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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

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ABSTRACT

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In order to better understand the mechanism of antimicrobial chemokine activity, including binding to and killing of bacteria, random transposon mutagenesis was performed in *Yersinia pseudotuberculosis*. Resulting mutants were screened for increased binding to chemokine and high binding clones were selected for further study. One mutant, designated mutant 27, was found to have a single insertion mutation in the *rfaD* gene. The *rfaD* gene product is involved in heptose biosynthesis, one of the sugars of the inner core oligosaccharide of Gram- negative lipopolysaccharide (LPS). Mutant 27 was found to bind both CCL25 and CCL28, two antimicrobial chemokines, more efficiently than the wild type bacteria. This clone was also found to be more susceptible to CCL28- mediated killing and polymyxin activity. Complementation with a plasmid bearing the full *rfaDFC* operon restored the wild type phenotype in both regards. These data suggest that normal LPS expression by *Y. pseudotuberculosis* serves to protect the bacteria from the antimicrobial function of chemokines and other antimicrobial proteins of the mammalian innate immune system.

Keywords: antimicrobial chemokines, *Y. pseudotuberculosis*, *rfaD*

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INTRODUCTION

1. Immune system

The human immune system is divided into two separate but closely interacting branches: natural, or innate immunity, and adaptive, or acquired immunity. The innate immune response is uniform throughout a whole species and similar elements or components are conserved even among vastly different species (e.g. leucine rich receptors (LRRs) in humans and flies) or kingdoms (e.g. RNA interference in plants and animals). Conversely, adaptive immunity seems to be characteristic to vertebrates only [1-3].

1.1 Innate Immunity

The innate immune response is faster reacting than the adaptive response but is less specific and relies primarily on physical, chemical, and cellular barriers. Physical barriers include the skin, the mucosal membranes of internal epithelia and the movement of cilia in the lungs. Other important components are soluble factors, such as proteins with antimicrobial activity (called antimicrobial peptides or, alternately, host defense peptides). Examples of these include lysozyme or defensins. Chemical barriers include stomach acid or bile salts. Leukocytes involved in the innate immune response are phagocytic cells, including blood monocytes, neutrophils and macrophages. Granulocytic neutrophils are generally the first cells at the site of infection and release a

variety of antimicrobial agents, many of which are antimicrobial peptides [4, 5]. Activated macrophages have increased phagocytic abilities and also secrete inflammatory mediators.

The innate immune system responds directly to pathogen invasion [6] using a variety of soluble factors and receptors. Due to the ancient roots and conservation of innate immune mechanisms in the animal kingdom, it is anticipated that the signals recognized would also be conserved by pathogens [7]. These conserved features are known as pathogen- associated molecular patterns (PAMPs). For example, the complement cascade can be triggered by recognition of bacterial or viral PAMPs including lipopolysaccharide (LPS), peptidoglycan, or viral protein coats. Complement activation leads to bacterial death via the formation of pores in the membrane (via the membrane attack complex). In addition to complement, pattern recognition receptors (PRRs), such as Toll- like receptors (TLRs) or Nod proteins recognize specific microbial ligands and initiate a signal transduction pathway that leads to cytokine production and other immune responses. These ligands may include bacterial LPS, peptidoglycan, dsRNA, ssDNA, and CpG unmethylated dinucleotide repeats, or other danger signals (sometimes called danger- associated molecular patterns (DAMPs)).

Both soluble factors and receptors play an important role in the actual innate immune response and in activating the adaptive immune response. Responses through

TLRs can increase cytokine production and lead to inflammation. Inflammation is important in pathogen clearance and in the activation of healing and rebuilding processes. Inflammation helps localize immune cells to the site of infection. Classical signs of inflammation are localized increases in redness, heat, swelling, pain, and loss of function. Complement connects the innate and adaptive immune responses through the complement fragments remaining after enzymatic cleavage (e.g. C3a or C5a). These are known as anaphylatoxins and can induce degranulation of mast cells and basophils as well as contribute to inflammation by increasing vascular permeability and inducing smooth muscle contraction [8]. Anaphylatoxins also act as chemoattractant signals [9] to further recruit immune cells, including helper T cells, an essential component of the adaptive immune response, helping contribute to the inflammatory response.

1.2 Adaptive Immunity

The adaptive immune response takes time to generate but the resulting response is specific and provides memory. The adaptive immune system responds in an antigen specific way to a wide variety of microbes, even those not previously encountered. Because of this specificity, it takes longer for the antigen to be found by its ideal (strongest) binding partner and for the proper response to develop. However, because of the memory capabilities of the adaptive immune response, the secondary response—the response developed after re- exposure to the same antigen—is much faster than the

primary response. The adaptive immune response includes the humoral response and the cell- mediated response.

The humoral response consists of antibodies that specifically recognize a particular antigen. Upon recognition of its antigen, the B cell expressing that antibody will clonally expand, producing both plasma cells, which continue to make antibodies with the same specificity, and memory cells, which retain their specificity but are not actively producing antibody. Upon re- exposure to the same antigen, these memory cells can be activated much quicker and produce the specific antibodies. In order to become fully active, B cells need CD4 T helper cells, which release cytokines that stimulate proliferation and promote class switching [10]. These are usually Th2 cells. The Th1 subset of CD4 T cells stimulates the cell- mediated response.

The effector cells of the cell- mediated adaptive immune response are cytotoxic T lymphocytes (CTLs). CTLs have T- cell receptors (TCRs) that recognize antigen presented by MHC class I molecules. Similar to B cells, upon specific binding CTLs will undergo clonal expansion, resulting in CTLs with the same antigen- specific TCR. CTLs kill by releasing perforins, which form holes in the microbial membrane [11] and granzymes, serine proteases that induce apoptosis [12]. They can also induce apoptosis via the Fas- FasL pathway. A subset of CTLs will also become memory cells that can be reactivated upon secondary exposure to antigen.

1.3 Cytokines and Cytokine Signaling

The innate and adaptive branches are connected through small signal molecules, known as cytokines. Immune cells of both branches produce and secrete cytokines in response to recognition of conserved sequences on pathogens by innate immune cells or antigen recognition by B or T cells.

Most cytokines can be grouped into one of six families based on structure and activity, and each family has a specific receptor type that recognizes it. Cytokines known as interleukins are responsible for cell- to- cell cytokine signaling. Cytokine signaling takes place in either an autocrine or paracrine fashion, allowing infected cells to upregulate their own defenses as well as warn neighboring cells. These cytokines may have pro- or anti- inflammatory effects, help in B and T cell activation, induce antibody class switching, or activate macrophages, eosinophils and mast cells. Binding of a cytokine to its reciprocal receptor triggers an intracellular signaling cascade that leads to activation or regulation of transcription. Another major subset of cytokines, a group of proteins called chemokines, is involved specifically in cell migration in a process known as chemotaxis.

2. Chemokines

Chemokines are cytokines that mediate chemotaxis, or cellular movement towards or away from a certain stimulus or signal. They are a superfamily of 8- 16kDa

proteins that share 20- 70% homology in their amino acid sequences. As signal molecules, chemokines have at least two different functions, often classified accordingly as homeostatic or inflammatory. Homeostatic chemokines are constitutively expressed and direct cells into and through lymphoid tissues and organs [13, 14], including bone marrow, spleen, thymus and lymph nodes. They also direct cells to mucosal tissues and the skin—surfaces that have contact with the outside environment [15]. These homeostatic chemokines have been found in blood, lymph nodes, sweat, saliva, milk, and tears (exocrine secretions) [15]. The inflammatory chemokines are inducible chemokines that attract immune cells to the site of infection. These chemokines are upregulated in response to immune stimuli, such as viral or bacterial infection [16]. The chemokines and receptors expressed by various cells affect the types of cells attracted to a specific site.

Chemokines are divided into the following four families, based on their amino acid sequence (specifically the spacing between the first two cysteine residues) and the immune cells they attract: CXC, CC, C and CXXXC [17]. Chemokines of the CXC and CC families are most common, but there are two examples of C chemokines and one example of CXXXC chemokines known. Almost all chemokines have four conserved cysteine residues (except for the C group) whose disulfide bonds help stabilize the Greek key structure common to the family (four adjacent anti- parallel β sheets

connected by two small hairpins and one longer loop) [18-21]. Chemokine nomenclature was standardized by Zlotnik and Yoshie in 2000 [16].

Chemokines have been shown to oligomerize, forming mostly homodimers. However, whether or not chemokines act as dimers is still uncertain. Chemokines can bind as monomers and it has been shown that the concentrations required for dimerization are lower than the concentrations found *in vivo* for optimal chemokine activity [21]. Additionally, even if dimerization does occur, it may not be essential for receptor binding and activation, as a mutant of monocyte chemoattractant protein- 1 (MCP- 1) without the ability to dimerize still showed wild type binding affinity and stimulatory activity [22].

Chemokine gradients are sensed by cell receptors and guide immune cells to the specific site of infection. Many of the cleavage products in the complement cascade (anaphylatoxins) also have chemotactic properties (C4a, C3a and C5a) [23]. Immune cells migrate up the concentration gradient, meaning they move towards the source of chemokine production as the concentration of chemokine increases. Different classes of human leukocytes have different chemokine receptor expression profiles.

2.1 Chemokine receptors

Chemokine receptors are seven transmembrane G- protein coupled receptors located on cell surfaces [24]. There are at least 18 chemokine receptors known and they

can be divided into classes that correspond to the class of chemokine that binds them [25]. Chemokine receptor- ligand binding is both specific and redundant, meaning one receptor can recognize multiple chemokines and that chemokines can have multiple cognate receptors. Binding of a chemokine to its receptor initiates a calcium dependent signal transduction pathway within the cell leading to upregulation of actin polymerization, adhesion, and cytoskeletal rearrangement—all modifications that are involved in chemotaxis.

3. Antimicrobial peptides

Antimicrobial peptides are important components of the innate immune response. They have an overall positive net charge, which is thought to allow them to interact, through electrostatic attraction, with the usually negatively- charged bacterial membranes (due to the lipid A of Gram- negative bacteria or the teichoic acids of Gram-positive bacteria) [26]. Of the more than 800 antimicrobial peptides found thus far, most are fairly small in size [27, 28]. Antimicrobial peptides are ancient mechanisms of defense and have a broad range of activity. Various antimicrobial peptides have been shown to have activity against both Gram- negative and Gram- positive bacteria, acid fast bacteria, fungi, parasites and even some viruses (for examples see [29-34]). As components of the innate immune system, antimicrobial peptides are a well conserved defense mechanism and have been found in numerous organisms of different kingdoms

and species, including humans, fish, plants, insects and others [35-38]. Antimicrobial peptides are rapidly expressed at the site of infection or damage. They can either be synthesized *de novo* or deposited at the site by some cell, usually an immune cell such as a neutrophil [39]. While not normally expressed at high levels, antimicrobial peptides can be upregulated or induced leading to an increased concentration at the site of an infection.

3.1 Mechanism and activity

There are many proposed mechanisms for antimicrobial peptide activity, many of which include membrane permeabilization via the formation of a pore, leading to cell death. Antimicrobial peptides may also have intracellular effects leading to cell death, such as binding to DNA, inhibition of transcription, translation and protein synthesis, inhibition of enzyme activity, inhibition of cell wall formation, alteration of cytoplasmic membrane, or activation of autolysin or other non- membrane external targets [26].

