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ABSTRACT

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Yersinia pseudotuberculosis is a foodborne pathogen that is the ancestral strain to *Yersinia pestis*, the causative agent of Plague. *Y. pseudotuberculosis* invades a host through the intestinal epithelium. The bacteria resist mucosal innate immune defenses including antimicrobial chemokines and phagocytic cells, and replicate in local lymph nodes. They cause Tuberculosis-like symptoms, including necrosis of local tissue and granuloma formation. Like all bacteria, *Y. pseudotuberculosis* has a net negative charge, which contributes to its susceptibility to some cationic antimicrobial peptides. *Y. pseudotuberculosis* is able to reduce this negative charge by adding 4-amino-4-deoxy-L-arabinose (L-Ara4N) to the lipid A portion of lipopolysaccharide. The production and addition of the L-Ara4N is coded for by the *pmrHFIJKLM* (*pmrF*) operon. A previous study has shown that the *Y. pseudotuberculosis pmrF* operon is important for resistance against polymyxin, but is not important for virulence in mice. Several previous reports have shown a strong influence of growth temperature on resistance to antimicrobial peptides and *pmrF* expression in pathogenic *Yersinia* species, but these studies also suggest significant variability between species, and even between strains of individual species. In particular, the regulation of the *Y. pseudotuberculosis pmrF* operon and its effect on bacterial interactions with mucosa-associated antimicrobial chemokines and neutrophils is not understood. In these studies, we investigated the environmental influences on *pmrF* expression in *Y. pseudotuberculosis.* We found that the promoter activity of the *pmrHFIJKLM* operon is increased at lower temperatures (21ºC) and in the presence of human serum. A Δ*pmrI* mutant strain of *Y. pseudotuberculosis* defective for addition of L-Ara4N was found to be more susceptible to killing by the antimicrobial chemokine CCL28 compared to wild-type. This suggests that this gene is important in the bacterial defense against antimicrobial chemokines. However, when the Δ*pmrI* mutant strain was exposed to human neutrophils, there was a decrease in phagocytosis as compared to wild-type bacteria. Our results suggest that the regulation of L-Ara4N modifications in *Yersinia* is more complex than previously appreciated and varies between species. Addition of L-Ara4N to *Y. pseudotuberculosis* appears to enhance resistance to some antimicrobial peptides like CCL28 and promote greater phagocytic engulfment by neutrophils. These opposing effects may partly explain why there is no net apparent survival defect in mutants lacking the *pmrF* operon during infection.

Keywords: *Yersinia pseudotuberculosis*, antimicrobial chemokine, phagocytosis, neutrophil, pathogenesis, immunology

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INTRODUCTION

The immune system

The human immune system consists of an innate and an adaptive immune response to protect our bodies against foreign invaders. The adaptive immune system is specifically tailored to adapt to individual pathogens and prevent future infections from the same specific organism. Conversely, the innate immune system is fast acting and often responds to general groups of pathogenic organisms. The innate immune system does not "learn" or improve over time. There are two types of response for the immune system; humoral and cellular. Humoral immunity includes the complement system, naturally occurring antibodies, and other soluble antimicrobial proteins [1]. These humoral immunity soluble proteins may be constantly circulating in the blood or are produced by cells in response to invading microorganisms. The cellular response of the innate immune system includes activation of phagocytic white blood cells. These include macrophages, granulocytes, and dendritic cells. The granulocytes are a subset of white blood cells that have multi-lobed nuclei and contain granules. Neutrophils, basophils, eosinophils, and mast cells make up this group. Basophils, eosinophils, and mast cells can initiate inflammatory pathways that result in allergies and asthma [2], however eosinophils are important for protection against parasitic infections [3, 4]. Neutrophils are the most abundant white blood cell and one of the initial responders during inflammation [5].

Neutrophils

Neutrophils remain inactive in the blood until external stimuli activate them to begin clearing an infection. When activated, neutrophils can aid in clearing an infection through phagocytosis, degranulation, and expelling Neutrophil Extracellular Traps (NETs). Phagocytosis

is the most common activity of neutrophils, and results in a target bacterium being engulfed it into a phagosome. Once the bacterium is in the neutrophil, granules bind to the phagosome and release large amounts of antimicrobial peptides and producing free radicals, resulting in death of the bacteria [5, 6]. Degranulation can also result in extracellular release of granules, which can cause tissue damage, but also results in death of extracellular invaders [6]. The last mechanism is the release of NETs. Neutrophils can release a network of granule proteins and chromatin that form a fibrous matrix that can trap and kill bacteria [7]. This can be activated in the presence of lipopolysaccharide or other soluble factors, such as interleukin-8 (IL-8) [7].

