predicted O-antigen mutants had the same phenotype. However, they did not test whether *rfaH* mutants were defective following oral infection, nor did they experimentally determine a mechanism for attenuation of *rfaH* transposon mutants. These previous studies suggest that further experiments investigating the effect of RfaH on virulence of *Yersinia* are warranted.

Experimental objectives

The accumulated evidence described above suggests that RfaH is likely required for production of O-antigen and possibly core oligosaccharide in *Y. pseudotuberculosis*. However, since *Y. pestis* fails to produce O-antigen, the role of RfaH in this species is difficult to predict. Further, any truncation of the LPS resulting from the loss of RfaH is likely increase susceptibility to antimicrobial peptides. Therefore, we hypothesized that a *Y. pseudotuberculosis rfaH* mutant will exhibit decreased virulence in an *in vivo* mouse infection. If so, then RfaH could be an attractive target for the development of antibacterial treatments, given its importance in a diverse group of pathogens.

The first objective in these studies was to identify the role that the *rfaH* plays in in the synthesis of the O-antigen and core oligosaccharide in *Y. pseudotuberculosis* serotype O:1b IP32953 and in *Y. pestis* KIM6+. We determined changes in the LPS structure in the wild-type and *rfaH* mutant strains using SDS-PAGE gel electrophoresis. We then sought to correlate the changes in LPS structure with transcriptional changes in LPS synthesis genes by quantitative RT-PCR in these strains. The second objective was to determine how resistance to host defense peptides is impacted by the loss of *rfaH*. We performed *in vitro* binding and killing assays with antimicrobial peptides and chemokines to quantify the differences between the wild-type and the *rfaH* gene mutants. The third objective was to identify any changes in virulence between the wild-type and *rfaH* mutant bacteria. We measured bacterial survival in mouse organs following oral or intravenous inoculation of wild type or *rfaH* mutant *Y. pseudotuberculosis* in both virulence plasmid P<sup>+</sup> and P<sup>-</sup> strains.

## MATERIALS AND METHODS

### Bacterial strains and growth conditions

The two wild-type bacterial strains used in this study were *Yersinia pestis* KIM6+ and *Yersinia pseudotuberculosis* serotype O:1b IP32953. Both virulent (P<sup>+</sup>) and non-virulent (P<sup>-</sup>) strains of *Yersinia pseudotuberculosis* were used which indicates the presence of the *Yersinia* virulence plasmid, pYV. Kanamycin (30 µg/mL) and chloramphenicol (10 µg/mL) were added as necessary when working with mutant strains. The strains were grown up in Terrific Broth (TB) at either 21°C or 37°C. *Escherichia coli* strains used were grown up in Luria Broth (LB) at 37°C.

### Gene deletions and complements

The *rfaH* gene was deleted from both *Y. pestis* and *Y. pseudotuberculosis* using a suicide plasmid, pRE112 (Figure 3) [78]. Primers were designed using Geneious (Biomatter Inc., Newark, New Jersey) to include 500 bp upstream and downstream homology of the *rfaH* gene in both *Y. pestis* and *Y. pseudotuberculosis*. The forward primer contained a SacI restriction site and the reverse primer contained a KpnI restriction site. The primers were ordered from Sigma-Aldrich (St. Louis, Missouri) and are shown in Table 1. A kanamycin resistance gene (Km<sup>R</sup>) was amplified using polymerase chain reaction (PCR) which included 500-bp upstream and downstream homology of the *rfaH* gene. The amplified product was purified using a DNA Clean and Concentrator Kit (Zymo Research, Irvine, California). The purified PCR product and the pRE112 plasmid were then cut using SacI and KpnI restriction enzymes (New England Biolabs, Ipswich, MA). They were ligated together using an Instant Sticky End ligase mix (New England Biolabs, Ipswich, MA), and transformed into a chemically competent *E. coli* MFDpir strain,

which are auxotrophic for diaminopimelic acid (DAP) [79]. The bacteria was plated on LB plates + DAP. These *E. coli* MFDpir were then mated with either *Y. pseudotuberculosis* or *Y. pestis*, and plated on LB + DAP. The mated cells were collected and plated on LB without DAP (to prevent growth of *E. coli* MFDpir) which had the antibiotic kanamycin (Km) added to select for successful transformations. Transformants were picked and then grown on LB + 10% sucrose plates were used to ensure double crossover events. Successful transformations were verified with PCR using the *rfaH* 500 F & R primers to show the change in product size which correlates with insertion of the larger Km<sup>R</sup> gene and the *rfaH* internal F& R primers (Table 1) to show that the *rfaH* gene is removed (Figure 3B). The newly created strains were called *Y. pseudotuberculosis*  $\Delta rfaH$  and *Y. pestis*  $\Delta rfaH$ .

The *rfaH* gene was reinserted via plasmid vector into  $\Delta rfaH$  mutant strains to create complemented strains. The *rfaH* gene and its native promoter from either *Y. pseudotuberculosis* or *Y. pestis* were amplified using *rfaH* forward and reverse primers which included 200 bp upstream and downstream of the *rfaH* gene. The primers designed using Geneious (Biomatter Inc., Newark, New Jersey) and ordered from Sigma-Aldrich (St. Louis, Missouri) and are shown in Table 1. The amplified *rfaH* gene was inserted using restriction enzymes Sall and XbaI (New England Biolabs, Ipswich, Maine) into a low copy plasmid, pACYC184 [80]. The plasmid was then electroporated into the  $\Delta rfaH$  bacteria. The electroporation involved performing a series of washes with cold water to make the *Y. pseudotuberculosis* or *Y. pestis* strains competent. The competent bacteria were mixed with 5  $\mu$ L of plasmid and were added to a 1mm cuvette (Genesee Scientific, San Diego, California) and placed in a MicroPulser Electroporation Apparatus (Bio-Rad, Hercules, California) and the Ec1 setting used to deliver the shock. Immediately after the shock, 500  $\mu$ L of Recovery Media (Lucigen, Middleton, Wisconsin) is added and the bacteria

were placed on the shaker for 1-2 hours. They are then plated on plates with antibiotics to select for positive electroporations. The new complement strains created were called *Y. pestis rfaH+* and *Y. pseudotuberculosis rfaH+*.

#### Lipopolysaccharide isolation and analysis

Bacteria were grown overnight (12-18 hours) at 21°C or 37°C in TB. The culture was then diluted to be OD 1.0 in 5 mLs. It was then pelleted and the pellet was resuspended in 1 x SDS-buffer (4%  $\beta$ -mercaptoethanol, 4% sodium dodecyl sulfate (SDS), in 0.1 Tris-HCL, pH 6.8 with bromophenol blue). The bacteria were boiled for 15 min and cooled at room temperature for 15 minutes. The samples were treated with proteinase K and incubated at 37°C overnight. Ice cold Tris saturated phenol is added and then vortexed. The samples were incubated at 65°C for 15 minutes and then diethyl ether is added. The samples are centrifuged and the bottom blue layer was collected which contained the isolated LPS.

The extracted LPS were run on 4-20% Tru-PAGE Precast Gels (Sigma-Aldrich, St. Louis, Missouri) at 95 V. The gel was stained using the Pro-Q Emerald 300 Staining kit following the manufacturer's protocol (Invitrogen, Carlsbad, California). The stained gel was visualized using a UV transilluminator.

#### RNA extraction and gene expression changes

The bacterial RNA was extracted using rBAC RNA Isolation Kit (IBI Scientific, Peosta, Iowa) following the manufacturer's instructions. The bacteria used were grown in TB at 21°C and 37°C for 16 hours to be in stationary phase for extraction. The presence of RNA was verified by running the RNA out on a bleach agarose gel. Any DNA contamination was then removed using the Amibon Turbo DNase Free Kit (ThermoFisher Scientific, Waltham, Maine). The

concentration of the RNA was checked using a Nanodrop ND-1000 (ThermoFisher Scientific, Waltham, Maine). cDNA was made from the RNA using a ProtoScript II First Strand Synthesis Kit (New England Biolabs, Ipswich, Maine). The concentration of the cDNA was checked on a Nanodrop and then all samples were diluted to 70 ng/ $\mu$ L and were then used in quantitative PCR reactions (qPCR).

qPCR primers were designed using Geneious (Biomatter Inc., Newark, New Jersey) and are listed in Table 2. Primers were ordered from Invitrogen (Carlsbad, California) or Sigma-Aldrich (St. Louis, Missouri). qPCR 2x Green Master Mix, High ROX (Genesee Scientific, San Diego, California) was used in 20  $\mu$ L reactions with 3  $\mu$ M each of forward and reverse primers and the reaction was run on a StepOne Real-Time PCR System machine and the data were analyzed using StepOne Software v2.1 (Applied Biosystems, Carlsbad, California). The two-step cycling conditions were the following: 95°C for 15 min, followed by 35 cycles of 95°C for 20 seconds then 60°C for 1 min. A melt curve analysis was then performed which included 95°C for 15 seconds followed by 60°C for 1 min. This temperature was increased in increments of 0.3°C each cycle. The resulting CT values were normalized to a control gene, *nlpD*, which has been used as a control gene in other studies [81]. Comparative  $\Delta\Delta$ CT values were calculated and used to show the fold changes as described previously [82].

#### Antimicrobial chemokine binding assay

The ability of CCL28 to bind to bacteria was tested using a flow cytometric assay designed in the Erickson and Wilson laboratories. Bacteria were grown up in TB broth at 37°C overnight. A subculture was started the next morning and grown to mid-logarithmic phase (OD 0.6). 1 mL was removed and centrifuged and resuspended in Phosphate Buffered Saline (PBS)



with 0.5 g bovine serum albumin added (PBSA). 5  $\mu$ L of the diluted bacteria was added to 100  $\mu$ L of PBSA and 10  $\mu$ L of CCL28 (0.5  $\mu$ g/ $\mu$ L). The sample was then vortexed and incubated at room temperature for 30 min. After 30 min the sample was washed in PBSA and then centrifuged. 5  $\mu$ L of biotinylated anti-CCL28 (0.5  $\mu$ g/ $\mu$ L; R&D Systems, Minneapolis, Minnesota) was added and incubated for 30 min. Another wash is performed and then Streptavidin-Allophycocyanin (APC; BD Biosciences, San Jose, California) is added and incubated for 1 hour. After a wash, the samples are run on a BD Accuri C6 Flow Cytometer (BD Biosciences, San Jose, California) to measure fluorescence of APC.

### Killing assays

The ability of CCL28 or polymyxin to kill bacteria was tested using a flow cytometric assay designed in the Erickson and Wilson laboratories. Bacteria were grown up in TB broth at 37°C overnight. A subculture was started the next day and grown to OD 0.6. The bacteria were then diluted 1:25 in potassium phosphate buffer (containing 0.2 M  $\text{KH}_2\text{PO}_4$  and 0.2 M  $\text{K}_2\text{HPO}_4$ ; PPB). CCL28 (0.5  $\mu$ g/ $\mu$ L), polymyxin (10  $\mu$ g/ $\mu$ L) or BSA (0.01g) were then mixed with the bacteria. The samples were incubated for 2 hours and then counting beads were added (diluted 1:62500, PolyBead Polystyrene 1 $\mu$ m Microspheres, Polysciences Inc., Warrington, Pennsylvania). 7  $\mu$ L of propidium iodide (PI) (Invitrogen, Carlsbad, California) was added before running the samples on the BD Accuri C6 Flow Cytometer. Percent survival was calculated by subtracting PI positive cells from the total number of treated bacteria (CCL28 or polymyxin) divided by PI positive cells from the total number of untreated bacteria (BSA) per 30,000 beads.

## Mouse infections

5 mL of P<sup>+</sup> or P<sup>-</sup> strains of wild-type and  $\Delta rfaH$  *Y. pseudotuberculosis* were grown up overnight at 28°C in TB broth. P<sup>-</sup> strains were then subcultured in 40 mLs (5 mLs for P<sup>+</sup> strains) of TB broth and grown until OD 1.0. Once the OD reached 1.0, the bacteria were spun down and resuspended in 1 mL of PBS. The inoculum was then centrifuged and resuspended in 1 mL of PBS. 100  $\mu$ L of each strain was infected orally using a tube and ball syringe to either a male or female BALB/c mouse that was approximately 3 months of age. The mice were separated into different cages according to the strain of bacteria used to infect. The CFU of the inoculum was measured by performing serial dilutions of the inoculum and plating 100  $\mu$ L onto Yersinia Selective Agar (YSA) plates (which contain bile salts, crystal violet, and irgasan).  $\sim 2 \times 10^8$  CFU/mL were used to infect for the P<sup>+</sup> strains and  $\sim 2 \times 10^9$  CFU/mL were used for the P<sup>-</sup> strains. The mice were fasted for 16 hours before infection and were then given food 2 hours after infection. The mice were observed for three days and on the third day the mice were sacrificed and the mesenteric lymph nodes, Peyer's patches, fecal pellets, spleen, and liver were collected. These organs were weighed and homogenized in PBS. Serial dilutions were performed and plated onto YSA plates. Colonies were counted and the CFU/g of each organ was calculated.