There are three main proposed pore forming mechanisms—the carpet model, the barrel- stave model, and the toroidal pore model. The carpet model entails the accumulation of peptides on the cell membrane. Cationic peptides are electrostatically attracted to the negative charges on the bacterial membrane and align parallel to the membrane [40]. Hydrophobic interactions are also important in antimicrobial peptide

binding [41]. When a certain threshold concentration is reached, it allows the peptide to disrupt the membrane in a detergent- like fashion [42-44].

Another pore- forming model is the barrel- stave model. In this model, antimicrobial peptides insert into the membrane, forming a pore lined with the peptide [45]. A similar mechanism is the toroidal- pore model. In this model, the membrane bends continuously so that the polar head groups of the lipids are continuously in contact with the α- helix of the antimicrobial peptide [46].

Some antimicrobial peptides have been shown to work together in a synergistic manner [47, 48]. In the presence or absence of other antimicrobial peptides, antimicrobial activity was impacted. In many cases, activity was increased more than expected when used with another antimicrobial peptide. This may have implications in the mechanism of different antimicrobial peptides as well as the conditions required for optimal activity.

3.2 Defensins

There are two well- studied groups of antimicrobial peptides in mammals—the defensins and the cathelicidins [49-52]. Studies of these two groups of antimicrobial peptides have led to increased understanding of the conditions necessary for antimicrobial activity, characteristics of antimicrobial peptides, and possible mechanisms of action.

There are two main classes of defensins in humans, α- and β- defensins. Both are composed of β- sheets linked by disulfide bonds between the conserved cysteine residues. The pattern of disulfide bridges determines whether a defensin is an α- defensin or a β- defensin. There are six known α- defensins and four known β- defensins [26]. α- defensins are primarily found in granules released by neutrophils or by the Paneth cells of the small intestine [53] and are, on average, slightly shorter than β- defensins. β- defensins are secreted in mucosal tissues, including in the eye, skin, oral mucosa, and urogenital and respiratory systems [35]. There is also a circular form of defensin, which is known as a θ- defensin, identified first in rhesus macaques and found only in non- human primates [54, 55]. Defensins are highly soluble in water [35] and are released upon tissue damage or injury [39]. The exact mechanism of defensin killing is still unknown but it most likely involves oligomerization [56, 57] and electrostatic attraction between the cationic peptide and the negatively charged bacterial membrane [35], possibly utilizing the carpet model.

3.3 Cathelicidins

Cathelicidins are another well studied group of antimicrobial proteins that all share a domain known as the cathelin domain. LL- 37, also known as hCAP18, or FALL- 39, is the only known human cathelicidin. It is an amphipathic α- helix and is

primarily expressed by neutrophils [58] and monocytes [59]. LL- 37 disrupts the bacterial lipid bilayer via the toroidal pore model [60] .

3.4 Other antimicrobial peptides

Other peptides have also been shown to have antimicrobial activity in humans. These include ribonucleases, phospholipase A2, histones, and platelet- derived factors. Antimicrobial activity as a secondary or additional function is a common theme amongst these. Peptides such as psoriasin or lysozyme also exhibit antimicrobial activity [61-65].

4. Antimicrobial chemokines

Defensins have been shown to bind, activate, and induce migration via the chemokine receptor CCR6, a receptor found on both immature dendritic cells and memory T cells [59, 66-68]. Although there is no clear sequence similarity between chemokines and defensins, these molecules resemble each other in charge, size and structure [69]. Although chemokines have been traditionally classified based on their ability to induce cellular chemotaxis, various chemokines, including CCL28 and CCL25, also show antimicrobial activity *in vitro* [70, 71].

4.1 CCL28

CCL28, previously known as mucosae- associated epithelial chemokine (MEC), was first identified and reported in 2000 by two independent groups [17, 72]. As described by Pan et al., it acts as a ligand for two separate receptors: CCR3 and CCR10. CCL28 also has 6 cysteine residues, whereas most chemokines contain 4 cysteines.

CCL28 is selectively expressed in mucosal tissues, such as the salivary and lactating mammary glands, trachea, stomach, and large intestine [71, 72]. The C- terminus of human CCL28 shows significant sequence homology to histatin- 5, another well-known antimicrobial peptide [71]. CCL28 attracts plasma cells, particularly IgA antibody secreting cells (ASCs) via CCR10 [71-74]. CCR3, the other cognate receptor, is present on eosinophils and activated T lymphocytes [75].

CCL28 shares significant homology (about 40% at the protein level), with CCL27, although CCL27 shows no antimicrobial activity [70]. CCL27, also known as cutaneous T- cell attracting chemokine, or CTACK, is expressed in the skin by keratinocytes and is another CCR10 ligand. As described above, CCL28 is expressed on mucosal surfaces and can attract IgA plasma cells, memory lymphocytes and eosinophils [72]. CCL28 is encoded on human chromosome 5, by at least 4 exons with large introns [72], while CCL27 is on chromosome 9 [76]. CCL28 as a protein is 127 amino acids long [17] with a

longer C- terminus than CCL27. This may contribute to their different properties, as the C- terminal end of chemokines has been shown to have α- helical structure.

Further studies with CCL28 have shown that antimicrobial activity is dependent on highly charged amino acids in the C- terminal region. Charge reversal and deletion mutations in the C- terminus led to the identification of a sequence of positively charged amino acids, conserved across species, that is necessary for microbe killing [77]. Studies have shown the C- terminus to be necessary for full antimicrobial activity but not sufficient by itself for effective antimicrobial activity. Fusion proteins consisting of the N- terminus of CCL27, which alone does not have any known antimicrobial properties but is highly homologous to the N- terminus of CCL28, and the C- terminus of CCL28 did show effective microbe killing. A fusion protein between the N- terminus of CCL5 and the C- terminus of CCL28 did not show killing similar to the full length CCL28, suggesting that some part of the N- terminus assists in bacterial killing [77].

Additional studies with CCL28 also show that osmotic pressure is important in antimicrobial activity. At normal concentrations, CCL28 fails to kill bacteria in high salt or sugar solutions [78]. Chemokines are still able to bind bacteria in such conditions but their antimicrobial properties are restricted. Whether CCL28 binds to a specific protein or receptor on the bacterial surface or if it binds through electrostatic and hydrophobic interactions is still unknown.

Binding of antimicrobial peptides—including antimicrobial chemokines—is the first step in membrane permeabilization and antimicrobial action. However, peptide binding does not always lead to cell death, as peptide binding may still occur without accompanying cell lysis (unpublished observation).

4.2 CCL25

CCL25 is another antimicrobial chemokine used in this study. It is highly expressed in the thymus and small intestine [79] and has also been shown to have antimicrobial activity [70]. Its receptor, CCR9, is expressed on IgA ASCs as well as some T cells [80-82].

4.3 *in vivo* vs. *in vitro* effects

The physiologic relevance of antimicrobial chemokines *in vivo* is less understood than their role in cell migration. Many antimicrobial chemokines have been shown to exhibit antimicrobial activity only at high chemokine concentrations or low salt concentrations. Similar to antimicrobial peptides, chemokines can also be upregulated or induced. This may enable them to create microenvironments in which inhibitory salt concentrations may become negligible [69].

5. Bacterial defense mechanisms

Despite the complex systems designed to maintain human health, diseases caused by bacteria continue to be a problem. Continued evolution in both the human immune system and bacterial defensive and offensive strategies make it so that, in the words of the Red Queen of Lewis Carroll's Wonderland, "it takes all the running you can do, to keep in the same place" [83].

One of the main defenses of bacteria is their outer wall. In Gram- positive bacteria, this consists of a thick layer of peptidoglycan, a chain of alternating N- acetylglucosamine (GlcNAc) and N- acetylmuramic acid (MurNAc) residues, cross-linked together by short peptide chains. Peptidoglycan is important in maintaining the structure of the cell wall and resisting osmotic pressure changes [84]. Gram- negative bacteria have only a thin layer of peptidoglycan, sandwiched between two phospholipid membranes. The inner membrane of Gram- negative bacteria is where major cellular functions occur, including electron transport, DNA synthesis, and nutrient transport. The outer membrane is polar, with the inner leaflet being similar to the inner membrane while the outer leaflet is primarily composed of LPS.

5.1 LPS

LPS constitutes ~75% of the outer leaflet of the outer membrane of Gram-negative bacteria [85]. It is composed of three distinct parts: Lipid A, the oligosaccharide

core and the repeating O- units that make up O- antigen. Lipid A consists of a β- 1, 6- linked, acylated glucosamine disaccharide. The fatty acids, which anchor lipid A and LPS into the phospholipid membrane, can vary between species in number and length [86]. Negatively charged phosphate groups on lipid A, at positions 1 and 4', provide anchors for divalent cations to bridge neighboring LPS molecules [87]. Phospholipid patches and porins make up the rest of the outer membrane outer leaflet.

Lipid A and the core oligosaccharides are generally conserved between species and seem to be the minimum requirements for bacterial survival. The oligosaccharide core can be divided into the inner and outer cores. The inner core is more tightly conserved between species, and usually includes 3- deoxy- D- *manno*- oct- 2- ulosonic acid (Kdo), L- *glycero*- β- D- *manno*- heptose (L, D- heptose or L, D- Hep) and other sugar residues. At the least, Kdo residues attached to lipid A can suffice as LPS. Kdo is characteristic and essential to LPS [88].

Major variation between species is usually within the O- antigen. O- antigen variation includes differences in the number of O- units added, the types of sugars incorporated, and the linkage between repeating units [86]. Regions internal to the outer core and O- antigen are not involved in surface interactions and so are not as prone to change [86]. O- antigen molecules also serve as bacteriophage receptors and may have evolved to become more resistant to bacteriophages [89]. Additionally, the importance

of lipid A and the inner core in maintaining outer membrane stability may make it less likely to change.

The immune response to LPS comprises TLR4 recognition, assisted by CD14 and MD- 2, triggering the production of pro- inflammatory cytokines, including TNF- α and IL- 1 [90, 91].

5.1.1 LPS modifications

Two component systems are commonly used by bacteria to rapidly adjust to changing environments and conditions. Two component systems consist of a sensor kinase and a response regulator. Upon sensing the presence or absence of certain ions or nutrients, the sensor kinase phosphorylates the response regulator, leading to an increase in transcription of certain genes.

Two such systems are particularly relevant to bacterial resistance to antimicrobial peptides. In *Salmonella enterica* serovar Typhimirium and *Pseudomonas aeruginosa*, the PhoP/PhoQ system responds to low calcium, low magnesium concentration, or low pH, typical of the microenvironment in the phagosome of a neutrophil. The presence of cationic antimicrobial peptides can also activate this system. Activation increases acylation of lipid A, specifically by adding palmitoyl groups [92]. This decreases membrane fluidity, making it more rigid and thus harder for antimicrobial peptides to penetrate [85]. PhoP/PhoQ activation also activates the

PmrA/PmrB two component system [93]. This system results in incorporation of aminoarabinose (Ara4N) or addition of ethanolamine (EtN) to lipid A, masking the negative charges of the phosphate groups [94-96]. This modification of LPS makes the overall charge of bacteria less negative, making it less attractive to antimicrobial peptides and more resistant to polymyxin. Polymyxin is an antibiotic that is also attracted to the negatively charged bacterial membrane and destabilizes the electrostatic and hydrophobic interactions of the membrane [97]. In *Yersinia*, this polymyxin-resistance operon, *pmrF*, is actually independent of PmrA/PmrB, instead being directly upregulated by PhoP/PhoQ [98].