While neutrophils can be activated by cytokines produced by other immune cells, the most potent way to activate neutrophils is by direct contact with a pathogen. Neutrophils are able to detect bacteria and other microorganisms by the use of toll-like receptors (TLRs). TLRs are pattern recognition receptors, and can detect microbial associated molecular patterns (MAMPs). Neutrophils express all but one of the ten TLRs, majority of which are found on the cell surface [8, 9]. They can be used to detect gram-positive and gram-negative bacteria, viruses, and fungi. Several of the TLRs are used to detect bacteria specifically. TLR2 can act together with TLR1 or TLR6 to detect di-acylated or tri-acylated peptides on gram-positive bacteria. TLR4 is able to detect the lipid A of LPS on gram-negative bacteria [10]. Neutrophils can also detect bacteria via TLR5 which can detect bacterial flagellin [11]. Upon TLR activation, a neutrophil can then produce pro-inflammatory cytokines, increase free-radicals, and initiate phagocytosis [9].

Another way that neutrophils can detect infection is through complement receptors on the cell surface. Complement proteins are soluble in the blood and work as a signaling cascade to initiate inflammation, opsonization, and pore formation in a cell surface. When the complement cascade is active, complement proteins C3a, C4a, and C5a participate in inflammation, C5b can

form a membrane attack complex leading to pore formation and cell death, while C3b participates in opsonization. Opsonization occurs when complement proteins coat a cell surface, marking it for phagocytosis [12]. Neutrophils have complement receptors C1qR, CR1, CR3, CR4, and C5aR on their surface. When the CR1 receptor binds to C3b, the neutrophils can more easily phagocytize the cell that the complement is bound to [13, 14].

Chemokines

Innate immune cells, including neutrophils, need chemokines to migrate to the location they are needed. Chemokines are low molecular weight signaling proteins used in chemotaxis. There are four subfamilies of chemokines, CXC, CC, CX3C and XC, organized by the spacing of their first cysteine residues [15]. Chemokines can be present to direct regular cell movement, and can recruit immune cells to a sight of infection [15]. Homeostatically expressed chemokines are produced by the thymus and lymphoid tissue for regular cell migration, however a wide range of cells produce specific chemokines after infection occurs [16].

Immune cells need chemokine receptors to be able to recognize and interact with a chemokine. Chemokine receptors are commonly found on hematopoietic cells, and offer redundancy where multiple chemokines may bind to one receptor, and one chemokine may bind to multiple receptors. Chemokine receptors are G protein-coupled receptors that have seven trans-membrane domains [17, 18]. Hematopoietic cells have several different responses to binding to a chemokine, which can include chemotaxis, cytotoxic response, or others [15-18]. Immune cells follow a chemokine gradient and go the direction of higher concentration, migrating to the location of chemokine origination and infection.

Antimicrobial peptides

Cationic antimicrobial peptides (AMPs) are an important part of the innate immune system due to their ability to directly kill pathogens. These short proteins have been shown to use electrostatic interactions to bind to negatively charged phosphate groups on bacterial surfaces. Once bound, the peptide can then form pores in the bacterial cell, resulting in ion channels and cell death [19]. AMPs can be used as broad-spectrum antimicrobials and have shown to be effective against bacteria, enveloped viruses, and fungi.

There are two major categories of cationic AMPs; cathelicidins and defensins. The cathelicidins are named for their conserved cathelin regions. Only one cathelicidin, LL-37, is found in humans and is produced in epithelial cells and leukocytes [19-22]. Cathelicidins contain α-helical structures, while the defensins are made up of anti-parallel β-sheets. The defensins are sub-categorized into two groups; the α-defensins and β-defensins. The α-defensins are typically found in neutrophils and NK cells. The β-defensins are more wide-spread and can be found in a wide variety of leukocytes and epithelial cells [23]. Defensins have a positively charged domain as well as a hydrophobic domain that allow them to penetrate the negatively charged bacterial membrane and cause pores, leading to death [21, 24]. The hydrophobic domain can be important for stabilizing the peptide on the target cell [23, 24]. The ability of defensins to form pores in a cell surface has been shown by visualizing with electron microscopy electron-dense deposits on the cell surface where pores have formed [25].