Intravenous infections were also performed and the bacteria were prepared in a similar method except the amount of bacteria used to infect was  $\sim 1 \times 10^5$  CFU of *Y. pseudotuberculosis* P<sup>-</sup>. The mice were infected retro-orbitally under anesthesia with 100  $\mu$ L of bacteria. After 3 days of infection, the mice were sacrificed and the liver and spleen were harvested, homogenized, and then plated on YSA plates. The colonies were counted and the CFU/g of each organ was calculated.

## RESULTS

The *rfaH* gene is deleted from *Y. pseudotuberculosis* and *Y. pestis* and complementation is successful in *Y. pseudotuberculosis* but not in *Y. pestis*

To test the function of the *rfaH* gene in *Y. pseudotuberculosis* and *Y. pestis*, the gene was removed from each bacterial genome and replaced with a kanamycin resistance gene. The *rfaH* gene in *Y. pseudotuberculosis* is 99.8% identical to the *rfaH* gene in *Y. pestis*, as shown in Figure 3C. The gene was first deleted from *Y. pestis* using lambda-red recombination because of its greater ability to take up DNA via electroporation. After the mutation was made in *Y. pestis*, the modified allele (*rfaH* upstream and downstream regions flanking the kanamycin resistance gene) was cloned into the suicide plasmid pRE112. The gene deletion strategy and result are shown in Figure 3A and 3B. The mutations were verified through PCR and gel electrophoresis which showed an appropriate change in size using primers that flank the *rfaH* gene and the absence of the *rfaH* gene using *rfaH* internal primers.

In order to show that any change observed in these strains was due to the loss of the *rfaH* gene and not any other downstream effects, functional *rfaH* genes were added back to the mutants. The *rfaH* gene was amplified from *Y. pseudotuberculosis* and *Y. pestis* using PCR and inserted into a low copy plasmid, pACYC184, which exists at about 15 copies per cell. The resulting plasmid was electroporated into the *Y. pseudotuberculosis* and *Y. pestis*  $\Delta rfaH$  strains. The presence of the plasmid with the *rfaH* gene insert within the  $\Delta rfaH$  strains was verified with PCR (Figure 3B). These complemented strains were named *Y. pseudotuberculosis rfaH*<sup>+</sup> and *Y. pestis rfaH*<sup>+</sup>. As will be discussed below, the *Y. pseudotuberculosis rfaH*<sup>+</sup> strain was able to

restore or mostly restore the wild-type phenotype. The *Y. pestis rfaH*<sup>+</sup> strain was unable to complement the phenotypes observed.

Another complementation attempt was performed using a site-specific Tn7 transposon to insert the *rfaH* gene back into the *Y. pseudotuberculosis* and *Y. pestis* bacterial chromosome without disrupting any genes [83]. An advantage of this method would be that the *rfaH* gene would be present as a single copy as opposed to multiple copies found in the pACYC184 plasmids. The successful insertion of the Tn7 into the bacterial chromosome was verified by PCR. As will be described below, this complementation strain was unable restore the wild-type phenotype in either *Y. pseudotuberculosis* or *Y. pestis*.

RfaH impacts lipopolysaccharide synthesis in *Y. pseudotuberculosis* and *Y. pestis* differently

As RfaH plays a role in the synthesis of the LPS in *E. coli* and *Salmonella*, we first wanted to determine if RfaH influences the synthesis of the LPS in *Y. pseudotuberculosis* and *Y. pestis*. In *Yersinia*, the composition of the sugars in LPS changes depending on the growth temperature of the bacteria (Figure 1). The LPS was extracted from cultures grown at two temperatures, 21°C and 37°C, to determine any changes that occur in the LPS based on temperature.

While examining the *Y. pseudotuberculosis* LPS, we found that the core oligosaccharide of the  $\Delta rfaH$  mutant is truncated in comparison to the wild-type strain at both temperatures (Figure 4). The *Y. pseudotuberculosis*  $\Delta rfaH$  strain also results in the partial loss of the O-antigen at 21°C as the band in that region is fainter. *Y. pseudotuberculosis* does not produce an O-antigen at 37°C which is observed as an absence of O-antigen bands. When the *rfaH* gene was placed in a plasmid and inserted into the *Y. pseudotuberculosis*  $\Delta rfaH$  strain creating a complemented

strain it restores the size of the core and presence of the O-antigen and resembled that of the wild-type strain.

For comparison, we included LPS from a *Y. pseudotuberculosis* *hldD* transposon mutant (*hldD*::Tn5) strain. The *hldD* gene functions to synthesize the first L-D Heptose I which is attached to the Kdo residue (see Figure 1 for *Y. pseudotuberculosis* LPS structure). Therefore, in the *hldD*::Tn5 lane the size of the two Kdo residues can be visualized in relation to the full core (containing 3 L-D Heptose residues, GlcNAc (N-acetylglucosamine), glucose, and D-D Hep or galactose, depending on the temperature). The truncation observed in the *Y. pseudotuberculosis*  $\Delta$ *rfaH* mutant is not as extreme as found in the *hldD*::Tn5, indicating that it likely has lost the heptose/galactose residue and one of the heptose residues.

In *Y. pestis*, the core region of strain KM218 was previously shown to be larger when the bacteria were grown at 25°C rather than at 37°C [84]. These researchers found that this was correlated with the production of glucose, galactose, 2 LD-Hep, DD-Hep, and 2-amino-2-deoxyglucose (GlcN) sugars at 25°C rather than the same monosaccharides except galactose at 37°C. In contrast, we found that the core oligosaccharide of *Y. pestis* KIM6+ is shorter when grown at 21°C than at 37°C (Figure 4). The *rfaH* gene is required for production of this longer form of core oligosaccharide at 37°C, since we saw no difference in the size of the LPS band when comparing the wild-type and  $\Delta$ *rfaH* mutant at 21°C whereas the core appeared shorter in the  $\Delta$ *rfaH* mutant strain at 37°C. At 37°C the *Y. pestis*  $\Delta$ *rfaH* core is likely missing the D-D Heptose/galactose residue and one or two of the L-D Heptose residues (Figure 1). We attempted to complement the phenotype of the  $\Delta$ *rfaH* mutant of *Y. pestis*. We transformed the  $\Delta$ *rfaH* mutant with the same plasmid containing the *rfaH* gene from *Y. pseudotuberculosis*. This plasmid failed to complement the wild-type phenotype in *Y. pestis*. Since the *Y. pestis* *rfaH*

sequence differs by one nucleotide we also created a plasmid containing the *Y. pestis* version of this gene. This plasmid also failed to restore the wild-type phenotype. We then attempted complementation of the mutant by chromosomal insertion of the *rfaH* gene at the *attTn7* insertion site which also failed to complement the phenotype (data not shown). In bacterial genetic studies, when phenotypes are unable to be complemented, this can be due to additional mutations in unknown genes. To see if this were happening in the *Y. pestis*  $\Delta rfaH$  mutant we re-made the mutation in *Y. pestis* KIM6+ wild-type strain using the pRE112 suicide plasmid. The same mutant phenotypes were observed in this strain but again, this mutation was not able to be complemented via expression of the gene from a plasmid.

Genes that function in lipopolysaccharide synthesis are downregulated with loss of *rfaH*

Since the loss of RfaH caused truncation of the core oligosaccharide as well as loss of O-antigen, we wanted to determine the corresponding changes in gene expression that were responsible. We isolated RNA from wild-type,  $\Delta rfaH$  mutant, and the complemented strains of both *Y. pseudotuberculosis* and *Y. pestis*, at both 21°C and 37°C. We then measured transcription of LPS-related genes using qPCR. Genes that function in either O-antigen or core oligosaccharide synthesis were selected for examination (Table 3). The *ddhD* gene cluster has an 8-bp ops sequence upstream and contains genes which are annotated to encode O-antigen synthesis proteins, as shown in Figure 2 (*ddhD-wbyK*). From this cluster, we selected *ddhD*, *ddhA*, *ddhB*, *ddhC*, *wbyJ*, and *wbyK* to examine. In addition to these genes, we also measured transcription of *waaQ*, *waaE*, and *waaC* which are not found in this region. These are separate genes found in different operons throughout the genome and are predicted to function in core oligosaccharide synthesis and do not possess an ops sequence. As an internal control, we also

measured transcription of the *dnaE* gene, which showed no significant differences in any of the comparisons we made (Table 4).

As shown in Table 4, at both 21°C and 37°C in *Y. pseudotuberculosis* (columns 2 and 4) the genes in the *ddhD* gene cluster are significantly downregulated (\* indicates  $p < 0.05$ ). The downregulation increases in genes furthest from the promoter (*wbyI* and *wbyK*). The genes which function in the synthesis of the core (*waaQ*, *waaE*, and *waaC*) were downregulated in the  $\Delta rfaH$  mutant even though they do not have a detectable ops sequence upstream. These results correlate with truncation we observed in the core region and the lack of O-antigen in the LPS isolated from the both the O-antigen and core regions of the LPS in *Y. pseudotuberculosis*  $\Delta rfaH$  mutant (Figure 4).

We also measured transcription of the same genes in *Y. pestis* at both temperatures in wild-type and  $\Delta rfaH$  mutant bacteria (Table 4). When grown at 37°C the only gene that appeared to be downregulated was *ddhD* (column 5). However, at 21°C we found downregulation in all of the *ddhD* gene cluster genes (*ddhD*, *ddhA*, *ddhB*, *ddhC*, *wbyI*, and *wbyK*). The core transferase genes (*waaQ* and *waaE*) were not downregulated in *Y. pestis* at either temperature, and the *waaC* gene was only affected by the  $\Delta rfaH$  mutation at 21°C. Thus, we could not associate reduced transcription of any of the LPS-related genes we tested in *Y. pestis* at 37°C with the altered core length observed in Figure 4.

We also calculated the fold change in expression of each gene in wild-type bacteria when grown at 21°C compared to the expression at 37°C (Table 5). There were only a few genes that are significantly downregulated at 37°C as compared to 37°C in the wild-type *Y. pseudotuberculosis*: *ddhD*, *ddhA*, *wbyI*, and *wbyK* (column 2). This reduction in transcription of

these genes may contribute to the loss of O-antigen production at 37°C. In *Y. pestis* wild-type only *ddhD* and *wbyK* are downregulated in 21°C as compared to 37°C (column 3). There are no differences in the expression of the LPS synthesis genes in the *Y. pestis*  $\Delta rfaH$  strain when grown at the different temperatures (column 5). This indicates that the RfaH protein is not active at 37°C or that the genes are transcribed hardly at all so that the differences are not detected.

RfaH is necessary for protection against antimicrobial chemokine binding in *Y.*

*pseudotuberculosis* but not *Y. pestis*

As the LPS plays an important role in the defense against host antimicrobial peptides [55], *Y. pseudotuberculosis* and *Y. pestis* strains were examined to see if the loss of *rfaH* decreased resistance of the bacteria to two peptides. We first measured the impact of *rfaH* deletion on binding to the antimicrobial chemokine CCL28 using flow cytometry. As shown in Figure 5A the *Y. pseudotuberculosis* wild-type displays low binding by CCL28 but when the *rfaH* gene is deleted there is a significant increase in binding. CCL28 requires an unknown surface protein for binding to the surface of *Y. pseudotuberculosis*. These results show that the *rfaH* deletion truncates the LPS barrier so that CCL28 has easier access to bind to the bacteria. When the *rfaH* gene is reinserted into the into the *Y. pseudotuberculosis*  $\Delta rfaH$  there is a significant reduction of binding by CCL28, although it does not completely restore the wild-type binding levels. The *Y. pseudotuberculosis* *hldD*::Tn5 strain, a positive control which has a truncation to the Kdo residues in the LPS, displays a similar level of high binding. We observed in the gel analysis that the *hldD*::Tn5 strain LPS is much shorter than the  $\Delta rfaH$  strain (Figure 4). As there is not a significant change in binding between the two strains, it appears that there is a certain threshold where more truncation in the LPS does not matter.



We also sought to measure the effect of *rfaH* mutation on binding to *Y. pestis*. Figure 5B shows that binding that the wild-type *Y. pestis* normally displays a 35% binding by CCL28. This binding does not change significantly in the *Y. pestis*  $\Delta rfaH$  or the *Y. pestis* *rfaH*<sup>+</sup> strains. The results show that when the LPS is truncated at 37°C in *Y. pseudotuberculosis* there is an increase in binding by CCL28 but it does not change binding in *Y. pestis*, which is unexpected. It is possible that *Y. pestis* has some other defenses apart from the LPS which protect against host defense peptides when grown at 37°C which are not found in *Y. pseudotuberculosis*.