5.1.2 LPS synthesis

LPS synthesis occurs in two general phases. Lipid A and the core oligosaccharides are synthesized and assembled on the cytoplasmic side of the inner membrane. The lipid A and core are translocated to the periplasm, where repeating O- units, making up the O- antigen are added by waaL, the O- antigen ligase. The number of O- units added, thus determining the length of the O- antigen, is determined by the *wzz* gene product, the O- antigen chain length determinant. The whole molecule is then transported to the outer membrane.

5.2 Other bacterial defenses

In addition to changing or masking surface charges, bacteria have developed a number of defense mechanisms to either avoid or overcome the immune system. These may include efflux pumps that rid the cell of antimicrobial peptides [99], steric hindrance [100], preventing binding by decoy trapping [101] or capsule or biofilm formation, secretion of proteases to directly degrade antimicrobial peptides [102], or preventing the host from making antimicrobials in the first place [103].

6. *Yersinia psuedotuberculosis* and the *rfaD* operon

Yersinia pseudotuberculosis is a Gram- negative bacterium that can cause a tuberculosis- like disease in animals, including beavers, hares, horses, goats and monkeys [104-107]. Symptoms may include local necrosis and granulomatous inflammation in the lymph nodes, spleen and liver. In humans, it usually causes a self-limiting, food- borne, gastroenteric disease that can mimic the pains of appendicitis [108, 109]. *Y. pseudotuberculosis*, similar to *Salmonella* or *Shigella*, travels to the lymph nodes by passing through the M cells of the small intestine. It is closely related to *Yersinia pestis*, which evolved from *Y. pseudotuberculosis* relatively recently (1500- 20000 years ago) [110]. *Y. pestis* is more virulent and causes more disease pathology than *Y. pseudotuberculosis*, including bubonic plague, or the Black Death, and pneumonic plague, a highly contagious, airborne disease with a fatality rate approaching 100%

[111]. A third species of the genus *Yersinia*, *Yersinia enterocolitica*, is also pathogenic to humans, but less so, causing a gastroenteric disease like *Y. pseudotuberculosis. Y. pestis* and *Y. pseudotuberculosis* have been shown to be more resistant to the activity of cationic antimicrobial peptides than *Y. enterocolitica* [112].

6.1 *Yersinia* LPS

Y. pseudotuberculosis lipid A acylation changes with temperature. At 21°C, *Y. pseudotuberculosis* produces tetra-, penta-, and hexa- acylated lipid A. At 37°C, it produces two major forms: a tetra- acylated form similar to *Y. pestis* and a penta-acylated form, which results from addition of a palmitoyl group (C16:0) [113]. Studies with a PhoP knockout in *Y. pestis* showed that this temperature dependent shift in acylation was, interestingly, PhoP independent. Ara4N addition still occurred in a PhoP- dependent manner. [113]

LPS consisting only of lipid A and Kdo are known as deep- rough mutants. Rough mutants include both lipid A and elements of the core oligosaccharide and have increased hydrophobicity. Bacteria with full length O- antigen LPS are considered to have smooth LPS.

Natural isolates of *Y. pestis* are rough, although they still carry a mutated O- antigen gene cluster [114, 115]. *Y. pseudotuberculosis* O- antigen expression is significantly reduced at 37°C [116, 117].

6.1.1 *Yersinia* inner core

Lipid A and the core oligosaccharide structures are generally conserved between members of the same genus. The chemical composition for the core regions of *Y. pestis, Y. pseudotuberculosis* and *Y. enterocolitica* were found to be the same [118], although the actual structures differ slightly. The core structure for *Y. pestis* LPS (KM218 grown at 25°C) was described in 2002, as determined by NMR spectroscopy and electrospray-ionization mass spectroscopy (ESI-MS) [119]. *Y. enterocolitica* and *Y. pestis* share similar but not identical cores [118]. The main core backbone, consisting of L, D- Hep III → L,D-Hep II → L, D-Hep I → Kdo, is conserved between *Y. pestis* and the three serotypes of *Y. enterocolitica* studied (O:3, O:8 and O:9) [118]. ESI-MS was also used to compare the LPS of *Y. pestis* and *Y. pseudotuberculosis* and data suggest that their core structures are virtually identical, excepting differences that occur as a result of temperature-dependent variations [120]. These studies used *Y. pseudotuberculosis* serotypes O:3 and O:4. The *Y. pseudotuberculosis* core is similar in all serotypes [121]. Additionally, studies using a *Y. pestis* specific bacteriophage found that the phage receptor, *Y. pestis* LPS core, was also present in *Y. pseudotuberculosis* [122]. This shared core structure includes at the least two Kdo residues (or Kdo and Ko) and three L, D-heptose residues [123]. While the core structure for the *Y. pseudotuberculosis* strain used in this study (O:1b) has not been described, from the data described above, we expect that the core structure is as shown in Figure 1. Figure 1 shows the *Y. pestis* core when grown at 25°C with potential

substitutions between D, D- heptose and galactose as well as between Kdo and Ko (adapted from [119]).

Growth at different temperatures affects what other sugars, including glucose, galactose, D, D- heptose and D- *glycero*- D-*talo*-oct-2-ulosonic acid (Ko), are incorporated in the inner core. Both *Y. pestis* and *Y. pseudotuberculosis* have temperature- dependent structure variations [120]. At 37°C, the core consists of D, D- Hep, L, D- Hep III, L, D- Hep II, L, D- Hep I, and a Kdo disaccharide with glucose branching from L, D- Hep I. At 6°C, D, D- heptose is replaced with β-D-glucose and one Kdo residue is replaced Ko. At 25°C, a mix of glycoforms containing D, D- Hep + Ko and Gal + Kdo are found [123]. Incorporation of galactose instead of D, D- heptose is under PhoP/PhoQ control [41, 123]. The biological role of these different glycoforms or substitutions is not exactly known [41, 120].

6.2 *rfaD* operon

rfaD, alternatively known as *gmhD*, *hldD* or *htrM*, encodes ADP- L- *glycero*- D-*manno*- heptose- 6- epimerase. This gene is responsible for the epimerization of L, D- heptose from D, D- heptose. L, D- heptose is the preferred isomeric form of heptose used for at least the first two heptose residues in the LPS inner core. While D, D- heptose can be used, studies have shown it has less activity and that the physiological donor is indeed L, D- heptose [85].

In *Yersinia*, *rfaD* is part of a three gene operon, also including, in order, *rfaF* and *rfaC* (alternatively known as *waaF* and *waaC*, respectively). Both are heptosyltransferases, with *rfaC* responsible for transferring the first L, D- heptose residue onto the Kdo and *rfaF* encoding the transferase adding on the second L, D- heptose residue.

A fourth gene, *rfaL* (or *waaL*), encoding the O- antigen ligase, is found in the homologous operon in *E. coli*. However, in *E. coli* the core biosynthesis genes are found right next to the O- antigen biosynthesis genes (*waaQ* operon) and the Kdo transferases. While the Kdo transferase does neighbor the *rfaD* operon, the O- antigen biosynthesis genes are found elsewhere in the *Yersinia* genome.

7. Experimental Approach

Using *Y. pseudotuberculosis* (IP 32953, serotype O:1b) and CCL28 as models, we sought to find genes that are important in bacterial resistance to antimicrobial peptides, specifically antimicrobial chemokines. To achieve this, we performed random transposon mutagenesis, screening for mutants with altered outer membrane composition and/or increased chemokine binding. Sequencing of the first high binding mutant identified, called mutant 27, revealed an insertion in the *rfaD* gene.

To further explore the role of *rfaD* in chemokine binding and chemokine-mediated killing, we transcomplemented the mutant and measured its effects. Because

these three genes are transcribed and translated as an operon, it was not clear if the mutation in *rfaD* was solely responsible for the observed high- binding phenotype or if the other two genes were somehow involved. Accordingly, the partial operon, including *rfaD* and *rfaF* (*rfaDF*), was also transcomplemented as was the whole operon (*rfaDFC*). Complemented strains were then compared to wild type bacteria and to mutant 27 bacteria in terms of chemokine binding and killing by CCL28 or polymyxin. qPCR was used to analyze gene expression differences in the mutant and complemented strains.

Additionally, we sought to physically characterize mutant 27 bacteria compared to wild type bacteria and complemented strains in terms of size, growth patterns, and LPS expression.

We hypothesize that *rfaD* and possibly *rfaF* or *rfaC* play a key role in preventing antimicrobial peptide binding and possibly chemokine- mediated killing. A defect in LPS synthesis may lead to greater accessibility to the cell wall, thus granting increased access to the bacterial membrane for antimicrobial chemokines. Through mutating different genes involved in LPS synthesis and measuring the differences in chemokine binding and killing ability, we may be able to find genes important in bacterial resistance to antimicrobial chemokines.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Y. pseudotuberculosis IP 32953, a fully virulent clinical isolate from a patient [124], was used as the wild type and transposon mutagenesis and subsequent complementation was performed in it. Unless otherwise mentioned, all *Yersinia* species were grown at room temperature in tryptic soy broth (TSB) with shaking or on tryptic soy agar (TSA). *Yersinia* cultures for RNA extraction were grown at 30°C on brain- heart infusion (BHI) plates. Ampicillin (100 μg/mL) or kanamycin (30 μg/mL) was added when necessary. *E. coli* SM10 carrying pRL27 [125] was used as the transposon donor in mutagenesis and *E. coli* DH5α was used as a cloning host. *E coli* strains were grown at 37°C.

Transposon mutagenesis

Random transposon mutagenesis in *Y. pseudotuberculosis* was performed by biparental mating with *E. coli* SM10 harboring pRL27, which contains a Tn5 element encoding kanamycin resistance [125]. Kanamycin resistant insertion mutants were selected and pre- screened in one of two ways: (1) on Congo red agar to test for altered outer membrane composition, or (2) by magnetic separation, to select mutants with increased chemokine binding. Mutants were then cycle sequenced to find the precise

location of the transposon insertion. All sequencing was done at the DNA Sequencing Center at Brigham Young University.

Flow cytometry binding assays

To assure logarithmic growth phase, 10 μL of overnight culture were inoculated into 3 mL TSB plus the appropriate antibiotic and grown for 3 hours. Bacteria were diluted 1:20 in filtered phosphate buffered saline (PBS) supplemented with 0.5 g bovine serum albumin (BSA) and 0.2 nM chemokine was added and incubated on ice for 30 minutes. Biotin- conjugated anti- chemokine antibody was added and incubated for 30 minutes. Finally, fluorescent streptavidin- conjugates were added, incubated for at least 30 minutes, washed and then fluorescence was measured using the BD FACSCanto II flow cytometer and analyzed using FACSDiva software (BD Biosciences, San Jose, California). Samples were washed between each step and all incubations were done on ice.

Complementation

Primers were designed to amplify *rfaD*, *rfaF*, and *rfaC (*see Table 1). A region of 310 base pairs upstream of *rfaD* was included so that the native promoter would also be cloned in complementation plasmids. PCR products were cloned using the Fermentas CloneJET cloning kit (Glen Burnie, Maryland) and transformed into DH5*α* competent cells (New England BioLabs, Ipswich, Massachusetts). Plasmids were isolated from

ampicillin resistant clones using the QIAprep Spin Miniprep Kit (QIAGEN, Valencia, California) and then cycle sequenced to confirm amplification of the proper gene. Once this was confirmed, complementation plasmids were transformed into mutant 27 via electroporation. Transformations were verified with colony PCR using Taq polymerase.