Killing mechanism

There have been three proposed methods for AMP pore formation; barrel-stave, carpet, and toroidal-pore. The barrel-stave method requires multiple peptides to penetrate the cell

surface, with the hydrophobic region facing out. When multiple peptides have formed a ring in the membrane, the pore is formed resulting in cell death [26]. The carpet method proposes that the AMPs align parallel to the membrane, creating a "carpet" covering the cell with the hydrophobic regions contacting the lipid bilayer. As the coating is made, sections of the peptide coated membrane become separated and a pore is formed [26]. The toroidal-pore method acts similarly to the barrel-stave method due to multiple peptides forming a ring in the cell membrane. However, in the toroidal-pore method, the AMPs are causing the lipid monolayers to bend continuously through the membrane until bound with the opposite lipid monolayer, forming a pore of AMPs and hydrophilic lipid heads [26]. It has also been proposed that AMPs could affect bacteria through inhibiting enzymatic activity or synthesis of key cellular elements such as the cell wall, nucleic acid, proteins, or the cytoplasmic membrane [27].

Antimicrobial chemokines

Several chemokines have been found to have a novel function as an antimicrobial peptide. The antimicrobial chemokines are cationic and similar in structure to defensins [28]. However, not all cationic chemokines have antimicrobial properties [28]. Different chemokines appear to require different regions of their structure to act as an antimicrobial, which may mean that different antimicrobial chemokines have different killing methods. It has been found in CXCL6 that the N-terminal domain has more antimicrobial properties than the C-terminal domain [29]. Conversely, in CCL28 and CXCL9 it has been seen that the C-terminal domain is necessary for antimicrobial properties, and the N-terminal domain does not have any antimicrobial activity on its own [30, 31]. Multiple studies have shown membrane disruption as a killing mechanism of antimicrobial chemokines through electron microscopy [29], as well as flow cytometric assays with propidium iodide entrance into the cell [32]. These antimicrobial

chemokines have shown to be effective against gram-positive and gram-negative bacteria, fungi, and parasites [16, 30, 32].

CCL28

The chemokine CCL28, previously referred to as mucosae-associated epithelial chemokine (MEC), is found in mucosal tissues, including lungs, intestines, mammary glands, and nasal passages [33]. The chemokine binds to chemokine receptors CCR3 and CCR10. While most chemokines have 4 cysteine residues, CCL28 has 6. It is responsible for recruiting IgA-producing plasma cells, eosinophils, and activated T lymphocytes to these mucosal tissues [16, 34]

CCL28 has recently been shown to have direct antimicrobial activity [34]. It has been shown that there is a positively charged C-terminus that is required for CCL28 mediated bacterial killing [30]. The structure of this chemokine is highly similar to the structure of β-defensins and so it is believed that they have a similar killing mechanism [21]. The positively charged C-terminus alone is not sufficient to cause cell death, however it is unknown what else is required [30]. While CCL28 can kill bacteria in low-osmolarity conditions in vitro, in higher osmolarity solutions the peptide can bind to the surface of bacteria without causing death. The impact of such binding on bacterial interaction with host tissues or immune cells is unknown but deserves further investigation.

Lipopolysaccharide

Gram-negative bacteria have an inner membrane, with a thin peptidoglycan cell wall, and an outer membrane [35]. The outer leaflet of the outer membrane contains lipopolysaccharide (LPS). LPS consists of three sections; the lipid A, the core oligosaccharide, and the O-antigen.

Even though LPS is highly conserved, there is variability between the exact structure of the LPS between different bacterial strains [36]. Lipid A consists of an acylated glucosamine disaccharide with flanking phosphate groups and fatty acid chains that anchor the LPS to the bacterial membrane. The negatively charged phosphate groups can aid in dipole interactions with other LPS molecules. LPS is beneficial to bacteria by adding to physical rigidity, as well as decreasing permeability to prevent entrance of antimicrobial compounds [37]. LPS is highly immunostimulatory, immune cells can produce very potent pro-inflammatory cytokines such as TNF-α and IL-1β when even small amounts of lipid A are detected [38-40].