Loss of *rfaH* increases susceptibility of *Y. pseudotuberculosis* to killing by antimicrobial peptides and chemokines but does not affect *Y. pestis*

Since a *rfaH* mutation increased the ability of CCL28 to bind to the surface of the bacteria, we reasoned that this would also increase susceptibility to antimicrobial chemokines. To test this, we measured bacterial survival in the presence of CCL28 or polymyxin B in low osmolarity conditions using flow cytometry. The *Y. pseudotuberculosis* wild-type strain was largely unaffected when incubated with CCL28, around 80% percent survival (Figure 6A). In the *Y. pseudotuberculosis*  $\Delta rfaH$  strain, the percent survival drops significantly to 55%, indicating that the bacteria had become more susceptible to killing by CCL28. The *Y. pseudotuberculosis* *rfaH*<sup>+</sup> strain has restored the percent survival values to wild-type levels. The effect of a *rfaH* mutation in *Y. pestis* was different, however. Figure 6B shows that *Y. pestis* wild-type has an 80% percent survival, similar to that of the *Y. pseudotuberculosis* wild-type. There is a slight decrease in the percent survival of the *Y. pestis*  $\Delta rfaH$  strain but it is not significant. These results support the results of the binding assay for *Y. pestis* as they both show no change in binding or killing by CCL28.

Susceptibility to polymyxin was also affected by *rfaH* mutation. *Y. pseudotuberculosis* is relatively resistant to polymyxin at 37°C. As shown in Figure 7, *Y. pseudotuberculosis*  $\Delta rfaH$  strain shows a dramatic decrease in percent survival as compared to the wild-type (Figure 7A). The complemented strain showed similar survival to the wild-type strain. In contrast, *Y. pestis* is very sensitive to polymyxin at 37°C (Figure 7B). There was a slight decrease in survival in the *Y. pestis* *rfaH* mutant strain but the difference was not statistically significant under these conditions.

RfaH does not affect the ability of *Y. pseudotuberculosis* to persist in *in vivo* oral and intravenous mouse infections

The truncated LPS and increased susceptibility to antimicrobial peptides and chemokines indicate that the *Y. pseudotuberculosis*  $\Delta rfaH$  strain might not survive as effectively and cause disease in mice. Previous experiments with pYV negative strains of *Y. pseudotuberculosis* have shown that an *rfaH* transposon mutant could not persist as long as the wild-type in mouse liver or spleen following an intravenous infection [18]. Another transposon mutagenesis experiment with *Y. pseudotuberculosis* containing pYV virulence plasmid showed in an orogastric infection that O-antigen synthesis gene mutations (*ddhC*) caused attenuation in the bacteria [85]. We first compared the ability of *Y. pseudotuberculosis* wild-type and  $\Delta rfaH$  cells that carry the pYV virulence plasmid (P<sup>+</sup>) to colonize after oral infection. After three days the mesenteric lymph nodes, Peyer's patches, spleen, liver, and fecal pellets were collected and the bacterial loads were determined. As shown in Figure 8A there was no significant difference in survival of *Y. pseudotuberculosis* wild-type P<sup>+</sup> and *Y. pseudotuberculosis*  $\Delta rfaH$  P<sup>+</sup>. Given this unexpected result, we reasoned that the effect of *rfaH* on bacterial survival *in vivo* might only be apparent in strains lacking the pYV virulence plasmid (P<sup>-</sup>). We performed similar oral infections (10<sup>9</sup> CFU)

in mice using plasmid-cured wild type and *rfaH* mutant strains. Similar to the P<sup>+</sup> strains, there were no significant differences in bacterial numbers in any organ between the  $\Delta rfaH$  and wild-type strains (Figure 8B). These results clearly indicate that *rfaH* is not required for virulence of *Y. pseudotuberculosis* strain IP32953 via oral infection.

The lack of *rfaH* function may have different impacts on bacterial survival depending on the route of 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 *Y. pseudotuberculosis* wild-type P<sup>-</sup> or *Y. pseudotuberculosis*  $\Delta rfaH$  P<sup>-</sup>. The bacterial loads in the liver and spleens of these mice were measured following three days of infection (Figure 8C). Similar to the oral infection results, survival of the *rfaH* mutant was not decreased compared to wild-type bacteria in any of the organs. These data shows that although a *rfaH* deletion alters LPS structure and increases susceptibility to antimicrobial peptides, these changes do not affect virulence via the oral or intravenous route in strains that contain the pYV virulence plasmid and in strains that do not.

## DISCUSSION

The *rfaH* gene was identified in a transposon mutagenesis screen for mutants of *Y. pseudotuberculosis* with increased susceptibility to antimicrobial chemokine binding. This mutagenesis used *Y. pseudotuberculosis* strain IP32953 and tested the resistance of each mutant to binding by CCL28 [55]. Transposon mutagenesis in mice also showed that *Y. pseudotuberculosis* YPIII P<sup>-</sup> *rfaH* mutants had decreased persistence in BALB/c mice after a co-infection with 20,000 transposon mutants in an intravenous infection for three days [18]. As RfaH has been identified in *E. coli* and *Salmonella* to function as a transcriptional antiterminator in the synthesis of the LPS, it was selected for further study. Our hypothesis was that RfaH performs a similar regulatory role in the synthesis of the LPS in two important human pathogens, *Y. pseudotuberculosis* and *Y. pestis* and that these changes would be important for bacterial survival *in vivo*.

We showed using SDS-PAGE gel analysis that loss of the *rfaH* results in no detectable O-antigen production in *Y. pseudotuberculosis*. Additionally, the core oligosaccharide of *Y. pseudotuberculosis* and *Y. pestis* are truncated in the  $\Delta rfaH$  strains, except when the *Y. pestis* is grown at 21°C. The core oligosaccharide of the *Y. pestis* wild-type is much shorter when grown at 21°C than 37°C. Other studies have shown that the core region in some strains of *Y. pestis* (such as in the KM218 strain) is larger when grown at 25°C than 37°C [84]. However, it has been found that there are structural differences between the LPS in different strains of *Y. pestis* whether they are from Eurasia or the Americas [86]. At 21°C it is possible that the KIM6+ strain does not produce the terminal D-D Heptose or galactose sugar which results in a shorter structure. There might also be changes in phosphorylation, aminoarabinose addition, or other modifications of the residues. In order to know the exact effect that RfaH has on the LPS structure and why

there are differences in the wild-type LPS in *Y. pestis*, it would be important to determine the structure of the *Y. pestis* KIM6+ LPS, which has not been done previously. The LPS structure of several strains of *Y. pestis* and *Y. pseudotuberculosis* has been determined by using high-resolution electrospray ionization mass spectrometry (ESI MS) [87]. The researchers discovered that the LPS from *Y. pestis* KM260 could have up to two 4-amino-4-dioxy-L-arabinose, up to three phosphate groups, and substitution of several sugars. By extracting the LPS from *Y. pestis* wild-type at 21°C and 37°C and performing ESI MS we would be able to identify what changes are occurring which cause this reduction in size.

Complementation with the *rfaH* gene in the pACYC184 plasmid was able to restore the wild-type phenotype in the *Y. pseudotuberculosis*  $\Delta rfaH$  strain. There is still a band in these *Y. pseudotuberculosis* *rfaH*<sup>+</sup> lanes that is the same size as the core in the  $\Delta rfaH$  strains indicating that there might not be complete complementation, and some truncated LPS are still being synthesized. This intermediate phenotype was also observed in chemokine binding and polymyxin susceptibility experiments. When the *rfaH* gene was added to the *Y. pestis*  $\Delta rfaH$  strain it did not detectably alter any of the phenotypes we measured. This could be due to the fact that perhaps RfaH functions slightly differently in these two stains, or that in *Y. pestis* the multiple copies of the *rfaH* gene is detrimental. It is also possible that secondary, non-target mutations arise extremely quickly in the *Y. pestis*  $\Delta rfaH$  strain that prevent restoration of RfaH function. A separate complementation strategy was attempted using a Tn7 transposon to insert the *rfaH* gene into the bacterial chromosome in the  $\Delta rfaH$  strains. The benefits of this type of complementation would be that there is a single copy in the chromosome and there should not be overexpression of the *rfaH* gene. However, despite successful insertion of the gene, this was also unable to complement the truncation LPS in the *Y. pestis*  $\Delta rfaH$  (data not shown). After multiple

attempts via different methods it remains unclear why complementation the *rfaH* gene in *Y. pestis* has not been successful.

We identified specific genes involved in LPS synthesis that were downregulated in the absence of RfaH which could cause of the alteration of the LPS. Several genes within the *ddhD* gene cluster were shown to be downregulated in the  $\Delta rfaH$  strains and they are annotated as functioning in the synthesis of the sugars of the O-antigen [9]. This was an expected result since we were able to identify a clear ops sequence upstream of this operon (Figure 2). The pattern of downregulation of these genes is consistent with other published studies and the mechanism of action of the RfaH protein [57, 88]. As the RNA polymerase progresses down the operon during transcription, it is more likely to prematurely release, causing polarity in  $\Delta rfaH$ . While all of the genes exhibit downregulation, the genes *wbyI* and *wbyK* which are located furthest from the promoter exhibit more drastic reduction of expression as compared to the genes in the beginning of the cluster.

Several genes we examined (*waaQ*, *waaE*, and *waaC*) do not have an ops sequence upstream and yet were downregulated in the *Y. pseudotuberculosis*  $\Delta rfaH$  strain. In *Salmonella*, the *waa* genes occur together in an operon and have an ops sequence upstream and are regulated by RfaH [76]. Nagy et al. [76] conducted a genome-wide expression experiment to identify all the genes in *Salmonella* whose expression is altered by loss of RfaH. They found numerous changes, despite the presence of only two ops sequences in the genome, including altered expression of non-LPS related virulence genes. They also showed that these other gene expression changes were an indirect effect of the LPS truncation in the *rfaH* mutant. The downregulation observed in the core genes in *Y. pseudotuberculosis* might also be an indirect effect of loss of *ddh* gene expression. To examine this, individual *ddh* gene deletions could be

made in the *Y. pseudotuberculosis* wild-type strain and expression of the *waaQ*, *waaE*, and *waaC* genes would be analyzed to see if they are reduced in response to the absence of the O-antigen genes. It is also possible that a modified ops sequence exists upstream of the *waa* genes that remains undetected.

It is interesting that although *Y. pestis* does not produce an O-antigen it still retains the *ddhD* gene cluster. The five genes in this cluster have been inactivated in *Y. pestis* are *ddhB*, *wbyI*, *wzy*, *gmd*, and *fcl* [9]. We observed that the transcription of these genes is regulated by the RfaH at 21°C in *Y. pestis*. However, transcription of the O-antigen or core synthesis genes was not affected by the loss of RfaH in *Y. pestis* at 37°C. This is confusing because LPS gel analysis showed truncation in the core region of the LPS at 37°C but not 21°C. As shown in Figure 1, there are many other genes that function in the synthesis of the core such as *waaA* and *waaF* which were not chosen for examination in this study. The synthesis of the entire LPS molecule is a complex process involving many proteins which all are essential to forming the completed structure. If one of these genes not examined was downregulated by the loss of *rfaH* it may be the cause of the truncation of the LPS seen at 37°C. Further experimentation would involve testing each of these genes for expression changes to see if they are causing the truncation observed. Another possible explanation is that there are some post-transcriptional functions that are affected by the loss of the *rfaH* gene. RfaH has been shown to also activate translation of certain genes by undergoing conformational changes and interacting with the ribosome [89]. Although transcription might not be influenced, the truncation observed in the LPS might be from the lack of translation initiation by RfaH. This could be tested by measuring the concentration of the LPS synthesis proteins in the *Y. pestis* wild-type and  $\Delta rfaH$  strains by

Western Blot analysis. If the LPS synthesis proteins were less concentrated in the  $\Delta rfaH$  strain it could indicate that translation might be affected.

We calculated the fold changes between each LPS synthesis gene when grown at either 21°C or 37°C to see if there was an effect of temperature on the activity of RfaH. There was a fairly uniform, albeit small, decrease in expression of the *ddh* cluster of O-antigen genes at 37°C compared to 21°C. There was no consistent change in expression of these genes in *Y. pestis* according to temperature. Additionally, in the *rfaH* mutants, there was also no clear pattern of temperature-dependent expression, which could be due to extremely low transcript accumulation in the absence of RfaH.

One of the functions of the LPS is to provide resistance against host defenses including complement and antimicrobial peptides. When the LPS is truncated it allows for antimicrobial peptides to more easily reach the bacterial membranes and exert their effect. Studies have shown previously that truncation in the core region of the LPS in *Burkholderia cenocepacia* results in increased susceptibility by antimicrobial peptides [34]. We found that when the LPS is truncated in *Y. pseudotuberculosis* at 37° C it dramatically increases the ability of polymyxin B to kill the bacteria. However the same concentration of polymyxin B used completely killed all the strains of *Y. pestis* regardless of the presence of *rfaH*. It has been shown that *Y. pestis* is more resistant to polymyxin B at 21°C than 37°C [31]. This due to higher outer membrane permeability which results from acyl chain fluidity. The difference between susceptibility between *Y. pseudotuberculosis* and *Y. pestis* is that *Y. pseudotuberculosis* contains more complex fatty acid chains and *Y. pestis* contains saturated fatty acids. While *Y. pestis* might reduce the number of acyl chains to avoid recognition by the immune system, it appears to increase its susceptibility to host defense peptides.