PCR cycling conditions were as follows: 98°C for 30 seconds, 30 cycles of 98°C for 10 seconds and 72°C for 60 seconds, then 72°C for 5 minutes. For colony PCR, a 5 minute denaturation step was included at the beginning followed by 30 cycles of 94°C for 30 seconds, 63°C for 30 seconds, 72°C for 90 seconds with a 5 minute extension step (at 72°C) at the end.

Killing assays

Bacteria were grown to mid- logarithmic phase (OD_{600} between 0.5- 0.6) and diluted 1:25 in filtered potassium phosphate buffer (PPB). After addition of chemokine (0.4 nM CCL28), polymyxin (initial concentration= 10 μg/μL) or BSA (0.01%), bacteria were incubated at room temperature until the appropriate time point. 20 μL of bacteria were removed at each time point and put on ice in PBS plus counting beads (diluted 1:62500, Polybead ® Polystyrene 1 μm Microspheres, Polysciences Inc., Warrington, Pennsylvania). Propidium iodide (PI) (Invitrogen, Carlsbad, California) was added just before reading on the flow cytometer. Viable bacteria were determined by subtracting PI positive bacteria from the total number of bacteria counted per 30000 beads. Percent

survival was calculated by dividing viable bacteria of the sample by viable bacteria in the BSA control.

Killing assay set up was the same for CCL28 and polymyxin but the concentrations and time points used were different. CCL28 assays were done over 5 hours with a consistent concentration. Polymyxin assays were performed with differing polymyxin concentrations at time points 0 hours and 2 hours.

Growth curves

Growth curves were performed using the same protocol as the killing assays except the initial subculture was diluted 1:1000 in TSB and there was no addition of chemokine or BSA. Results were plotted as fold- increase (compared to the number of cells at time zero) over time.

qPCR cDNA preparation and cycling conditions

RNA extraction from overnight cultures was done using the Direct- zol RNA MiniPrep Kit (Zymo Research, Irvine, California) following the protocol for cell suspensions as instructed by the manufacturer. DNAse treatment was done using the Ambion TURBO DNA- free Kit (Invitrogen, Carlsbad, California). cDNA synthesis was done using the SuperScript VILO cDNA Synthesis Kit or SuperScript VILO MasterMix, both from Invitrogen (Carlsbad, California).

qPCR primers were designed using the PrimerQuest tool at Integrated DNA Technologies (idtdna.com) and are listed in Table 2. Primers were ordered from Invitrogen (Carlsbad, California) and the Maxima SYBR Green/ROX qPCR Master Mix (2x) from Fermentas Molecular Biology Tools (Glen Burnie, Maryland) was used. 3 μM each forward and reverse primers (1.5 μM for *dnaE* primers) were added to the master mix and DNA. The comparative C_T method was used, with *dnaE* as the endogenous control. ROX, included in the master mix, was used as the passive reference dye.

qPCR cycling conditions were as follows: 10 minute initial denaturation at 95°C followed by 40 cycles of 95°C for 15 seconds then 60°C for 1 minute for annealing and extension. The melt curve analysis at the end included 95°C for 15 seconds, 60°C for 1 minute followed by the temperature increasing in increments of 0.3°C. The reaction was performed on a StepOne Real- Time PCR System and analyzed using StepOne Software v2.1 (Applied Biosystems, Carlsbad, California).

Reactions were done in duplicate and results are the average of 5 independent experiments (or 2 experiments for DFC (forward)). Results are representative of 3 independent RNA extractions.

LPS isolation and analysis

1 mL of overnight culture (OD_{600} between 1- 2) was pelleted and resuspended in 100 μL Tris/Tricine sample buffer. After boiling for 10 minutes, proteinase K was added

and samples were allowed to incubate for 1 hour at 60°C. After another 10 minute boiling step, samples were separated by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS- PAGE; 10- 20%). LPS staining was done using the Pro- Q Emerald 300 LPS Gel Stain Kit (Invitrogen, Carlsbad, California) with the manufacturer's directions followed exactly. Briefly, this included overnight fixation in 50% methanol/5% acetic acid at room temperature followed by two 20 minute washes with 3% glacial acetic acid. Oxidation for 30 minutes with periodic acid was followed by three additional washes. The gel was stained with a freshly prepared staining solution, as directed by the manufacturer, for 2 hours at room temperature, washed twice more and then visualized on a UV transilluminator.

Scanning electron microscopy sample preparation

Scanning electron microscopy (SEM) was used to measure wild type and mutant 27 bacteria. A vacuum filter system was used to mount bacterial samples (sub- cultured to represent mid- logarithmic phase growth) suspended in filtered PPB on a membranous filter. Polycarbonate track etch filters with a pore size of 0.4 μm were purchased from Whatman (GE Healthcare, Kent, United Kingdom). Samples were fixed in 1% buffered glutaraldehyde for at least 1 hour. Filters were then washed in PPB four times, 10 minutes each. The filters were then left in a secondary fixative (osmium tetraoxide, OsO_4) for at least 2 hours. Distilled water was then used to wash the OsO_4

out of the sample (six washes, 10 minutes each). Following the distilled water washes, samples went through an ethanol dehydration series (10%, 30%, 50%, 70%, 95%, and 100%). Filters were washed three times in 100% ethanol and then once in 100% acetone. Critical point drying was performed with at least four wash/purge cycles (with liquid CO_2), followed by mounting on clean 1" stubs and sputter coating with gold. Samples were viewed in the environmental scanning electron microscope (ESEM) in hi- vacuum mode. Accelerating potential, spot size, magnification and working distance were all optimized. All samples were measured at a magnification between 1500X- 38000X and only cells laying flat were measured. ImageJ image processing and analysis software was used for all measurements (http://rsbweb.nih.gov/ij/).

RESULTS

Binding assays

Yersinia pseudotuberculosis mutants were initially screened based on their ability to bind chemokine better than wild type bacteria. Antimicrobial chemokine binding is regarded to be the first step toward cell death. Mutant 27 consistently showed better binding of both CCL28 and CCL25 than the wild type, over 50- fold and 100- fold, respectively. Somewhat surprisingly, addition of the *rfaD* complementation plasmid failed to restore the wild type phenotype. Complementation with *rfaDF* appeared to partially restore wild type binding while the full length operon completely restored wild type binding (Figure 2A). CCL28 binding was not statistically different between mutant 27, *rfaD*+ and *rfaDF*+ strains ($p > 0.01$). There was also no statistical difference between wild type and *rfaDFC*+ binding ($p = 0.09$). *rfaF* and *rfaC*, in individual transcomplementation assays showed no effect on chemokine binding (data not shown).

CCL25 binding patterns mirrored CCL28 binding (Figure 2B), suggesting that the two chemokines may bind in similar fashions. Mutant 27 binding was not statistically different from *rfaD*+ or *rfaDF*+ binding ($p > 0.01$) but the rest of the samples were statistically different from each other. These results suggest that a fully functional L, D-heptose epimerase along with appropriately spaced heptosyltransferases is important for bacteria in avoiding binding by antimicrobial chemokines.

Killing assays

In order to see if, and how closely, chemokine- mediated killing mirrors chemokine binding patterns, killing assays using CCL28 were performed on wild type, mutant 27, *rfaD*+, *rfaDF*+, and *rfaDFC*+ bacteria. A similar pattern of complementation was seen as in the chemokine binding assays. Figure 3A, which presents bacterial survival data, shows that the complete operon fully restores wild type resistance to CCL28 at all time points ($p > 0.01$) whereas *rfaD*+ and *rfaDF*+ show no difference from mutant 27 susceptibility compared to the wild type (also at all time points, $p > 0.01$).

In our assay, chemokine- mediated killing and bacterial survival are functions of the total number of cells counted and the number of PI positive cells. Thus, we also looked at both of these parameters individually for additional insight. Experiments in which BSA was used as a negative control (in place of CCL28) (Figure 3B) showed no significant difference between the number of cells counted in any sample at all time points ($p > 0.01$). In the CCL28 treated bacteria (Figure 3C), at all time points, mutant 27 and *rfaD*+ results were significantly different from the wild type cell count ($p < 0.01$). At time points 3 hours and 5 hours, *rfaDF*+ was also significantly different from the wild type. At all time points, there was no difference between the number of cells counted in the mutant 27 sample compared to *rfaD*+ bacteria ($p > 0.01$). Sheer number of surviving bacterial cells increased with the number of genes complemented.

The number of PI positive cells in both the BSA treated (Figure 3D) and CCL28 treated samples (Figure 3E) are in the hundreds (out of thousands of cells counted per 30000 beads counted). At time points 3 hours and 5 hours, *rfaD*+ and *rfaDFC*+ were found to have significantly different numbers of PI positive cells compared to the wild type, while the mutant and *rfaDF*+ bacteria were not different (using p= 0.01 for significance).

We next designed experiments to determine if resistance to a different antimicrobial peptide would be similarly affected by the *rfaD* mutation and subsequent complementations. In these experiments, the well characterized antimicrobial peptide polymyxin was used. Although the assays were performed following the same protocol as described above for the CCL28 assays, instead of analyzing survival at different time points, different dilutions of polymyxin were used. Time points 0 hours and 2 hours were used in all experiments.

Killing assays with polymyxin, which disrupts the bacterial membrane, were performed on wild type, mutant 27, and all three complemented strains. As seen in Figures 4A and 4B (time points 0 hours and 2 hours, respectively), mutant 27 was extremely sensitive to polymyxin and much more susceptible than all of the other strains tested. For example, at a concentration where more than 85% of the wild type survives after 2 hours (1:500 dilution), mutant 27 showed less than 1% survival.

Transcomplemented strains, while being less susceptible to polymyxin activity, still show only between 35- 50% survival. At the concentration where mutant 27 survival exceeds 50% (between 1:4000 and 1:8000 dilutions), the survival rate for the complemented strains are close to the wild type. At time point 2 hours, mutant 27 showed significantly lower survival compared to the wild type through the 1:4000 polymyxin dilution. *rfaD+* differs from the wild type through the 1:2000 dilution while *rfaDF+* differs at the only the first two dilutions (1:500 and 1:1000 dilutions). Mutant 27 is also different from the three complemented strains through the 1:4000 dilution ($p< 0.01$), as seen in Figure 4B.

We also looked at cells counted and PI positive cells for the polymyxin assays. Figure 4D shows that, at time point 2 hours, mutant 27 showed, at all concentrations of polymyxin except 1:16000, significantly fewer cells counted compared to the wild type. *rfaD+* and *rfaDF+* were also different from the wild type at higher concentrations of polymyxin (1:500 and 1:1000 dilutions) ($p< 0.01$).

Mutant 27 showed significantly more PI positive cells than the wild type, through the 1:8000 dilution, or any of the complemented strains, through the 1:4000 dilution ($p< 0.01$). All strains, at the highest concentration of polymyxin, show upwards of 500 PI positive cells (Figure 4F), more than the general number of PI positive cells in the CCL28 treated samples.