Yersinia pseudotuberculosis

Yersinia pseudotuberculosis is a gram-negative pathogenic bacterium that is ancestral to *Yersinia pestis*, the causative agent of bubonic and pneumonic plague [41, 42]. *Y. pseudotuberculosis* is less virulent than *Y. pestis* and causes Tuberculosis-like symptoms such as granuloma formation and local necrosis, as well as gastroenteritis and lymphadenitis with possible complications of reactive arthritis and erythema nodosum [41, 43, 44]. Even though *Y. pseudotuberculosis* and *Y. pestis* are so closely related, the infection methods between these two bacteria are very different. While *Y. pestis* can infect hosts by fleas and aerosols, infection by *Y. pseudotuberculosis* typically occurs through contaminated food and water. *Y. pseudotuberculosis* invade the intestinal epithelium through M cells into Peyer's patches or directly through the epithelial layer. They can colonize Peyer's patches within hours of infection or they can be engulfed by macrophages, where they can survive intracellularly, and taken to the lymph nodes to colonize. Pathogenic *Yersinia* species have a plasmid (pYV in *Y. pseudotuberculosis* and pCD1 in *Y. pestis*) that codes for necessary virulence factors, including a type III secretion system (TTSS) [44-47]. This TTSS is required for survival and replication in the host's lymphoid

tissue [46]. Through the TTSS *Yersinia* can secrete virulence factors known as Yersinia outer proteins (Yops) into a target host cell. Some Yops have been shown to induce apoptosis in phagocytic cells [45, 46].

While *Yersinia* can induce apoptosis in macrophages and dendritic cells, neutrophils are resistant to pYV+ *Yersinia* induced apoptosis [48, 49]. Phagocytosis of the bacteria is increased in pYV-deficient *Yersinia*; however neutrophil cell death occurs in higher levels due to reactive oxygen species production by neutrophils in response to phagocytosis of the bacteria [49]. It has also been shown that *Y. pseudotuberculosis* and *Y. pestis* are resistant to killing by neutrophils through use of the Yops [44, 47, 50]. In *Y. pestis* it has been shown that the PhoP-PhoQ two component system is important for neutrophil intracellular survival of the bacteria [48]. Although *Yersinia* devote considerable resources to surviving against neutrophil attack, they are critically important in early defenses against *Y. pestis* lung infections. *Y. pestis* that produce YopJ are able to suppress mouse neutrophil chemotaxis during the first 24 hours after infection. However, when neutrophils are induced to migrate to the lung prior to infection the numbers of bacteria are significantly decreased and mouse survival is enhanced [51]. However, a separate study found that *Y. pestis* was equally virulent in mice with or without neutrophils [52]. The interactions between *Yersinia* and neutrophils are important to study and understand since neutrophils are such a critical part of the innate defense system.

LPS modifications

Many pathogenic bacteria have defense mechanisms that can be used to evade a host's immune system during an infection. Since the LPS is so immunostimulatory due to the lipid A, many gram-negative bacteria have specific mechanisms to conceal LPS from being detected. For

example, *Yersinia* are able to alter their fatty acid chain composition when grown at various temperatures. When grown at 21ºC, *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* all produce hexa-acylated fatty acid chains on the lipid A. All three strains convert to a tetra-acylated lipid A when they are grown at 37ºC (Fig. 1) [53]. Having greater hexa-acylation patterns can aid in lower cell permeability and greater protection from antimicrobials that may cross the membrane. The bacteria benefit in mammalian infection with a tetra-acylation because it is less immunostimulatory. When monocytes are presented with the hexa-acylated lipid A, they respond by producing proinflammatory cytokine TNF-α. The same response was not observed when the tetra-acylated lipid A was used [53]. In addition to showing how bacteria can make physical alterations to be more clandestine, it shows that sometimes bacteria sacrifice some aspects of their physical fitness (in this instance, sacrificing membrane defenses) to increase probability of survival in a host.

The innate immune system frequently detects gram-negative bacteria due to the net negative-charge caused by the negatively charged phosphate groups on the lipid A structure. This negative charge facilitates cell recognition by cationic antimicrobial peptides, ultimately causing death to the bacteria by pore formation in the membrane [19]. Many gram-negative bacteria have the ability to modify the lipid A structure to conceal the negative charges that are present, resulting in decreased detection by cationic AMPs [40]. One mechanism used to accomplish this is the addition of phosphoethanolamine [54-56]. This addition causes a slightly less negative charge of the outer bacterial membrane by addition of phosphoethanolamine to either the lipid A or the core oligosaccharide. It has been shown that this mechanism does happen in *Yersinia*, and can be increased in low temperatures in *Y. pestis* [57]. Another defense mechanism is the addition of a positively charged amino sugar 4-amino-4-deoxy-L-arabinose (L-Ara4N) to the

lipid A structure at the negatively charged phosphate groups (Fig.1). This occurs in *E. coli, Salmonella, Yersinia*, and other gram-negative bacteria [56, 58]. This mechanism is carried out through the *pmrHFIJKLM* (*pmrF*) operon (Fig. 2a), sometimes referred to as the *arn* operon [59-61].