We observed similar trends with binding and killing by the antimicrobial chemokine, CCL28. In *Y. pseudotuberculosis* we see that both binding and killing by CCL28 are increased in the  $\Delta rfaH$  strains. In *Y. pestis* we see no change in the ability of CCL28 to bind to or kill the bacteria in the  $\Delta rfaH$  strains. The results are consistent for both experiments because if there was no increase in binding, we would not expect to see a change in killing by CCL28. The lifestyle of *Y. pestis* differs from that of *Y. pseudotuberculosis* in that it must be able to change expression of its genes to successfully survive in a flea vector and then a human host. Perhaps some features *Y. pestis* expresses at 37°C provide additional protection against host peptides that *Y. pseudotuberculosis* does not. Such defenses could include the formation of capsule or the Pla protease by *Y. pestis* (carried on plasmids pPCP1 and pMT1), which are not found in *Y. pseudotuberculosis* [24].

After testing *in vitro* resistance of the  $\Delta rfaH$  strains in *Y. pseudotuberculosis* and finding that they become more susceptible, we expected that they would not survive as well in an *in vivo* infection. We examined the survival of the *Y. pseudotuberculosis* strains in an *in vivo* mouse infections. We performed two types of infections: oral and intravenous which both showed no significant changes in survival between the wild-type and the  $\Delta rfaH$  strains. Other mouse infections in *E. coli* and *Salmonella* have shown that the *rfaH* mutant strains become attenuated [74, 76]. Another study showed that in a *Y. pseudotuberculosis* P<sup>-</sup> intravenous transposon mutagenesis infection in an *rfaH* mutant did not persist as long in the liver or spleen as the wild-type [18]. In these studies, the bacteria were usually co-infected in mice with the wild-type and *rfaH* mutant bacteria together, or in pools with 20,000 mutants infecting at once. In the present study, only single strain infections were performed and it is possible that competition studies would give a more sensitive measure of the defects caused by *rfaH* mutation. It could also be

possible that when *rfaH* mutants infect on their own (with altered LPS) they do not invoke as strong of an immune response, but when mixed with other bacteria that do trigger a stronger inflammatory response, they are preferentially removed. Our strain of *Y. pseudotuberculosis* is also different than the strain used in the previously published transposon mutagenesis study. Further experimentation with a co-infections with our strains might help to distinguish between these possibilities

While *Y. pseudotuberculosis* uses the virulence plasmid pYV and Yops proteins to efficiently establish infection in many organs, other studies have shown that the YPIII P<sup>-</sup> strains can also colonize the mesenteric lymph nodes, liver, and spleen [19]. However, we found that the *rfaH* gene has no effect on persistence in either plasmid carrying or plasmid absent strains of *Y. pseudotuberculosis*. This indicates that *rfaH* does not play a role in helping the plasmid deficient bacteria to reach and persist in any of the organs tested because there was still no difference between the wild-type and  $\Delta rfaH$  strains.

Overall our research has helped to identify the role the *rfaH* gene plays in the synthesis of the LPS in *Y. pseudotuberculosis* and *Y. pestis*. This is important information because *rfaH* deficient *Salmonella* strains have been explored as possible vaccine candidates against more virulent forms [76]. While our initial results show that there is no attenuation in single infection *rfaH* deficient *Y. pseudotuberculosis* this research is only in the beginning stages. Additional experiments might demonstrate ways that these *rfaH* deficient strains can potentially be used as vaccines for *Y. pseudotuberculosis*. Our work with antimicrobial chemokines helps to increase our knowledge of these important proteins and how they interact with and kill bacteria based on the length of the LPS. Antibiotic resistance is on the rise in many human pathogens including

methicillin-resistant *Staphylococcus aureus* (MRSA). Antimicrobial chemokines might prove to be an effective alternative method that could be used to treat antibiotic resistant strains.

TABLES AND FIGURES

Table 1. Primers used in the *rfaH* gene deletions and *rfaH* complementation plasmids.

<i>rfaH</i> 500 F SacI	5'- GCC GAG CTC GCC AAT AAT TTA CCA ACA TCT CGC-3'
<i>rfaH</i> 500 R KpnI	5'- CCG GGT ACC GCT AAG TAT GCG TCT TGC TAC TAT G-3'
<i>rfaH</i> F	5' - GCC GCG TCG ACT TCG GCG GCT ATG GGA TGC G - 3'
<i>rfaH</i> R	5' - CCG CCT CTA GAG TGC AGG TGC GGA TGG CGT A - 3'
<i>rfaH</i> internal F	5'-GGC TCA AGC TTC CCT TAG GAC ACC TTC ATG GAC- 3'
<i>rfaH</i> internal R	5'-GCG TGT CTA GAG GCG TTT AGT TTT CGT TAA CTT ATC TGG GC-3'

Table 2. Primers used in qPCR reactions

ddhD F	5' – TAT TGG GGA ATG CCA GCA GG - 3'
ddhD R	5' – TGG CTC CGG TCC AAG TAC TA - 3'
ddhA F	5'- TCT ACT TGG GAG CAG GAA CC-3'
ddhA R	5'- CAC GCA ACG TAT CCA TTG GC-3'
ddhB F	5'- CGA TGC TGA GGC TAC TCC AGG-3'
ddhB R	5'- ATG AGG GTG AGC ATT GCC AT-3'
wbyI F	5'- GCG GTA CAC TCG GAG TTG TA-3'
wbyI R	5'-TCC AAA AAT CAT AAT CAC TGA CAG GA-3'
wbyK F	5'- TGC GCG TGG TGG TTA TAA GA-3'
wbyK R	5'- ACT AGT GAA TGC CCC TCC AC-3'
waaQ F	5'- AAC GGC ACA CGC TAC TCA AT-3'
waaQ R	5'- CTC GAT ATC ATG CGG CCC AT-3'
waaE F	5'- CAG CGT CAT TGT CCC CGT AT-3'
waaE R	5'- AGA GCC ATC GTT AAC GGC AA-3'
rfaC F	5'-CGT GTG GTA CAA CAA CGC AG-3'
rfaC R	5'-ACT TTT ACA ATC CGG CCC GT-3'
dnaE F	5'-TTT CGT CCC GGT CCG TTA CAA- 3'
dnaE R	5'-ACA AGA TTA TGC CGT AGG TCG-3'
nlpD F	5' – TTC TCG TGG GCA ACC TAT CC - 3'
nlpD R	5' – TGC GTT CCC AGC ATA CAC AA - 3'

Table 3. List of genes selected for examination by qPCR

Function	Gene	Product
O-antigen synthesis	<i>ddhD</i>	CDP-6-deoxy-delta-3,4-glucoseen reductase
	<i>ddhA</i>	glucose-1-phosphate cytidyltransferase
	<i>ddhB</i>	CDP-glucose 4,6-dehydratase
	<i>ddhC</i>	CDP-4-keto-6-deoxy-D-glucose-3-dehydratase
	<i>wbyI</i>	putative glycosol transferase
	<i>wbyK</i>	putative mannosyltransferase
Core oligo synthesis	<i>waaQ</i>	predicted <i>waaQ</i> gene, coding for heptosyltransferase III enzyme
	<i>waaE</i>	predicted <i>waaE</i> gene, coding for glycosyl transferase family 2
	<i>waaC</i>	lipopolysaccharide heptosyltransferase
Control	<i>dnaE</i>	DNA polymerase III $\alpha$ subunit
	<i>nlpD</i>	Outer membrane lipoprotein

Table 4. Fold changes in gene expression compared between wild-type and  $\Delta$ *rfaH* strains in *Y. pseudotuberculosis* (*pstb*) and *Y. pestis* at 21°C and 37°C. Results are the average with standard error of the mean of 3 independent RNA isolations. \* = significantly different from 1 (p-value < 0.05).

Gene	$\Delta$ <i>rfaH</i> <i>Y. pstb</i> /wild-type <i>Y. pstb</i> 21°C		$\Delta$ <i>rfaH</i> <i>Y. pestis</i> /wild-type <i>Y. pestis</i> 21°C		$\Delta$ <i>rfaH</i> <i>Y. pstb</i> /wild-type <i>Y. pstb</i> 37°C		$\Delta$ <i>rfaH</i> <i>Y. pestis</i> /wild-type <i>Y. pestis</i> 37°C	
<i>ddhD</i>	0.03 ± 0.01	*	0.10 ± 0.01	*	0.02 ± 0.009	*	0.48 ± 0.09	*
<i>ddhA</i>	0.07 ± 0.02	*	0.17 ± 0.05	*	0.20 ± 0.06	*	0.97 ± 0.22	
<i>ddhB</i>	0.12 ± 0.04	*	0.37 ± 0.09	*	0.21 ± 0.08	*	0.97 ± 0.14	
<i>ddhC</i>	0.17 ± 0.05	*	0.23 ± 0.04	*	0.24 ± 0.04	*	0.69 ± 0.15	
<i>wbyI</i>	0.001 ± 0.0003	*	0.07 ± 0.04	*	0.006 ± 0.002	*	0.55 ± 0.29	
<i>wbyK</i>	0.007 ± 0.002	*	0.02 ± 0.003	*	0.005 ± 0.002	*	0.57 ± 0.15	
<i>waaQ</i>	0.05 ± 0.01	*	0.60 ± 0.15		0.03 ± 0.01	*	0.92 ± 0.26	
<i>waaE</i>	0.05 ± 0.01	*	0.66 ± 0.24		0.08 ± 0.01	*	1.23 ± 0.23	
<i>waaC</i>	0.37 ± 0.12	*	2.44 ± 0.14	*	0.65 ± 0.07	*	3.21 ± 2.24	
<i>dnaE</i>	1.27 ± 0.40		0.88 ± 0.5		2.29 ± 0.56		0.80 ± 0.08	

Table 5. Fold changes in gene expression compared between wild-type or  $\Delta rfaH$  *Y. pseudotuberculosis* (*pstb*) and *Y. pestis* at 21°C and 37°C. Results are the average with the standard error of the mean of 3 independent RNA isolations. \* = significantly different from 1 (p-value < 0.05).

Gene	wild-type <i>Y. pstb</i> 37°C/ wild-type <i>Y. pstb</i> 21°C		$\Delta rfaH$ <i>Y. pstb</i> 37°C/ $\Delta rfaH$ <i>Y. pstb</i> 21°C		wild-type <i>Y. pestis</i> 37°C/ wild-type <i>Y. pestis</i> 21°C		$\Delta rfaH$ <i>Y. pestis</i> 37°C/ $\Delta rfaH$ <i>Y. pestis</i> 21°C	
<i>ddhD</i>	0.39 ± 0.08	*	0.27 ± 0.10	*	0.16 ± 0.02	*	0.78 ± 0.18	
<i>ddhA</i>	0.34 ± 0.05	*	1.30 ± 0.43		0.58 ± 0.20		3.21 ± 0.81	
<i>ddhB</i>	0.52 ± 0.15		0.87 ± 0.18		1.34 ± 0.61		3.21 ± 0.82	
<i>ddhC</i>	0.66 ± 0.15		1.09 ± 0.27		1.01 ± 0.12		3.85 ± 1.91	
<i>wbyI</i>	0.32 ± 0.02	*	2.45 ± 1.48		0.73 ± 0.29		6.26 ± 1.36	
<i>wbyK</i>	0.25 ± 0.03	*	0.21 ± 0.005	*	0.05 ± 0.02	*	1.02 ± 0.12	
<i>waaQ</i>	1.00 ± 0.19		0.77 ± 0.16		1.94 ± 0.56		2.87 ± 0.68	
<i>waaE</i>	1.20 ± 0.42		1.56 ± 0.36		1.46 ± 0.39		3.51 ± 1.46	
<i>waaC</i>	0.80 ± 0.10		1.93 ± 0.96		3.08 ± 1.33		1.66 ± 0.22	
<i>dnaE</i>	1.34 ± 0.38		2.65 ± 1.09		1.02 ± 0.17		1.50 ± 0.57	