In these assays, the term "percent viability" integrates the number of PI negative cells and the number of total cells present (as determined by using counting beads as an internal reference). In short, this number reflects bacteria that have had their membranes permeabilized (PI positive) as well as cells that have lysed or failed to divide (total cells counted) and is an overall measure of the antimicrobial activity of the chemokine or antimicrobial peptide. These data suggest some interesting characteristics which may hint at possible differences in mechanism between CCL28 and polymyxin. With CCL28, inhibition of growth or cell lysis occurs (manifest as fewer numbers of cells counted). For polymyxin, membrane permeability seems to be more important. This is seen by the increase in PI uptake in polymyxin treated cells. Ultimately, both antimicrobial peptides result in fewer bacterial cells in a solution. However, these data suggest that CCL28 may act by inhibiting cell proliferation whereas polymyxin may act by permeabilizing the bacterial cell membrane.

qPCR gene expression analysis

Sequencing of the transposon insertion site of mutant 27 showed that the *rfaD* gene was interrupted; however, an *rfaD* complemented strain failed to restore the wild type phenotype for binding or killing. Because *rfaD* is part of a three gene operon, it was not clear if the mutation in *rfaD* was solely responsible for the observed high- binding phenotype or if the other two genes were also involved. Binding and killing data,

described above, suggested that each of the other genes in the operon play an important role. This would seem to indicate that the insertion mutation was a polar mutation, disrupting the downstream reading frames. To address this issue, we next used qPCR to assess gene expression in all complemented strains. Results are shown in Figure 5. Interestingly, the *rfaD*+ strain showed about equal expression of all three genes, despite not restoring the wild type phenotype.

There are two separate *rfaDFC*+ clones. When first cloning the full operon, we had clones where the operon was in "reverse," ("Rev") being expressed only under the control of its endogenous promoter. A later clone ("Fwd") had the full operon under control of its endogenous promoter as well as the promoter on the cloning plasmid. These two clones showed drastically different expression patterns. The Fwd clone showed high levels of expression for all three genes, with the highest expression for *rfaD*. The Rev clone also showed high *rfaD* expression but minimal or no expression of *rfaF* and *rfaC*. Both clones restore wild type binding and killing phenotypes.

There are a few possible reasons *rfaD*+ showed high expression but failed to complement the phenotypes. These possibilities include some sort of protein- protein interaction which may be needed, but because the genes were expressed from a plasmid compared to the chromosome, they might not have been in the correct spatial

orientation to interact appropriately. Also, because the cloning vector was a multicopy plasmid, this could abnormally affect the level of expression of plasmid encoded genes.

Growth curve analysis

In order to investigate if the *rfaD* mutation affected bacterial growth as well as bacterial death and susceptibility to antimicrobial chemokines, a growth curve was performed. Growth was measured as fold increase over the number of bacteria in the culture at time point 0 hours. Growth curve analysis over 5 hours (Figure 6) showed no significant difference between the wild type, the mutant, and the *rfaD*+, *rfaDF*+, or *rfaDFC*+ complemented strains. At time point 5 hours, mutant 27 did show slightly lower growth but it is not significantly different from the wild type.

Morphological characterization

Colony morphology

Consistent with similar growth patterns, there were also no obvious physical differences between the wild type, mutant 27, or any of the complemented strains in colonies plated on TSA plates (Figure 7). However, mutant 27 did precipitate out of solution faster than the wild type or any of the complemented strains (data not shown). While the *rfaD*+ strain was similar to the mutant 27 phenotype in other characteristics

measured, including binding and killing, the mutation did seem to affect the precipitation rate.

SEM analysis

Another aspect of physical characterization of the mutant is the size of individual bacterial cells. Initial flow cytometry experiments suggested that mutant 27 was bigger than the wild type, based on forward light scatter. However, SEM showed no obvious differences between wild type and mutant 27 bacteria in size. Measurement of hundreds of bacteria (WT, n= 228; mutant 27, n= 301) showed that mutant 27 is significantly bigger ($p= 0.0035$) but, as shown in Figure 8, the size difference does not seem to be relevant functionally (difference between the means= 0.148 μm). Mutant 27 did tend to be less uniform in size and may be tend to be slightly bigger, but the data was not statistically significant (data not shown). SEM also did not show any obvious differences in membrane surface characteristics between wild type bacteria (Figure 9A) and mutant 27 bacteria (Figure 9B).

LPS analysis

Lastly, LPS expression in the mutant bacteria was examined to assess how a mutated *rfaD* affects LPS composition. The predicted core structure for Mutant 27 is shown in Figure 10. SDS-PAGE followed by LPS specific staining (Figure 11) showed that *rfaDFC* fully and completely complemented the mutant and restored wild type LPS

expression and composition. It appears that some O- antigen is added with the successive restoration of each gene in the operon as the amount of "normal" lipid A plus core grows (lane 2- *rfaD*+; lane 3- *rfaDF*+). A faster migrating band, possibly representing lipid A alone, decreased in amount as the lipid A plus core band grows. *Y. pseudotuberculosis* grown at 37°C (lane 6) and *Y. pestis* (lane 7) were included as controls because previous research has shown that neither of these strains express O- antigen [115, 126, 127].

Interestingly, the core size of *Y. pseudotuberculosis* and *Y. pestis* differed, despite expectations that their compositions are identical (see Figure 1 which shows the *Y. pestis* core). This may be because they were grown at room temperature, at which multiple glycoforms are usually present [123]. However, even the core structure of the wild type *Y. pseudotuberculosis* still appears larger than the *Y. pestis* core. This could be due to differences in sugar substitutions, resulting from slightly different growth conditions. Alternatively, the rough version of *Y. pseudotuberculosis* may still have some part of the first O-unit attached, whereas *Y. pestis* has only the core sugars. Different serotypes (*Y. pseudotuberculosis*) and different strains (*Y. pestis*) also may have slight differences in their cores. While the basic frame is conserved (two Kdo residues and three heptoses), other sugars may or may not be included.

DISCUSSION

Using *Y. pseudotuberculosis* IP 32953 and CCL28 as models, we sought to find genes that are important in bacterial resistance to antimicrobial peptides, specifically antimicrobial chemokines. Following random transposon mutagenesis, mutants were screened for their antimicrobial chemokine binding abilities and their susceptibility to antimicrobial chemokine-mediated killing. Many of the genes found in our screen to increase antimicrobial chemokine binding were involved in O- antigen biosynthesis, core biosynthesis, LPS regulation or in the *pmr* operon, which is responsible for the incorporation of Ara4N to lipid A, aiding in resistance to cationic antimicrobial peptides (see Table 3 for other mutants identified).

Specifically, we have shown that bacteria with a mutation in *rfaD*, whose gene product is responsible for the epimerization of D, D- heptose to L, D- heptose, binds CCL28 much more efficiently than the wild type. Additionally, this mutant is more susceptible to CCL28- mediated killing as well as polymyxin killing. We have also characterized the *rfaD* mutant strain, mutant 27, based on size, growth patterns, and LPS expression.

To confirm that *rfaD* was responsible for the observed mutant phenotypes, we transcomplemented the mutant with *rfaD* alone, *rfaDF* and *rfaDFC. rfaD* alone is not sufficient to restore the wild type binding and killing phenotypes. It appears that the

whole operon needs to be present and transcribed from the same piece of DNA for complete complementation to occur. Because *rfaD* is responsible for the last step of heptose biosynthesis, the known roles of *rfaF* and *rfaC* as heptosyltransferases make sense in this context. Heptose may need to be readily available or physically on hand for the transferases to function appropriately and build the "normal" core. As seen on LPS gels, the more "normal" core present, the more O- antigen can be added.

These results demonstrate that the *rfaD* operon is important in bacterial resistance to a model antimicrobial chemokine, CCL28, and a prototypical membrane-binding antibiotic, polymyxin. Increased chemokine binding seems to be correlated with increased susceptibility, although antimicrobial peptide binding may not always be related to cell death.

Inactivation of the *rfaD* gene prevents full length O-antigen from being added to the LPS of *Y. pseudotuberculosis.* Absence of smooth LPS may relieve steric hindrance that, in turn, may be preventing cationic antimicrobial peptides and chemokines from reaching their primary target- the bacterial outer membrane. Mutant 27 binds antimicrobial chemokine better and, presumably, this binding is at the surface of the outer membrane. As this is assumed to be the first step in antimicrobial chemokine-mediated killing, it is also likely that the lack of full length O-antigen contributes to the increased susceptibility of mutant 27 to killing. Other antimicrobial peptides that bind

to the surface of the outer membrane and then form pores would also have increased access to the surface and thus increased antimicrobial activity. While this model is consistent with our data, it is also possible that other outer membrane proteins are involved in chemokine binding and/or killing. Lack of full-length O-antigen may affect the conformation and activity of these proteins [128]. If there is a specific binding site for CCL28, it is still unknown.

Mutant 27 is not different in size or growth pattern from the wild type, as determined by SEM, growth curves, and observation of colony growth on TSA plates. We used SEM to visualize any differences in appearance between wild type bacteria and mutant 27 bacteria. There were no visible differences. In the future, immunoelectron microscopy may be used to visualize chemokine binding and any resulting membrane effects. Experiments with different antimicrobial chemokines may show differences in binding patterns (i.e. generalized or localized on the membrane surfaces). Other mutants may likewise show different binding patterns with various antimicrobial chemokines. Additionally, TEM may be used to study intracellular chemokine targets and localization.

Many questions still remain about the nature of antimicrobial chemokines and their *in vivo* activity. Killing assays with other antimicrobial chemokines, like CCL25, can help generalize patterns of chemokine- mediated killing and determine if antimicrobial chemokines as a class act in similar manners. Binding and killing assays

done using the concentrations and conditions that would be found in *in vivo* microenvironments can help elucidate the role of antimicrobial chemokines *in vivo.* Some of these microenvironments may contain other antimicrobial peptides which may work in synergy with antimicrobial chemokines. In this case, the lethal dose for each may be reduced in the presence of the other. Likewise, multiple antimicrobials may be able to overcome inhibitory salt or osmotic effects. However, they may also work antagonistically. Preliminary results in our lab show that CCL28 actually protects bacteria from the activity of penicillin. Penicillin acts on the cell wall of bacteria by preventing cross- linking between molecules of peptidoglycan. Further studies are needed to determine how CCL28 and other antimicrobial chemokines may interact with other antimicrobials or antibiotics *in vivo*.

We have identified a gene that, when mutated, makes bacteria more susceptible to the natural antimicrobial compounds of the human innate immune system. Following this same experimental approach, we can find other genes important in chemokine recognition and binding.

Genes that do affect antimicrobial binding and subsequent killing may be used as targets for antibiotics in the future. Antibiotic resistant bacteria are an increasing concern in the medical industry. Overuse or misuse of antibiotics has led to selection of resistant bacteria that are no longer responsive to traditional antibiotics, including

penicillin and other β-lactams. The time and cost required to develop a new drug is ever increasing. Additionally, continual misuse of antibiotics may make these novel drugs obsolete much sooner than anticipated or desired. Drug design is also increasingly difficult as the number of new targets unique to bacteria shrinks. Changing the way drugs are designed may provide a new avenue for drug research and development. Current drug therapy focuses on inhibiting bacterial cell growth or some other aspect of bacterial survival. This research suggests that instead of trying to create drugs to kill bacteria, it may be possible to enhance the efficiency of our immune defenses. This could be done by targeting bacterial proteins that are important for resistance to antimicrobial peptides and/or chemokines. Drugs targeting proteins such as those involved in LPS biosynthesis would make it easier to eliminate bacteria that normally would not be susceptible to antimicrobial peptide killing. Proteins involved in LPS biosynthesis are ideal drug targets because there are no counterpart proteins in humans. Moreover, most of them are highly conserved between many species of bacteria. Efforts are already being made to create chemical inhibitors of key enzymes involved in LPS biosynthesis [88]. Our screen and accompanying assays may provide additional appropriate targets for such drug design.