L-Ara4N modification carried out by the *pmrHFIJKLM* operon

The process of L-Ara4N production and addition is started when Ugd (encoded by the *pmrE* gene, which is not part of the *pmrF* operon) converts UDP-glucose to UDP-GlcUA [62]. The *pmrI* gene, sometimes referred to as *arnA*, is responsible for a large portion of the creation and addition of L-Ara4N to the bacterial surface (Fig. 2b). The PmrI protein has two domains that are necessary for L-Ara4N addition (Fig. 2b) [63], an N-terminal formyltransferase and a C-terminal decarboxylase. The C-terminus catalyzes the change of UDP-GlcUA to UDP-Ara4O [64]. PmrH then facilitates the change to UDP-L-Ara4N [65]. The N-terminal formyltransferase domain of PmrI transfers a formyl group to the UDP-L-Ara4N [64]. PmrF then transfers this molecule to the inner membrane by removing UDP and attaching the molecule to undecaprenyl phosphate in the inner membrane [65, 66]. PmrJ then removes the formyl group and the molecule is transferred to the outer membrane [67]. While it is still unknown exactly which proteins are responsible for the transport of the molecule to the outer membrane, it has been shown that PmrL and PmrM may play a role in this [68]. However, loss of the *pmrL* and *pmrM* genes does not prevent L-Ara4N addition, and there is evidence that these genes can be supplemented if function is lost [69]. Finally, PmrK transfers L-Ara4N to the lipid A structure in a well-characterized reaction [70, 71].

In *Y. pestis* it has been shown that in the absence of *arnB* (*pmrH*) there is a compensatory gene, *wecE*, which partially makes up for the loss of the gene [72]. L-Ara4N was still added to the Lipid A in lower levels even after deletion of the *arnB* gene. The *wecE* gene is known for its role in biosynthesis of enterobacterial common antigen (ECA). ECA is found on all members of the *Enterobacteriaceae* family of bacteria. ECA is found on the outer leaflet of the outer membrane, similar to LPS. ECA is partially composed of an aminosugar heteropolymer [73]. L-Ara4N is also an aminosugar so it is reasonable that a gene helping the production of ECA could also help the production of L-Ara4N. After the loss of *wecE* and *arnB*, the L-Ara4N was no longer added [72]. This results in maintaining a higher level of resistance to cationic antimicrobial peptides even if there are conditions that prevent the original L-Ara4N addition mechanism to be functional. Specifically, the *wecE* gene codes for TDP-4-oxo-6-deoxy-D-glucose transaminase. There is a gene in *Y. pseudotuberculosis*, *rffA*, that is assumed codes for the same product, based on 99.73% gene homology [74]. There have been no studies on this gene, however if the assumption is correct that this gene is the same as *wecE*, then the *rffA* gene may be able to aid in the addition of L-Ara4N in *Y. pseudotuberculosis* as well.

The impact of L-Ara4N lipid A modification on bacterial fitness in host environments is not straightforward. While in *S. typhimurium* it has been shown that loss of the *pmrF* operon results in decreased virulence of the bacteria during infection [75], in *Y. pseudotuberculosis* a loss in *pmrF* function does not affect virulence in mice when infected by either intravenous or via gastric inoculation [60]. However, L-Ara4N has been implicated in the ability of *Y. pestis* to survive in fleas [71]. Interestingly, recent evidence also suggests that L-Ara4N modification may be detrimental to bacteria in some instances. Researchers investigating *Burkholderia* infection in Cystic Fibrosis patients were able to synthesize L-Ara4N modified and unmodified *Burkholderia*

lipid A. Their study showed that when the lipid A was modified with L-Ara4N, HEK cells were stimulated to produce NF-κB and macrophages were stimulated to produce IL-8, which would result in increased inflammation. Neither of these responses occurred when the lipid A was not modified [76]. Others have shown that *Burkholderia* penta-acylated lipid A is still highly immunostimulatory compared to hexa-acylated lipid A due to the L-Ara4N residues on the lipid A [77]. It's possible that the positive charges of the L-Ara4N are responsible for activating parts of the TLR4-MD2 complex, resulting in activated immune cells. These studies suggest that L-Ara4N modified lipid A may be a detriment to bacteria trying to evade detection by the immune system.