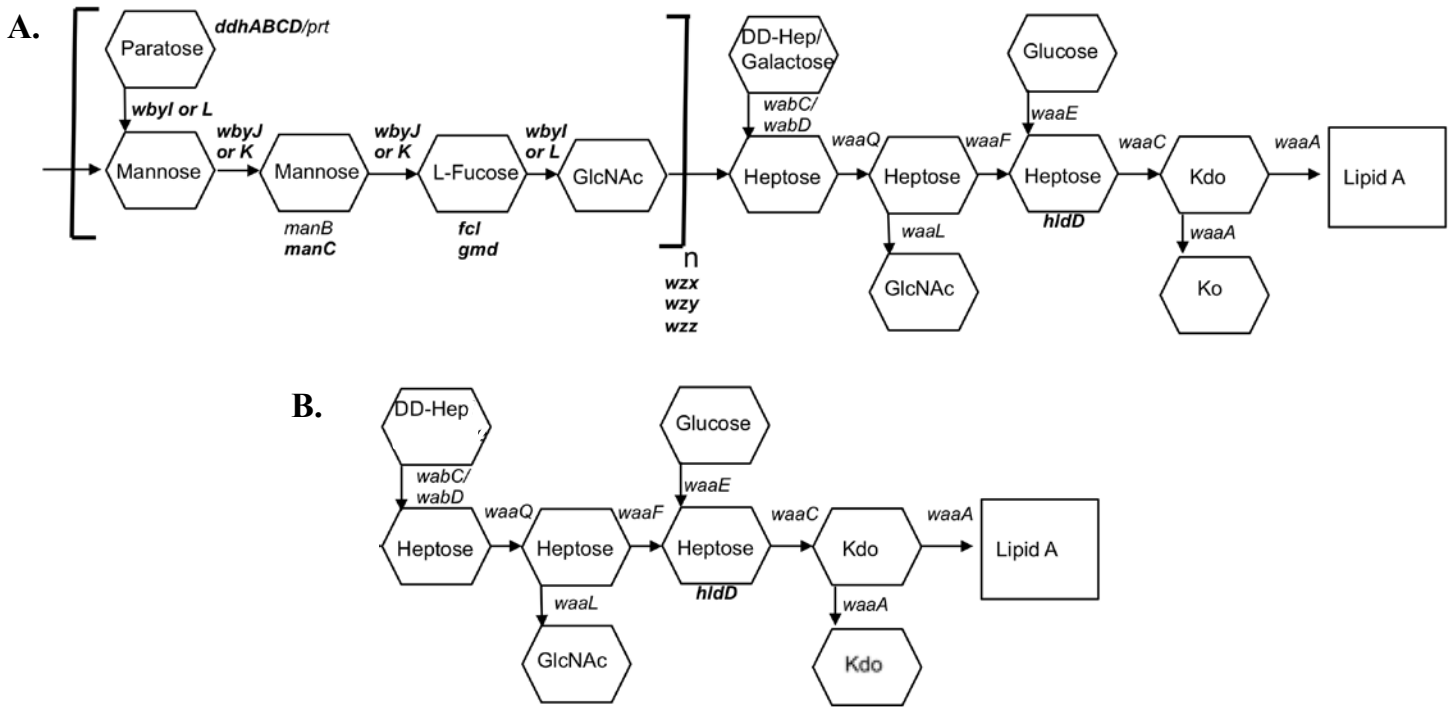


Figure 1. The lipopolysaccharide structure and temperature related changes in *Y. pseudotuberculosis* and *Y. pestis*. Genes that function in the synthesis or addition of different sugars are displayed. A. Putative *Yersinia pseudotuberculosis* serotype O:1b IP32953 lipopolysaccharide core and O-antigen grown at 21°C. Derived from elucidated structure of *Y. pestis* core. B. Putative *Yersinia pestis* KIM6+ lipopolysaccharide core grown at 37°C. Changes in structure due to temperature include two Kdo residue instead of a Ko residue at 37°C. The outer most residue is always a D-D Heptose, whereas at 21°C it could be either a D-D Heptose or a galactose. Kdo = 3-Deoxy-D-manno-oct-2-ulosonic acid; Ko = D-glycero-D-talo-oct-2-ulosonic acid; GlcNAc = N-Acetylglucosamine.

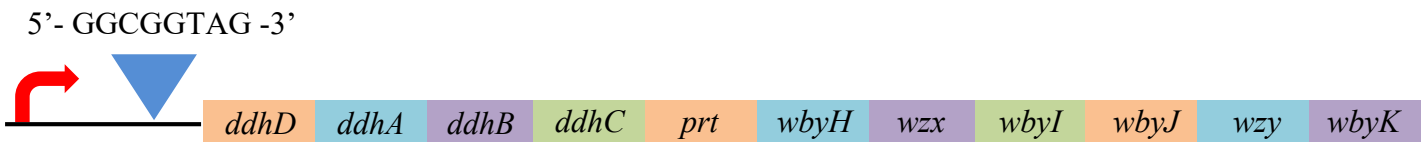
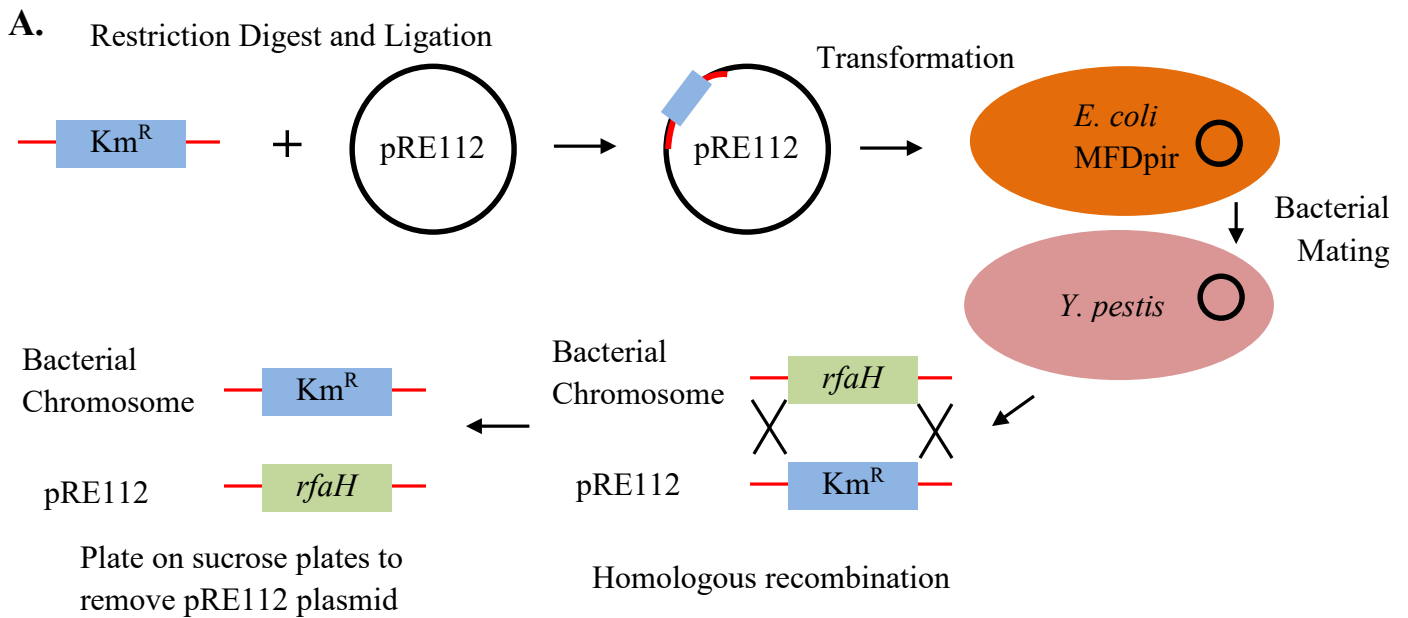
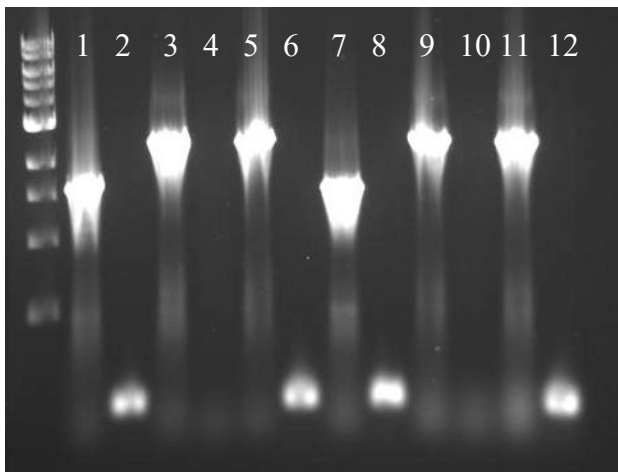


Figure 2. *ddhD* gene cluster, whose genes are annotated to function in O-antigen synthesis. Putative ops sequence indicated. Red arrow indicates promoter. There are more genes in this cluster than are indicated in this figure.



**B.**



wt  $\Delta rfaH$  *rfaH*<sup>+</sup> wt  $\Delta rfaH$  *rfaH*<sup>+</sup>  
*Y. pstb* *Y. pestis*

**C.**

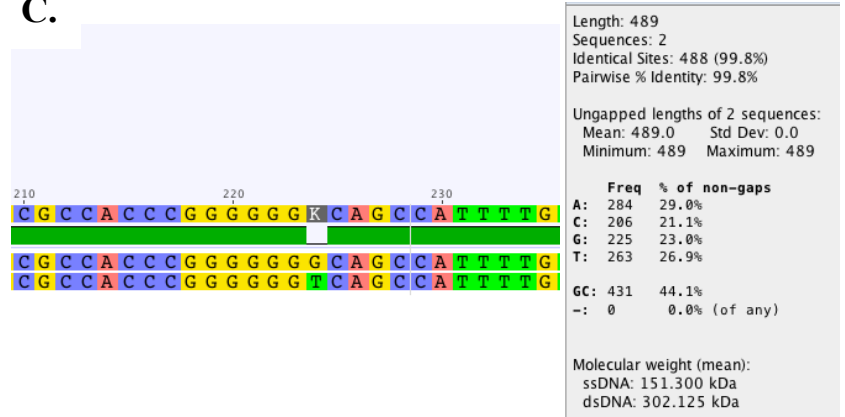


Figure 3. The *rfaH* gene is deleted from *Y. pseudotuberculosis* and *Y. pestis*. A. Gene deletion strategy utilizing suicide plasmid pRE112. Kanamycin resistance gene ( $Km^R$ ) with 500 bp upstream and downstream homologous regions of *rfaH* gene inserted into pRE112. pRE112 is transformed into *E. coli* MFDpir. The *E. coli* strain is mated with either *Y. pseudotuberculosis* or *Y. pestis*. Homologous recombination switches out *rfaH* gene from bacterial chromosome and the pRE112 plasmid is removed by plating on 10% sucrose plates. For details, see Materials and Methods. B. Evidence of successful *rfaH* gene deletion. Lanes 3, 5, 9, 11 are the *rfaH* 500 F & R primers which show increased product size in  $\Delta rfaH$  and *rfaH*<sup>+</sup> strains in both *Y. pstb* and *Y. pestis* as compared to the wild-type (Lanes 1 and 7) indicating the  $Km^R$  gene was inserted. Lanes 2, 6, 8, 12 are the *rfaH* internal primers which show *rfaH* gene present in wild-type and *rfaH*<sup>+</sup> complemented strains, but not  $\Delta rfaH$  (Lanes 4 and 10). C. Comparison between the *rfaH* gene in *Y. pseudotuberculosis* and *Y. pestis*. They share a 99.8% identify with only one nucleotide change.