TABLES AND FIGURES

***rfaD* (F)**	ACG AGT CG TGT CGA GTT GTT GTT C
***rfaD* (R)**	CAT GGC CTA ACG GC ATT GGA ATT G
***rfaF* (F)**	AAG AAT ATC TGG CCT GGC TCA ATC GC
***rfaF* (R)**	CGT CAG CGC GGG TAA GGT ATG TAA A
***rfaC* (F)**	TAC CGG CTA TCA CAA AGT CCG CAA
***rfaC* (R)**	AGC ACC ACA TTG AGC CGT GGT TAT

Table 1. Primers used for construction of complementation plasmids. All primers are written 5′- 3′.

***rfaD* (F)**	TGT GCG TGA AAT TCT GCC ACA AGC
***rfaD* (R)**	ATG GCC TTC ACG TGG ACC ATA AAC
***rfaF* (F)**	GCC CAA CTC ATT TAA ATC CGC GCT
***rfaF* (R)**	AGG AAA TAA CGC ATT TCG CCA CGC
***rfaC* (F)**	AGC GGA ACA TTT CCC ACA CGT AGA
***rfaC* (R)**	AAC AGC GTA ATG TTT GGG CGA TCC

Table 2. qPCR primers (5′- 3′).

Hits	Name	Function	% positive CCL25 binding	Mean fluorescence of positive cells
	N/A	Wild type	4.0	111
2	*pmrK*	dolichyl- phosphate- mannose- protein mannosyltransferase	78.8	714
16	*fcl* *	GDP- fucose synthetase	69.8	761
1		Xanthine/uracil permease family protein	69	816
7	*gmd**	GDP- D- mannose dehydratase	67.2	1068
3	*ddhD**	CDP- 6- deoxy- delta- 3,4- glucoseen reductase	44.7	1118
2	***rfaD***	***ADP- L- glycero- D- manno- heptose- 6- epimerase***	***44***	***1673***
5	*wbyK**	putative mannosyltransferase	40.2	723
1	*pmrI*	probable formyl transferase	35.6	1798
16	*wbyL**	probable glycosyltransferase	35.4	504
3	*rfaH*	transcriptional regulation of capsule/LPS	26	287
22	*manC**	mannose- 1- phosphate guanylyltransferase	20.5	385

Table 3. Selected *Y. pseudotuberculosis* Tn5 mutants with increased binding affinity to CCL25. Many insertions occurred in lipopolysaccharide synthesis genes. Hits refer to the number of individual Tn5 mutants found in the specific gene. * denotes genes within the O- polysaccharide cluster.

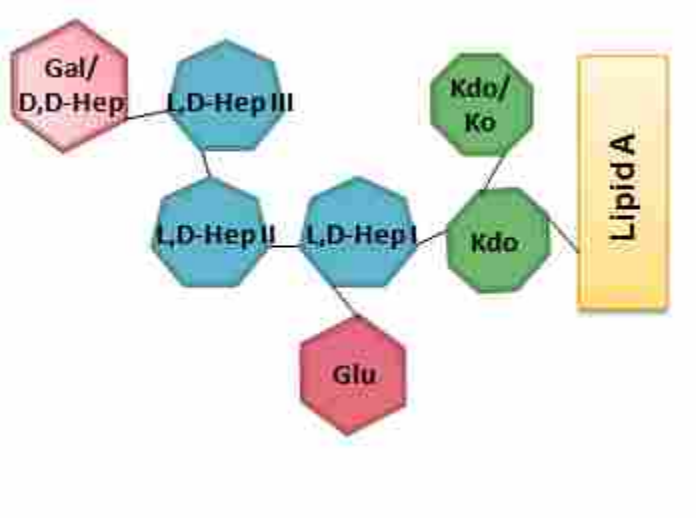
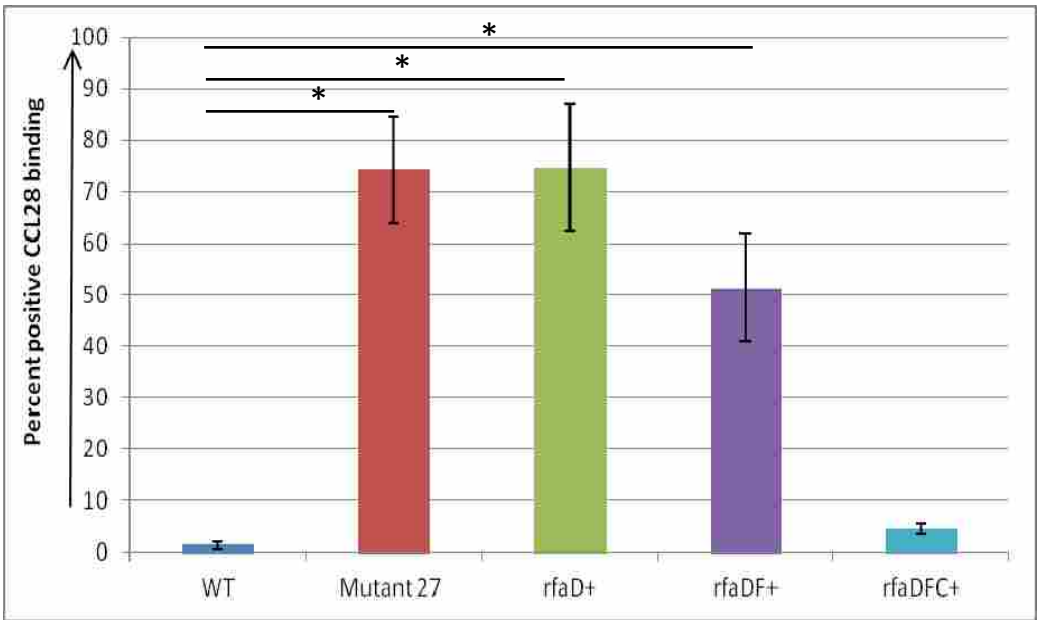


Figure 1. The inner core structure of *Y. pestis* grown at 25°C (adapted from [119]). Under different growth condition, different glycoforms of the core sugars can be found (see text for details) ([120, 123]). Kdo= 3- deoxy- D- *manno*- oct- 2- ulosonic acid. Ko= D- *glycero*- D-*talo*-oct-2-ulosonic acid. L, D- /D, D- Hep= ADP-L/D-*glycero*-β-D-*manno*-heptose. Glu= β-D- glucose. Gal= galactose

A.



B.

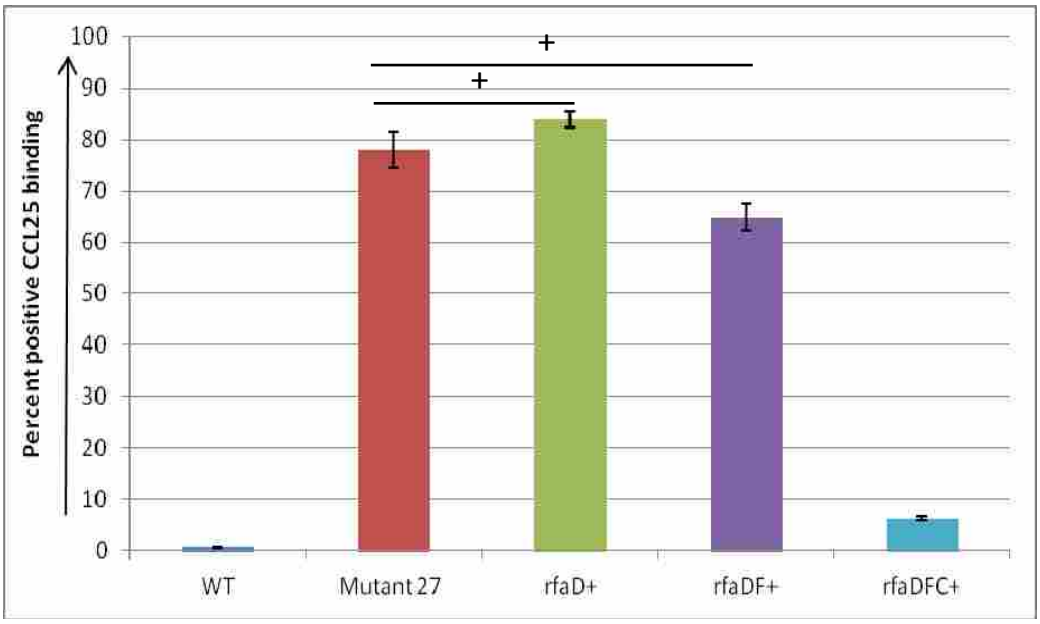


Figure 2. Chemokine binding, as measured by flow cytometry on wild type, mutant 27, and complemented strains. **A.** CCL28 binding. Mutant 27, *rfaD+*, and *rfaDF+* show significantly higher binding than the wild type. *rfaDFC+* restores wild type binding. *= $p < 0.01$ compared to the wild type. **B.** The same pattern holds true for CCL25 binding, another antimicrobial chemokine. += $p > 0.01$ compared to Mutant 27. Bars represent standard error of the mean. n= 3 for both experiments.