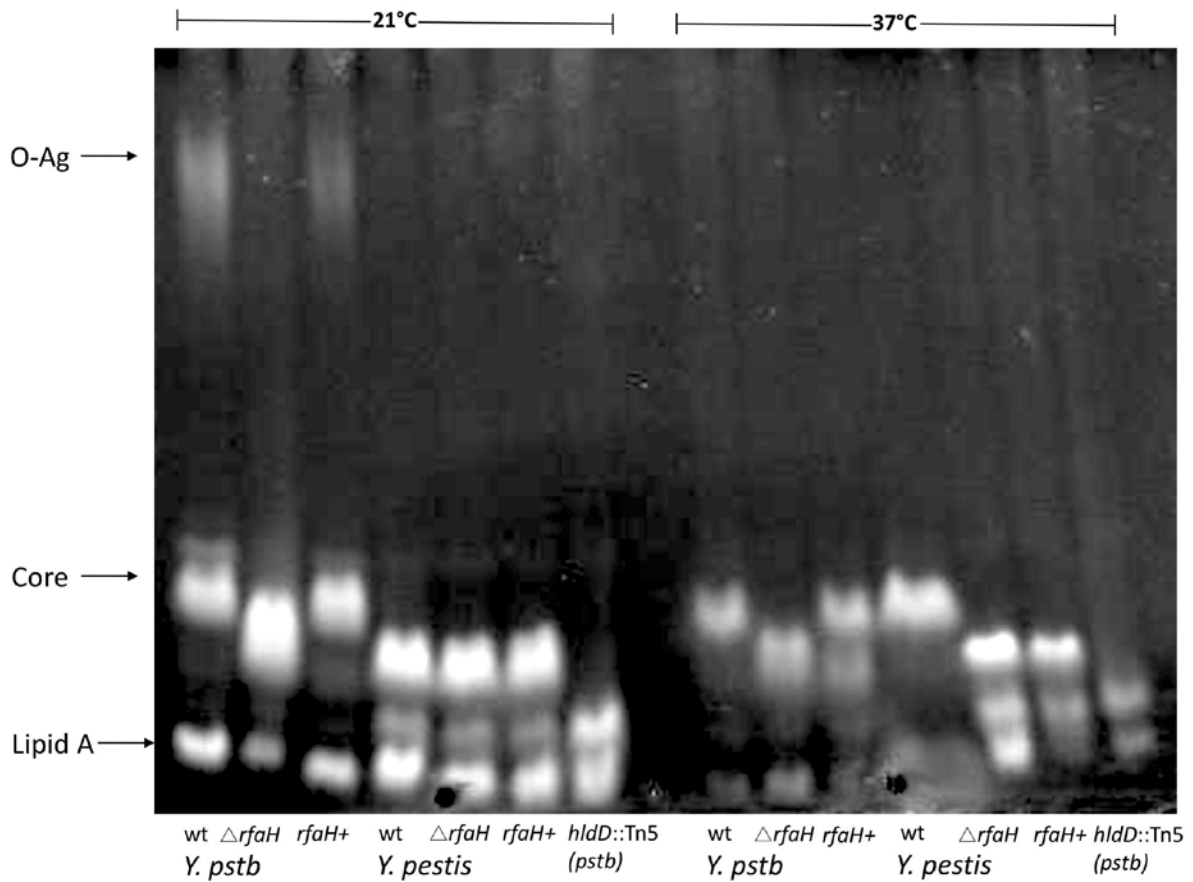


Figure 4. RfaH impacts lipopolysaccharide synthesis differently in *Y. pseudotuberculosis* and *Y. pestis*. *Y. pseudotuberculosis*  $\Delta rfaH$  displays a truncated core oligosaccharide structure at both 21°C and 37°C compared to the wild-type. The core of *Y. pestis*  $\Delta rfaH$  is truncated only at 37°C, as the core of the wild-type *Y. pestis* is smaller at 21°C. The complementation of the *rfaH* gene (*rfaH+*) appears successful in *Y. pseudotuberculosis* strains but not in *Y. pestis*. *hldD::Tn5* strain is shown as a positive control.

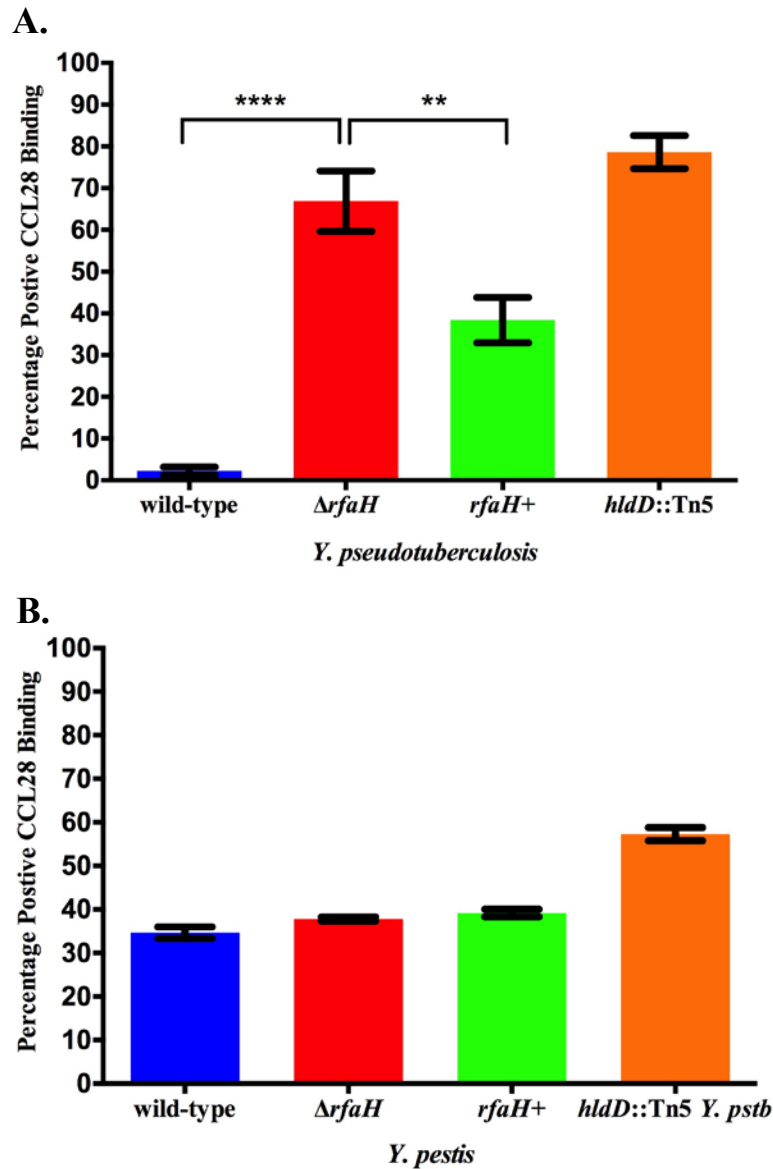


Figure 5. RfaH provides protection against CCL28 binding in *Y. pseudotuberculosis* but has no effect in *Y. pestis* at 37°C A. CCL28 binding with *Y. pseudotuberculosis* wild-type,  $\Delta rfaH$ , and complement strains (*rfaH*<sup>+</sup>). The ability of CCL28 to bind is greatly increased in the  $\Delta rfaH$  strain as compared to the wild-type. The complement *rfaH*<sup>+</sup> reduces the binding of CCL28. A positive control, *hldD*::Tn5, is shown which displays higher binding by CCL28. B. CCL28 binding with *Y. pestis* wild-type,  $\Delta rfaH$ , and complement (*rfaH*<sup>+</sup>) strains. There is no significant difference in binding between the wild-type,  $\Delta rfaH$ , and complement strains. \*\*\*\* = p-value < 0.0001. \*\* = p-value < 0.05. These are the results of a single experiment done in triplicate, but repeat experiments show similar trends.

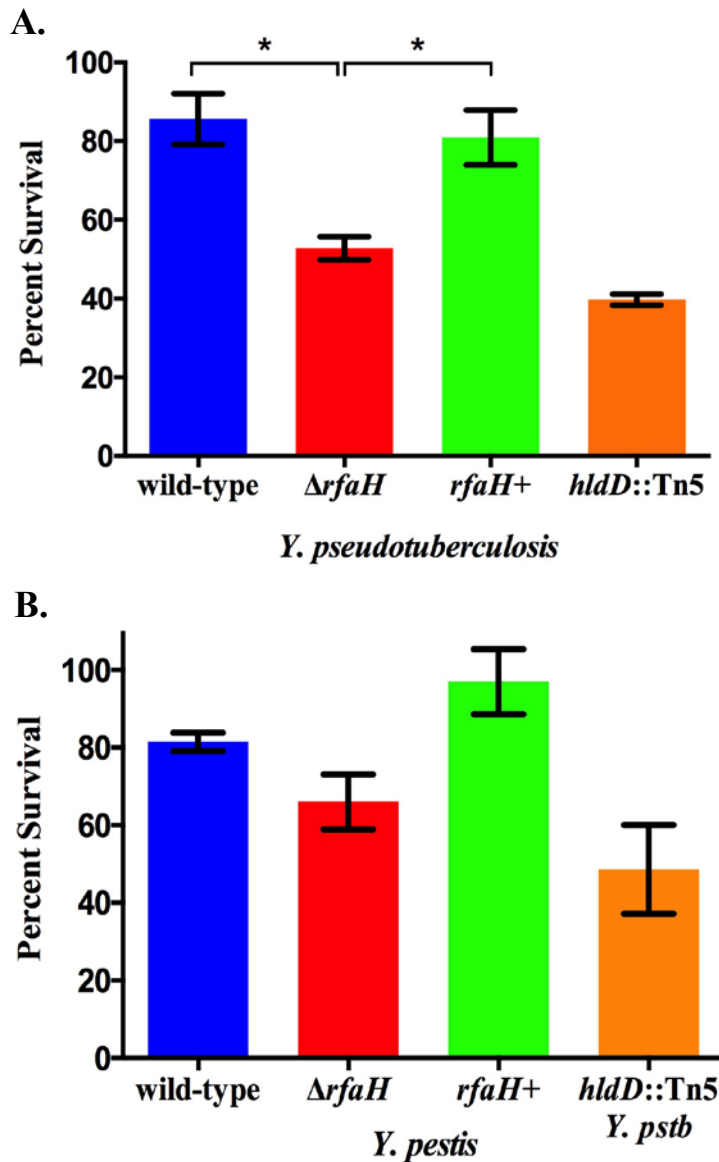


Figure 6. Loss of RfaH increases susceptibility of *Y. pseudotuberculosis* but not *Y. pestis* to killing by CCL28 at 37°C. A. Percent survival of *Y. pstb* wild-type,  $\Delta rfaH$ , and complement strains (*rfaH*<sup>+</sup>) after 2 hour incubation with CCL28. The ability of CCL28 to kill is significantly increased in the  $\Delta rfaH$  strain as compared to the wild-type. The complement *rfaH*<sup>+</sup> reduces the killing of CCL28, similar to wild-type levels. A positive control, *hldD*::Tn5, is shown which displays low percent survival when mixed with CCL28. B. Percent survival of *Y. pestis* wild-type,  $\Delta rfaH$ , and complement strains (*rfaH*<sup>+</sup>) after incubation with CCL28 for 2 hours. There are no significant differences in percent survival between the wild-type, *rfaH*<sup>-</sup>, and complement strains. \* = p-value <0.05. These are the results of a single experiment done in triplicate, but repeat experiments show similar trends.

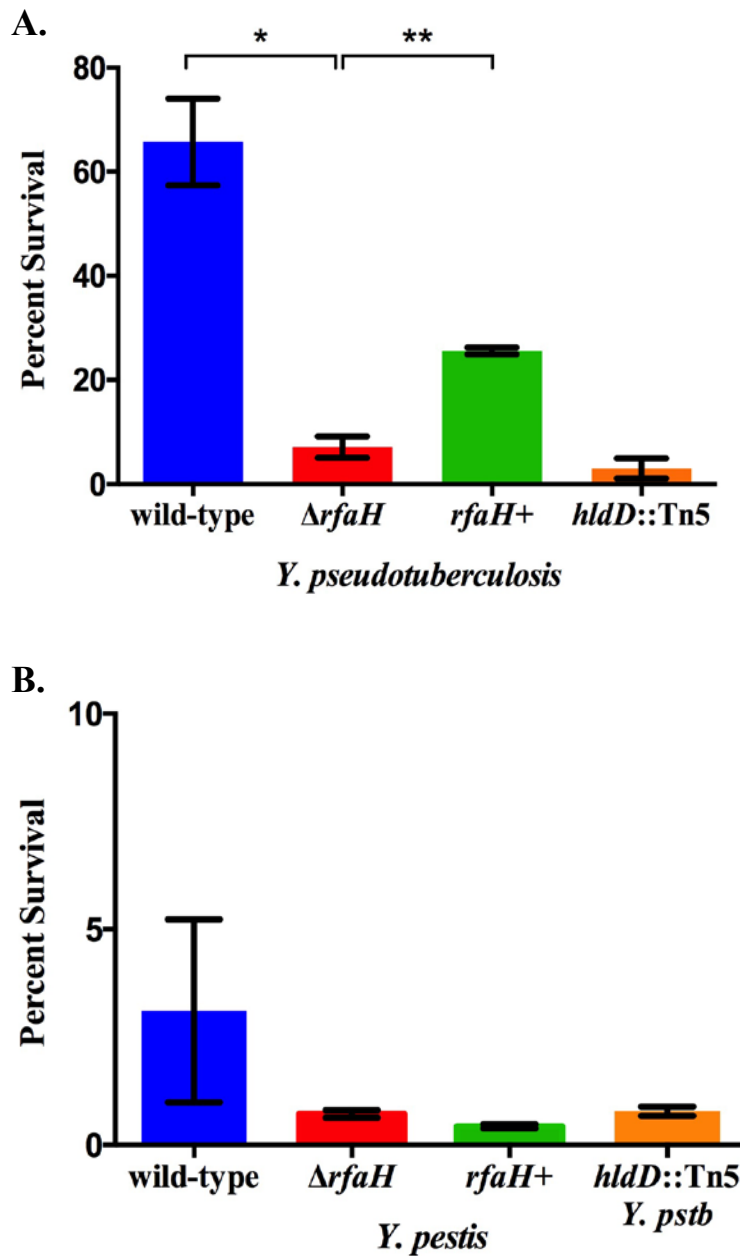


Figure 7. Loss of *rfaH* increases susceptibility to polymyxin B in *Y. pseudotuberculosis* but *Y. pestis* displays high sensitivity at 37°C A. Percent survival of *Y. pseudotuberculosis* wild-type,  $\Delta rfaH$ , and complement (*rfaH*<sup>+</sup>) strains after 2 hour incubation with polymyxin. The ability of polymyxin to kill is significantly increased in the  $\Delta rfaH$  strain as compared to the wild-type. The complement (*rfaH*<sup>+</sup>) significantly reduces the killing of polymyxin, but not quite to wild-type levels. A positive control, *hldD*::Tn5, is shown which displays low percent survival when mixed with polymyxin. B. Percent survival of *Y. pestis* wild-type,  $\Delta rfaH$ , and complement strains after incubation with polymyxin for 2 hours. There are no significant differences in percent survival between the wild-type,  $\Delta rfaH$ , and complement strains. \* = p-value < 0.05, \*\* = p-value < 0.005. These are the results of a single experiment done in triplicate, but repeat experiments show similar trends.

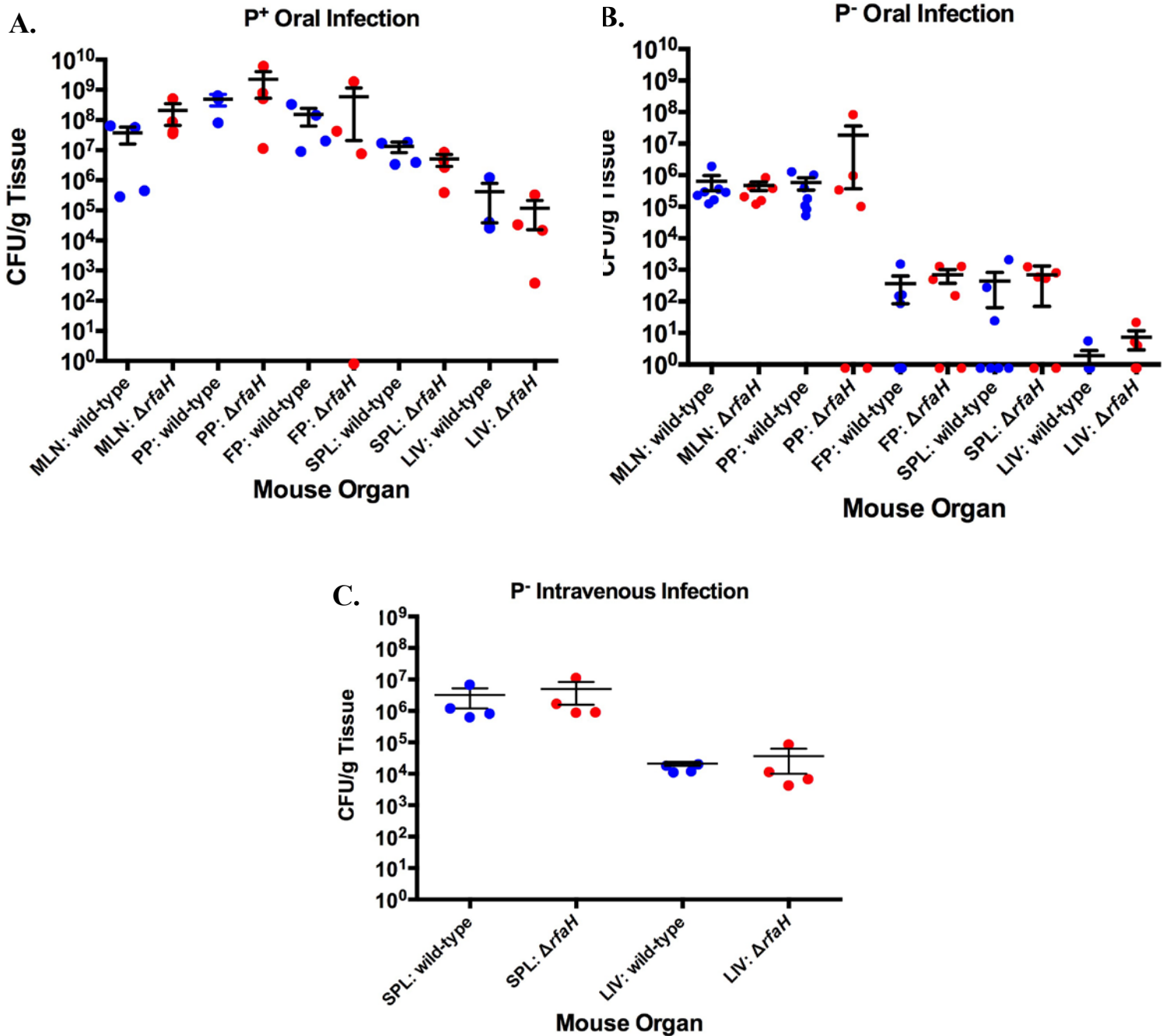


Figure 8. RfaH does not affect persistence of *Y. pseudotuberculosis* IP32953 in single strain *in vivo* mouse infection. A. Oral infection with *Y. pseudotuberculosis* P<sup>+</sup> wild-type and  $\Delta rfaH$ . All 5 organs examined showed no significant differences between the CFU/g tissue between wild-type and  $\Delta rfaH$  strains. Mice were infected with  $2 \times 10^8$  CFU. N = 4. B. Oral infection with *Y. pseudotuberculosis* P<sup>-</sup> wild-type and  $\Delta rfaH$ . All 5 organs examined showed no significant differences in CFU/g tissue between the wild-type and the  $\Delta rfaH$  strains. Mice were infected with  $2 \times 10^9$  CFU. N = 7. C. Intravenous infection with *Y. pseudotuberculosis* P<sup>-</sup> wild-type and  $\Delta rfaH$ . There was no significant difference between the CFU/g tissue in either organ examined. Mice were infected with  $1 \times 10^5$  CFU. N = 4. MLN = mesenteric lymph nodes, PP = Peyer's patches, FP = fecal pellets, SPL = spleen, and LIV = liver.

## REFERENCES

1. Perry, R.D. and J.D. Fetherston, *Yersinia pestis - Etiologic agent of plague*. Clinical Microbiology Reviews, 1997. **10**(1): p. 35-66.
2. Morelli, G., et al., *Yersinia pestis genome sequencing identifies patterns of global phylogenetic diversity*. Nature Genetics, 2010. **42**(12): p. 1140-1143.
3. Sun, Y.-C., et al., *Retracing the Evolutionary Path that Led to Flea-Borne Transmission of Yersinia pestis*. Cell Host & Microbe, 2014. **15**(5): p. 578-586.
4. Pechous, R.D., et al., *Pneumonic Plague: The Darker Side of Yersinia pestis*. Trends in Microbiology, 2016. **24**(3): p. 190-197.
5. Barnes, P.D., et al., *Yersinia pseudotuberculosis disseminates directly from a replicating bacterial pool in the intestine*. The Journal of Experimental Medicine, 2006. **203**(6): p. 1591-1601.
6. Amphlett, A., *Far East Scarlet-Like Fever: A Review of the Epidemiology, Symptomatology, and Role of Superantigenic Toxin: Yersinia pseudotuberculosis-Derived Mitogen A*. Open Forum Infectious Diseases, 2016. **3**(1).
7. Nuorti, J.P., et al., *A widespread outbreak of Yersinia pseudotuberculosis O : 3 infection from iceberg lettuce*. Journal of Infectious Diseases, 2004. **189**(5): p. 766-774.
8. Tseneva, G.Y., et al., *Pseudotuberculosis in the Russian Federation*, in *Advances in Yersinia Research*, A.M.P. DeAlmeida and N.C. Leal, Editors. 2012. p. 63-68.
9. Skurnik, M., A. Peippo, and E. Ervela, *Characterization of the O-antigen gene clusters of Yersinia pseudotuberculosis and the cryptic O-antigen gene cluster of Yersinia pestis shows that the plague bacillus is most closely related to and has evolved from Y. pseudotuberculosis serotype O:1b*. Mol Microbiol, 2000. **37**(2): p. 316-30.
10. Marra, A. and R.R. Isberg, *Invasin-dependent and invasin-independent pathways for translocation of Yersinia pseudotuberculosis across the Peyer's patch intestinal epithelium*. Infection and Immunity, 1997. **65**(8): p. 3412-3421.
11. Reboldi, A. and J.G. Cyster, *Peyer's patches: organizing B-cell responses at the intestinal frontier*. Immunological reviews, 2016. **271**(1): p. 230-45.
12. Heel, K.A., et al., *Peyer's patches*. Journal of Gastroenterology and Hepatology, 1997. **12**(2): p. 122-136.
13. Cornelis, G.R., et al., *The virulence plasmid of Yersinia, an antihost genome*. Microbiology and Molecular Biology Reviews, 1998. **62**(4): p. 1315-+.
14. Viboud, G.I. and J.B. Bliska, *Yersinia outer proteins: Role in modulation of host cell signaling responses and pathogenesis*, in *Annual Review of Microbiology*. 2005, Annual Reviews: Palo Alto. p. 69-89.
15. Viboud, G.I., et al., *Proinflammatory signalling stimulated by the type III translocation factor YopB is counteracted by multiple effectors in epithelial cells infected with Yersinia pseudotuberculosis*. Molecular Microbiology, 2003. **47**(5): p. 1305-1315.
16. Grosdent, N., et al., *Role of Yops and adhesins in resistance of Yersinia enterocolitica to phagocytosis*. Infection and Immunity, 2002. **70**(8): p. 4165-4176.
17. Logsdon, L.K. and J. Meccas, *Requirement of the Yersinia pseudotuberculosis effectors YopH and YopE in colonization and persistence in intestinal and lymph tissues*. Infection and Immunity, 2003. **71**(8): p. 4595-4607.
18. Crimmins, G.T., et al., *Identification of MrtAB, an ABC Transporter Specifically Required for Yersinia pseudotuberculosis to Colonize the Mesenteric Lymph Nodes*. Plos Pathogens, 2012. **8**(8).
19. Balada-Llasat, J.M. and J. Meccas, *Yersinia has a tropism for B and T cell zones of lymph nodes that is independent of the type III secretion system*. Plos Pathogens, 2006. **2**(9): p. 816-828.

20. Achtman, M., et al., *Yersinia pestis, the cause of plague, is a recently emerged clone of Yersinia pseudotuberculosis*. Proceedings of the National Academy of Sciences, 1999. **96**(24): p. 14043-14048.
21. Cui, Y., et al., *Historical variations in mutation rate in an epidemic pathogen, Yersinia pestis*. Proceedings of the National Academy of Sciences of the United States of America, 2013. **110**(2): p. 577-582.
22. Morelli, G., et al., *Yersinia pestis genome sequencing identifies patterns of global phylogenetic diversity*. Nature Genetics, 2010. **42**(12): p. 1140-1143.
23. Hinnebusch, B.J., et al., *Role of Yersinia murine toxin in survival of Yersinia pestis in the midgut of the flea vector*. Science, 2002. **296**(5568): p. 733-735.
24. Chain, P.S.G., et al., *Insights into the evolution of Yersinia pestis through whole-genome comparison with Yersinia pseudotuberculosis*. Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(38): p. 13826-13831.
25. Chouikha, I. and B.J. Hinnebusch, *Silencing urease: A key evolutionary step that facilitated the adaptation of Yersinia pestis to the flea-borne transmission route*. Proceedings of the National Academy of Sciences of the United States of America, 2014. **111**(52): p. 18709-18714.
26. Suomalainen, M., et al., *Temperature-Induced Changes in the Lipopolysaccharide of Yersinia pestis Affect Plasminogen Activation by the Pla Surface Protease*. Infection and Immunity, 2010. **78**(6): p. 2644-2652.
27. Sun, Y.-C., et al., *The Yersinia pestis Rcs Phosphorelay Inhibits Biofilm Formation by Repressing Transcription of the Diguanylate Cyclase Gene hmsT*. Journal of Bacteriology, 2012. **194**(8): p. 2020-2026.
28. Knirel, Y.A. and A.P. Anisimov, *Lipopolysaccharide of Yersinia pestis, the Cause of Plague: Structure, Genetics, Biological Properties*. Acta Naturae, 2012. **4**(3): p. 46-58.
29. Raetz, C.R.H., et al., *Lipid a modification systems in gram-negative bacteria*, in *Annual Review of Biochemistry*. 2007, Annual Reviews: Palo Alto. p. 295-329.
30. Bruneteau, M. and S. Minka, *Lipopolysaccharides of bacterial pathogens from the genus Yersinia: a mini-review*. Biochimie, 2003. **85**(1-2): p. 145-152.
31. Rebeil, R., et al., *Variation in lipid A structure in the pathogenic yersiniae*. Molecular Microbiology, 2004. **52**(5): p. 1363-1373.
32. De Castro, C., et al., *Genetic characterisation and structural analysis of the O-specific polysaccharide of Yersinia pseudotuberculosis serotype O:1c*. Innate Immunity, 2011. **17**(2): p. 183-190.
33. Qingke, K., et al., *Effect of deletion of genes involved in lipopolysaccharide core and O-antigen synthesis on virulence and immunogenicity of Salmonella enterica serovar Typhimurium*. Infection and Immunity, 2011. **79**(10): p. 4227-4239.
34. Loutet, S.A., et al., *A complete lipopolysaccharide inner core oligosaccharide is required for resistance of Burkholderia cenocepacia to antimicrobial peptides and bacterial survival in vivo*. Journal of Bacteriology, 2006. **188**(6): p. 2073-2080.
35. Anderson, M.S., C.E. Bulawa, and C.R.H. Raetz, *The Biosynthesis of Gram-Negative Endotoxin - Formation of Lipid A Precursors from UDP-GLCNAC in Extracts of Escherichia coli*. Journal of Biological Chemistry, 1985. **260**(29): p. 5536-5541.
36. Belunis, C.J. and C.R.H. Raetz, *Biosynthesis of Endotoxins - Purification and Catalytic Properties of 3-Deoxy-D-Manno-Octulosonic Transferase From Escherichia coli*. Journal of Biological Chemistry, 1992. **267**(14): p. 9988-9997.
37. Whitfield, C. and M.S. Trent, *Biosynthesis and Export of Bacterial Lipopolysaccharides*, in *Annual Review of Biochemistry, Vol 83*, R.D. Kornberg, Editor. 2014, Annual Reviews: Palo Alto. p. 99-128.
38. Gronow, S. and H. Brade, *Lipopolysaccharide biosynthesis: which steps do bacteria need to survive?* Journal of Endotoxin Research, 2001. **7**(1): p. 3-23.