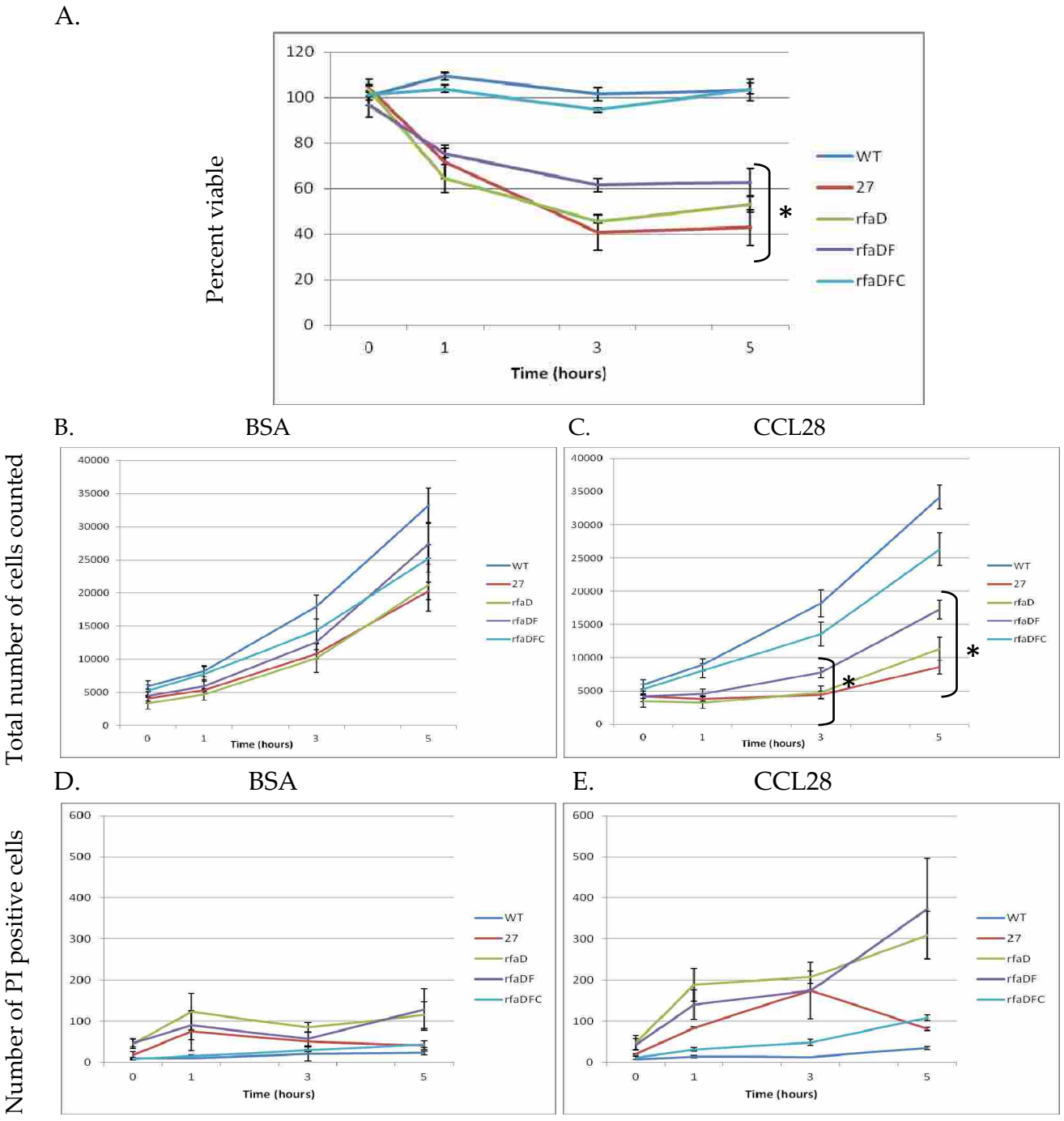


Figure 3. CCL28 killing assays. **A.** Overall survival. *rfaDFC*+ shows wild type resistance while mutant 27 and *rfaD*+ and *rfaDF*+ strains show reduced survival compared to the wild type. *= p< 0.01 **B.** Total cells counted in BSA (negative) controls. **C.** Total cells counted when treated with 0.4 nM CCL28. *= p< 0.01 compared to the wild type. **D.** Number of propidium iodide (PI) positive cells in BSA controls. **E.** PI positive cells, CCL28 treated (0.4 nM). Bars represent standard error. n= 3 for all experiments.

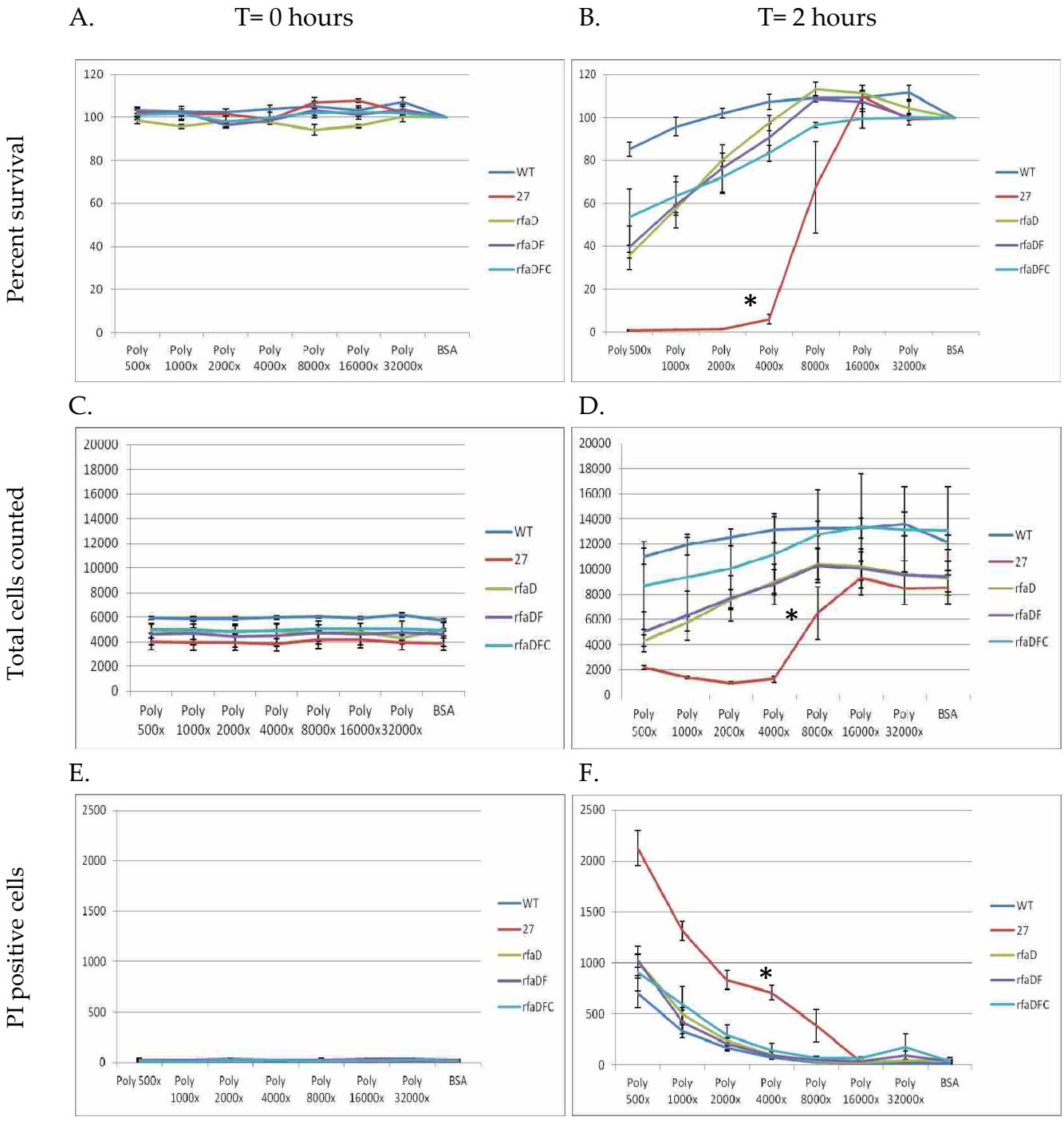


Figure 4. Polymyxin dilution killing assays. Overall survival at 0 hours (**A**) or 2 hours (**B**). *= p< 0.01 between Mutant 27 and wild type through the 1:4000 dilution. Total cells counted, 0 hours (**C**) and 2 hours (**D**). *= p< 0.01 between 27 and wild type through 1:8000 . PI positive cells, 0 hours (**E**) and 2 hours (**F**). *= p< 0.01 between 27 and wild type through 1:4000. Bars represent standard error. n= 3 for all experiments.

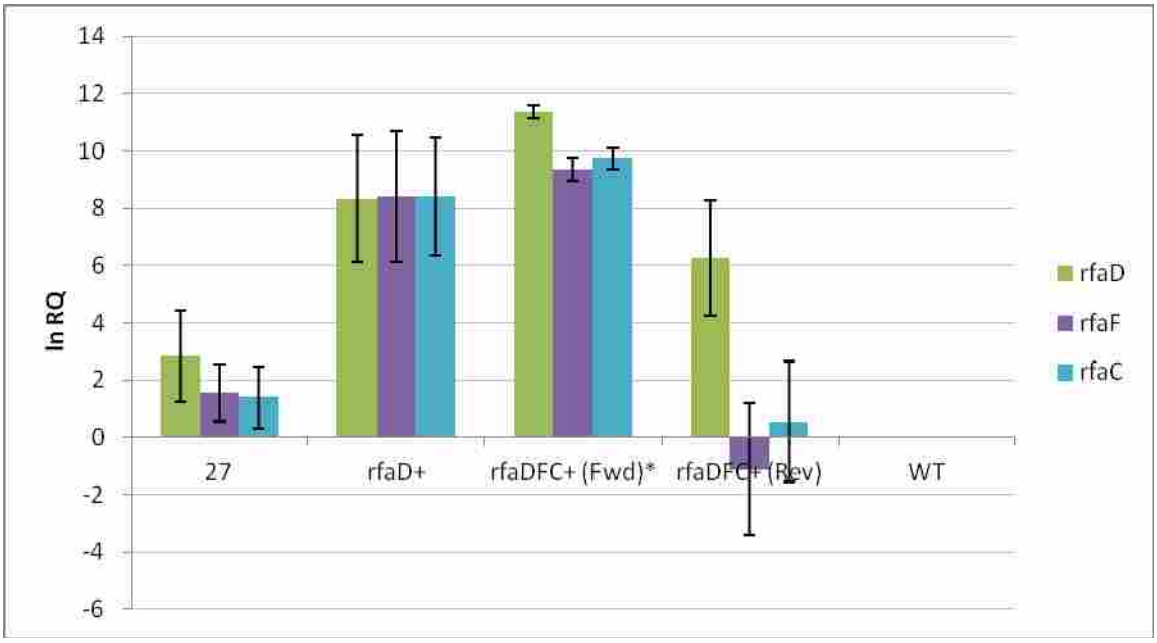


Figure 5. Relative gene expression in mutant 27 and complemented strains. Despite not showing complementation of the mutant phenotype, mutant 27 shows relatively high expression of all 3 genes within the *rfaDFC* operon. The complete operon in the "forward" direction shows high expression of all 3 genes, as expected. The "reverse" operon, surprisingly, shows high *rfaD* expression but low *rfaF* and *rfaC* expression. Bars represent standard error. n= 5 except for * n= 2.

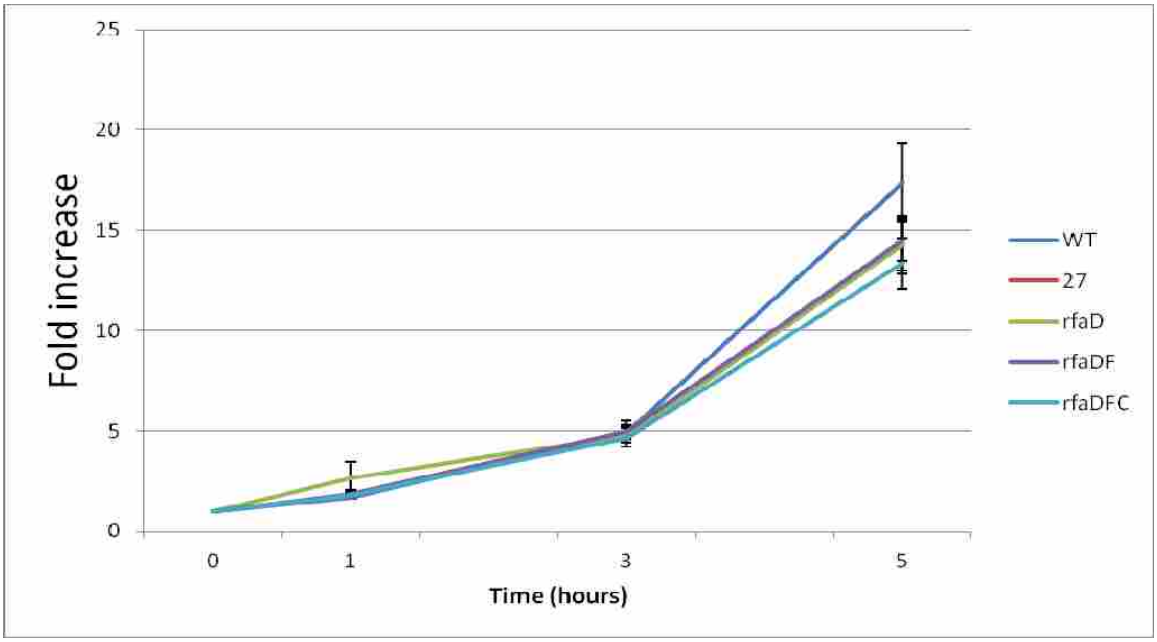


Figure 6. Growth curve over 5 hours. Bacterial growth is shown as fold increase compared to the initial cell count at 0 hours. There is a slight difference in growth of the mutant 27 and the wild type at 5 hours. Bars represent standard error. n= 3.

Figure 7. Cultures grown to mid-logarithmic phase plated on TSA show no obvious difference in colony shape or morphology after growth overnight at room temperature.

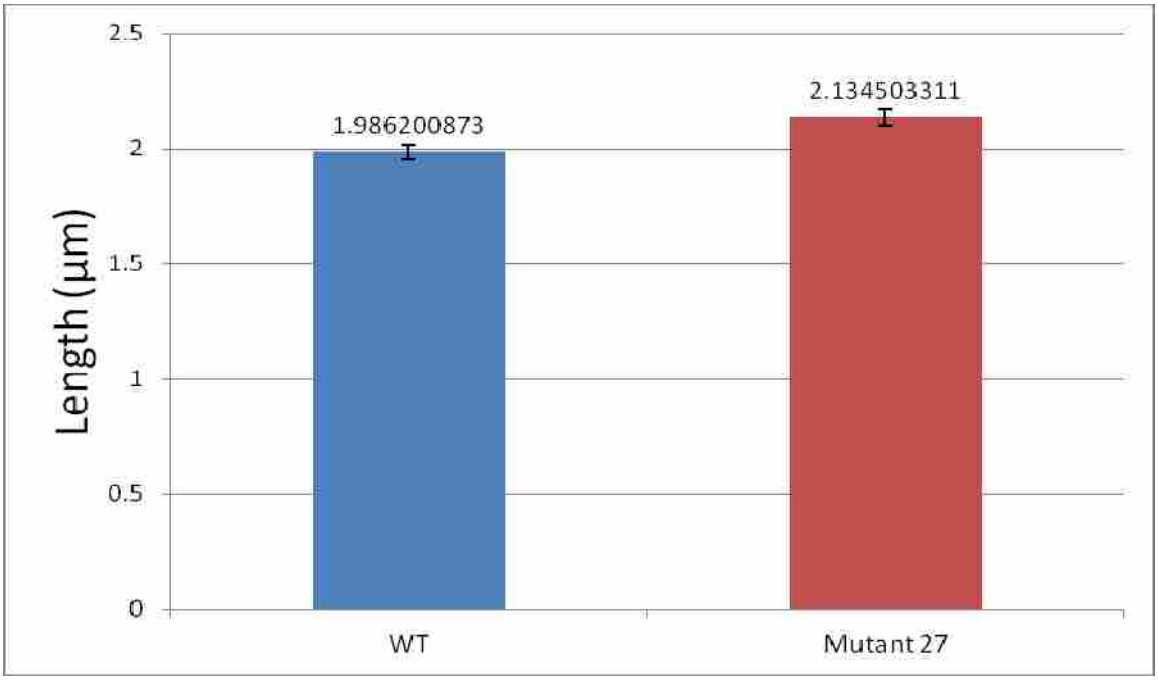
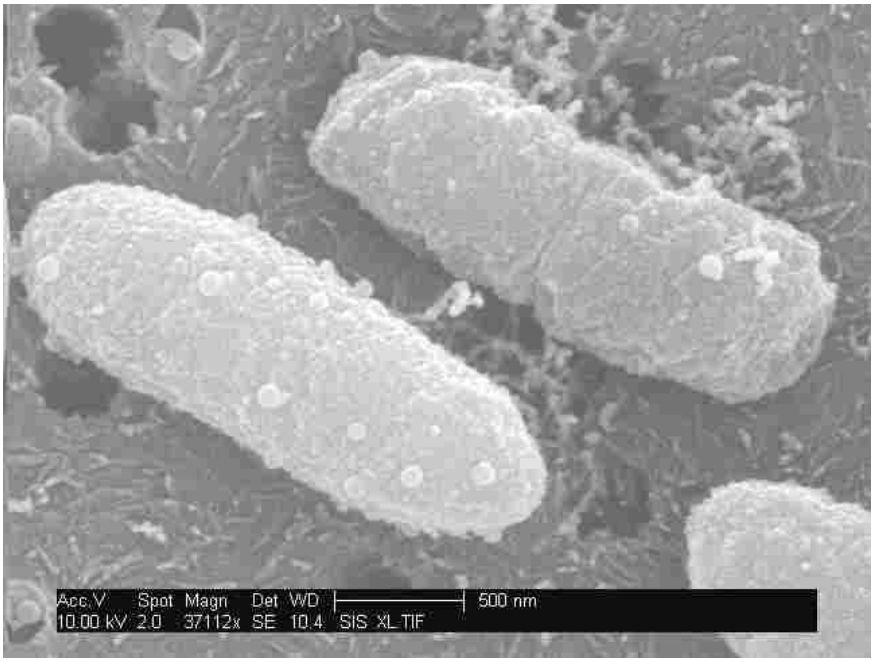


Figure 8. Comparison of average size as measured from SEM images. Mutant 27 is significantly different in size, statistically (p= 0.035), but not functionally, from the wild type. Bars represent standard error; wild type, n= 228, mutant 27, n= 301.

A.



B.

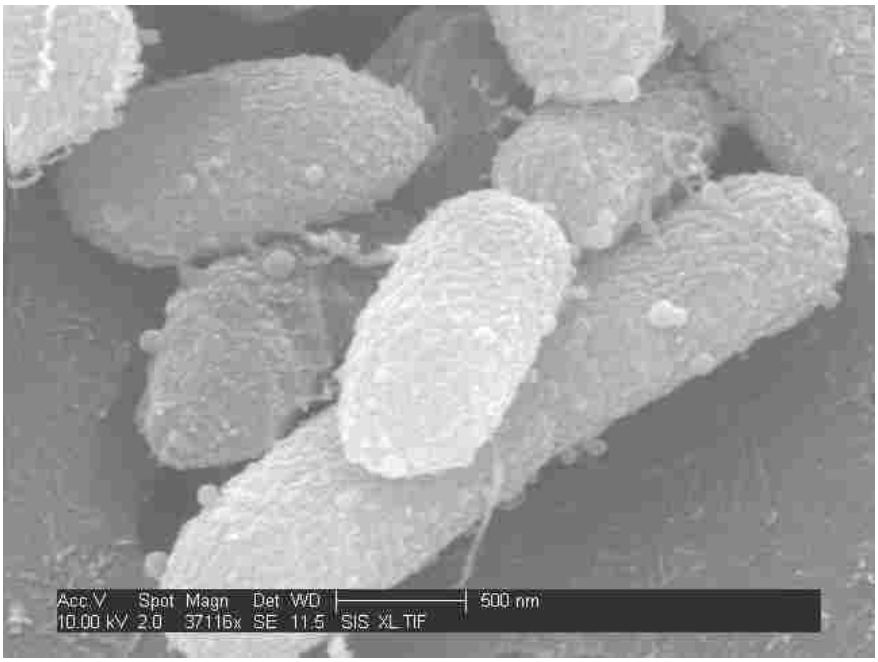


Figure 9. A. *Y. pseudotuberculosis* IP 32953 bacteria (wild type). **B** Mutant 27 bacteria. Sample preparation was performed as described in the text.

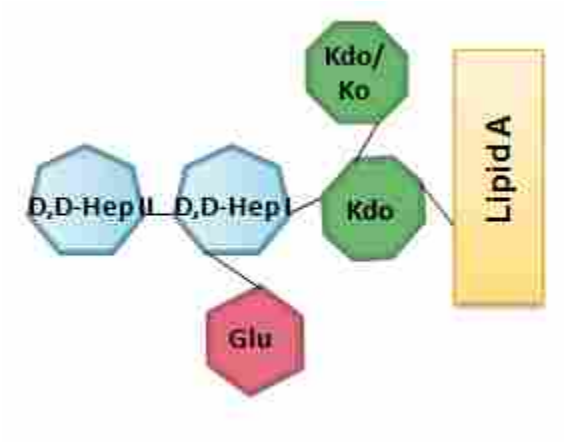


Figure 10. Predicted core structure for Mutant 27 (Δ*rfaD*). D, D- heptose can be used as a substrate for the heptosyltransferases encoded by *rfaF* and *rfaC,* though with reduced activity *[85]*. In *Y. pseudotuberculosis*, O-units are added to the second heptose unit [122].

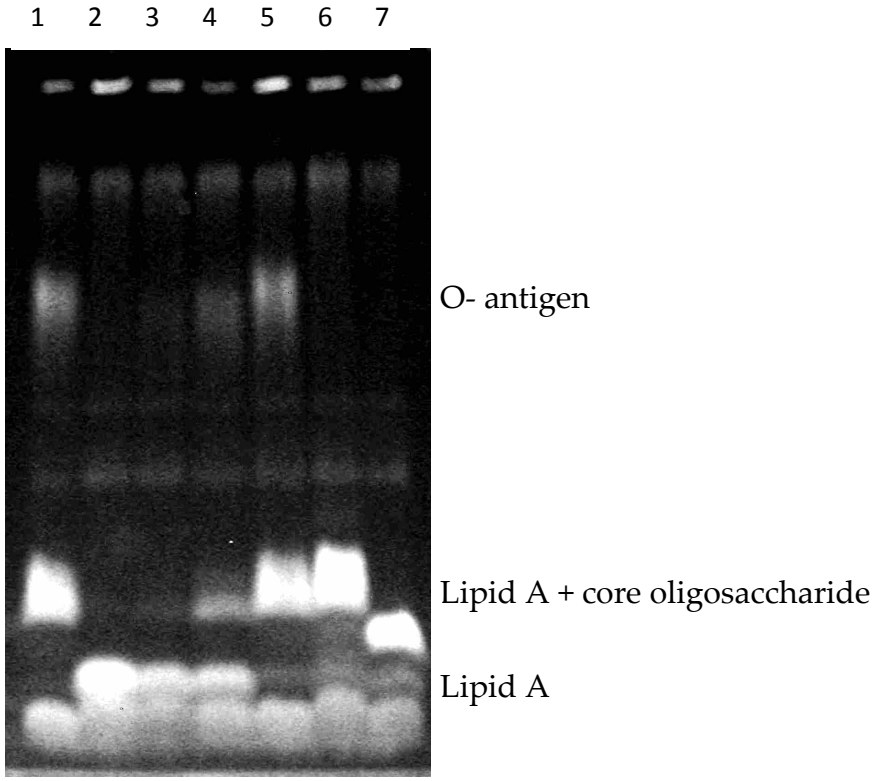


Figure 11. LPS analysis. Mutant 27 (lane 2) shows no O- antigen expression and the presence of a bright, fast- migrating band. Addition of the complementation plasmids (lanes 3-5) shows that with each successive gene, the fast- migrating band grows dimmer, the band representing lipid A plus the normal core grows brighter and the O- antigen band increases. As expected, neither *Y. pseudotuberculosis* grown at 37°C or *Y. pestis* shows O- antigen expression (lanes 6-7). Lane 1- Wild type Y. *pseudotuberculosis* IP 32953 (grown at 25°C). Lane 2- Mutant 27. Lane 3- *rfaD*+ complement. Lane 4- *rfaDF*+ complement. Lane 5- *rfaDFC*+ complement. Lane 6- Wild type Y. *pseudotuberculosis* grown at 37°C. Lane 7- *Y. pestis* KIM6.

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