39. Doerrler, W.T., H.S. Gibbons, and C.R.H. Raetz, *MsbA-dependent translocation of lipids across the inner membrane of Escherichia coli*. Journal of Biological Chemistry, 2004. **279**(43): p. 45102-45109.
40. Samuel, G. and P. Reeves, *Biosynthesis of O-antigens: genes and pathways involved in nucleotide sugar precursor synthesis and O-antigen assembly*. Carbohydrate Research, 2003. **338**(23): p. 2503-2519.
41. Skurnik, M., *Molecular genetics, biochemistry and biological role of Yersinia lipopolysaccharide*, in *Genus Yersinia: Entering the Functional Genomic Era*, M. Skurnik, J.A. Bengoechea, and K. Granfors, Editors. 2003, Kluwer Academic/Plenum Publ: New York. p. 187-197.
42. Ruiz, N., D. Kahne, and T.J. Silhavy, *TIMELINE Transport of lipopolysaccharide across the cell envelope: the long road of discovery*. Nature Reviews Microbiology, 2009. **7**(9): p. 677-683.
43. Simpson, B.W., et al., *Lipopolysaccharide transport to the cell surface: biosynthesis and extraction from the inner membrane*. Philosophical Transactions of the Royal Society B-Biological Sciences, 2015. **370**(1679).
44. May, J.M., et al., *Lipopolysaccharide transport to the cell surface: periplasmic transport and assembly into the outer membrane*. Philosophical Transactions of the Royal Society B-Biological Sciences, 2015. **370**(1679).
45. Pasupuleti, M., A. Schmidtchen, and M. Malmsten, *Antimicrobial peptides: key components of the innate immune system*. Critical Reviews in Biotechnology, 2012. **32**(2): p. 143-171.
46. Malmsten, M., *Antimicrobial peptides*. Upsala Journal of Medical Sciences, 2014. **119**(2): p. 199-204.
47. Ganz, T., *Defensins: Antimicrobial peptides of innate immunity*. Nature Reviews Immunology, 2003. **3**(9): p. 710-720.
48. Cole, A.M., et al., *Cutting edge: IFN-inducible ELR- CXC chemokines display defensin-like antimicrobial activity*. Journal of Immunology, 2001. **167**(2): p. 623-627.
49. Wang, W., et al., *Identification of a novel chemokine (CCL28), which binds CCR10 (GPR2)*. Journal of Biological Chemistry, 2000. **275**(29): p. 22313-22323.
50. Hieshima, K., et al., *CCL28 has dual roles in mucosal immunity as a chemokine with broad-spectrum antimicrobial activity*. J Immunol, 2003. **170**(3): p. 1452-61.
51. Pallister, K.B., et al., *Bovine CCL28 Mediates Chemotaxis via CCR10 and Demonstrates Direct Antimicrobial Activity against Mastitis Causing Bacteria*. Plos One, 2015. **10**(9).
52. Liu, B. and E. Wilson, *The antimicrobial activity of CCL28 is dependent on C-terminal positively-charged amino acids*. European Journal of Immunology, 2010. **40**(1): p. 186-196.
53. Johnson, L.E., *The pmrHF1JKLM Operon in Yersinia pseudotuberculosis Enhances Resistance to CCL28 and Promotes Phagocytic Engulfment by Neutrophils*. Brigham Young University, 2016.
54. Lew, C.S., *Loss of the Lipopolysaccharide Core Biosynthesis rfaD Gene Increases Antimicrobial Chemokine Binding and Bacterial Susceptibility to CCL28 and Polymyxin: A Model for Understanding the Interface of Antimicrobial Chemokines and Bacterial Host Defense Avoidance Mechanisms*. Brigham Young University, 2012.
55. Erickson, D.L., et al., *Lipopolysaccharide biosynthesis genes of Yersinia pseudotuberculosis promote resistance to antimicrobial chemokines*. PLoS One, 2016. **11**(6): p. e0157092.
56. Leskinen, K., et al., *Expression of the Yersinia enterocolitica O:3 LPS O-antigen and outer core gene clusters is RfaH-dependent*. Microbiology-Sgm, 2015. **161**: p. 1282-1294.
57. Bailey, M.J.A., C. Hughes, and V. Koronakis, *Increased distal gene transcription by the elongation factor RfaH, a specialized homologue of NusG*. Molecular Microbiology, 1996. **22**(4): p. 729-737.
58. Yakhnin, A.V., K.S. Murakami, and P. Babitzke, *NusG Is a Sequence-specific RNA Polymerase Pause Factor That Binds to the Non-template DNA within the Paused Transcription Bubble*. Journal of Biological Chemistry, 2016. **291**(10): p. 5299-5308.



59. Artsimovitch, I. and R. Landick, *Pausing by bacterial RNA polymerase is mediated by mechanistically distinct classes of signals*. Proceedings of the National Academy of Sciences of the United States of America, 2000. **97**(13): p. 7090-7095.
60. Wilkinson, R.G. and B.A.D. Stocker, *Genetics and Cultural Properties of Mutants of Salmonella Typhimurium Lacking Glucosyl or Galactosyl Lipopolysaccharide Transferases*. Nature, 1968. **217**(5132): p. 955-957.
61. Beutin, L. and M. Achtman, *Two Escherichia coli chromosomal cistrons, sfrA and sfrB, which are needed for expression of F factor tra functions*. Journal of Bacteriology, 1979. **139**(3): p. 730-737.
62. Bailey, M.J.A., et al., *Escherichia coli HlyT protein, a transcriptional activator of haemolysin synthesis and secretion, is encoded by the rfaH (sfrB) locus required for expression of sex factor and lipopolysaccharide genes*. Molecular Microbiology, 1992. **6**(8): p. 1003-1012.
63. Stevens, M.P., et al., *Regulation of Escherichia-coli K5 Capsular Polysaccharide Expression - Evidence for Involvement of RfaH in the Expression of Group-II Capsules*. Fems Microbiology Letters, 1994. **124**(1): p. 93-98.
64. Beutin, L., et al., *SfrA and SfrB Products of Escherichia-coli K-12 are Transcriptional Control Factors*. Journal of Bacteriology, 1981. **145**(2): p. 840-844.
65. Vicari, D. and I. Artsimovitch, *Virulence regulators RfaH and YaeQ do not operate in the same pathway*. Molecular Genetics and Genomics, 2004. **272**(5): p. 489-496.
66. Svetlov, V., et al., *Allosteric control of the RNA polymerase by the elongation factor RfaH*. Nucleic Acids Res, 2007. **35**(17): p. 5694-705.
67. Carter, H.D., V. Svetlov, and I. Artsimovitch, *Highly divergent RfaH orthologs from pathogenic proteobacteria can substitute for Escherichia coli RfaH both in vivo and in vitro*. Journal of Bacteriology, 2004. **186**(9): p. 2829-2840.
68. McGary, K. and E. Nudler, *RNA polymerase and the ribosome: the close relationship*. Current Opinion in Microbiology, 2013. **16**(2): p. 112-117.
69. Vassilyeva, M.N., et al., *Crystallization and preliminary crystallographic analysis of the transcriptional regulator RfaH from Escherichia coli and its complex with ops DNA*. Acta Crystallographica Section F-Structural Biology and Crystallization Communications, 2006. **62**: p. 1027-1030.
70. Santangelo, T.J. and J.W. Roberts, *RfaH, a bacterial transcription antiterminator*. Molecular Cell, 2002. **9**(4): p. 698-700.
71. Artsimovitch, I. and R. Landick, *The transcriptional regulator RfaH stimulates RNA chain synthesis after recruitment to elongation complexes by the exposed nontemplate DNA strand*. Cell, 2002. **109**(2): p. 193-203.
72. Sevostyanova, A., et al., *The elongation factor RfaH and the initiation factor or bind to the same site on the transcription elongation complex*. Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(3): p. 865-870.
73. Nagy, G., et al., *Expression of hemin receptor molecule ChuA is influenced by RfaH in uropathogenic Escherichia coli strain 536*. Infection and Immunity, 2001. **69**(3): p. 1924-1928.
74. Nagy, G., et al., *Loss of regulatory protein RfaH attenuates virulence of uropathogenic Escherichia coli*. Infection and Immunity, 2002. **70**(8): p. 4406-4413.
75. Nagy, G., et al., *Transcriptional regulation through RfaH contributes to intestinal colonization by Escherichia coli*. Fems Microbiology Letters, 2005. **244**(1): p. 173-180.
76. Nagy, G., et al., *Down-regulation of key virulence factors makes the Salmonella enterica serovar Typhimurium rfaH mutant a promising live-attenuated vaccine candidate*. Infection and Immunity, 2006. **74**(10): p. 5914-5925.
77. Nagy, G., et al., *Oral immunization with an RfaH mutant elicits protection against salmonellosis in mice*. Infection and Immunity, 2004. **72**(7): p. 4297-4301.
78. Edwards, R.A., L.H. Keller, and D.M. Schifferli, *Improved allelic exchange vectors and their use to analyze 987P fimbria gene expression*. Gene, 1998. **207**(2): p. 149-157.

79. Ferrieres, L., et al., *Silent Mischief: Bacteriophage Mu Insertions Contaminate Products of Escherichia coli Random Mutagenesis Performed Using Suicidal Transposon Delivery Plasmids Mobilized by Broad- Host- Range RP4 Conjugative Machinery*. Journal of Bacteriology, 2010. **192**(24): p. 6418-6427.
80. Chang, A.C.Y. and S.N. Cohen, *Construction and Characterization of Amplifiable Multicopy DNA Cloning Vehicles Derived From P15A Cryptic Miniplasmid*. Journal of Bacteriology, 1978. **134**(3): p. 1141-1156.
81. Chauvaux, S., et al., *Transcriptome analysis of Yersinia pestis in human plasma: an approach for discovering bacterial genes involved in septicemic plague*. Microbiology-Sgm, 2007. **153**: p. 3112-3123.
82. Livak, K.J. and T.D. Schmittgen, *Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2- $\Delta\Delta$ CT Method*. Methods, 2001. **25**(4): p. 402-408.
83. McKenzie, G.J. and N.L. Craig, *Fast, easy and efficient: site-specific insertion of transgenes into Enterobacterial chromosomes using Tn7 without need for selection of the insertion event*. BMC Microbiology, 2006. **6**.
84. Knirel, Y.A., et al., *Temperature-dependent variations and intraspecies diversity of the structure of the lipopolysaccharide of Yersinia pestis*. Biochemistry, 2005. **44**(5): p. 1731-1743.
85. Meccas, J., I. Bilis, and S. Falkow, *Identification of attenuated Yersinia pseudotuberculosis strains and characterization of an orogastric infection in BALB/c mice on day 5 postinfection by signature-tagged mutagenesis*. Infection and Immunity, 2001. **69**(5): p. 2779-2787.
86. Anisimov, A.P., L.E. Lindler, and G.B. Pier, *Intraspecific diversity of Yersinia pestis*. Clinical Microbiology Reviews, 2004. **17**(3): p. 695-695.
87. Knirel, Y.A., et al., *New Features of Yersinia Lipopolysaccharide Structures as Revealed by High-Resolution Electrospray Ionization Mass Spectrometry*. Advanced Science Letters, 2008. **1**(2): p. 192-198.
88. Bailey, M.J.A., C. Hughes, and V. Koronakis, *RfaH and the ops element, components of a novel system controlling bacterial transcription elongation*. Molecular Microbiology, 1997. **26**(5): p. 845-851.
89. Svetlov, V. and E. Nudler, *Unfolding the Bridge between Transcription and Translation*. Cell, 2012. **150**(2): p. 243-245.