Regulation of the *pmrF* operon

In *Salmonella* and *Y. pestis*, the *pmrF* operon is regulated by the PmrA-PmrB and the PhoP-PhoQ two-component systems [60, 61, 78, 79]. These systems have been best studied in *Salmonella enterica* serovar *typhimurium* in response to high iron and/or low magnesium levels. In this *Salmonella* strain, PmrB is the sensor kinase that is activated by high Fe^{3+} levels, which in turn activates the regulatory protein PmrA to directly control *pmrF* expression [60]. It has also been shown that PmrA activation occurs in low Mg^{2+} concentrations when the PhoP-PhoQ system is activated. This leads to an increase in production of PmrD, which activates PmrA at a post-transcriptional level [60] (Fig. 3A). High levels of Fe^{3+} also reduce PmrD transcription [80] thus reducing the input from the PhoP/Q system. *Y. pestis* does not have PmrD, but can still use both two-component systems to regulate *pmrF* expression (Fig. 3B) [48]. *Y. pseudotuberculosis* also lacks PmrD, and unlike in *Y. pestis*, *pmrF* expression is independent of PmrA-PmrB (Fig. 3C) despite a conserved PmrA binding site [60, 61]. Polymyxin resistance has been shown to increase in *Y. pseudotuberculosis* in low Fe^{3+} levels [60], and a second LysR-type regulator for

the *pmrF* operon was identified [69]. This second regulator is not present in *Y. pestis* and is significantly different in *Y. enterocolitica*.

These discrepancies among the ways closely related *Yersinia* regulate the *pmrF* operon are interesting and suggest a need for clarification of the exact factors that control *pmrF* expression and polymyxin resistance. The influence of temperature has been especially difficult to establish. For instance, *Y. pestis* strain KIM resistance to polymyxin was found to be unchanged at various temperatures [81], while another group found *Y. pestis* strain KIM6+ resistance to polymyxin is increased at 21ºC compared to 37ºC [53]. *Y. enterocolitica* is more susceptible to polymyxin than either *Y. pestis* or *Y. pseudotuberculosis* under every condition [53, 81]. However, non-pathogenic *Y. enterocolitica* strain PR serotype O:1,6 resistance to polymyxin is decreased at 37ºC [81] as is L-Ara4N modification in Y. *enterocolitica* strain 8081 [79]. Conversely, most previous studies have shown that *Y. pseudotuberculosis* resistance to polymyxin is decreased at temperatures lower than 37ºC (21ºC, 26ºC) [53, 81], even though *Y. pseudotuberculosis* strain WS 66/89 serogroup III exhibited diminished resistance at 37ºC [81]. One report suggested that *pmrF* transcript levels in *Y. pseudotuberculosis* were nonexistent at temperatures below 37ºC [60], however L-Ara4N addition has been measured at higher and lower temperatures in both *Y. pseudotuberculosis* and *Y. pestis* [53]. The exact mechanisms contributing to polymyxin resistance need to be clarified due to such high diversity between *Yersinia* species and strains. Further, the differences that are seen in *pmrF* expression patterns suggest that *pmrF* regulation is more complex than one would assume. Even significant differences are seen between strains of *Yersinia* that are very closely related. This highlights the need for further study of more isolates so that L-Ara4N regulation can be better understood.

EXPERIMENTAL APPROACH

Due to the dramatic differences in polymyxin resistance between *Y. pseudotuberculosis* and the other *Yersinia* species, the exact conditions of *pmrF* regulation need to be studied in greater detail. We determined to do this by focusing on three objectives.

The first objective of this study was to characterize the expression of the *pmrF* operon in *Y. pseudotuberculosis* IP32953 under various environmental conditions. To do this, we cloned the *pmrF* promoter in front of a promoterless green fluorescent protein (GFP) gene to create a reporter plasmid. We then used flow cytometry to measure expression changes in response to various external factors. We wanted to test different environmental conditions that the bacteria may encounter before and during an infection, so expression was measured in rich and minimal media, high (37ºC) and low (21ºC) temperatures, and in the presence of human serum and antimicrobial chemokine CCL28. We predicted that expression of the operon would increase at 37ºC, as well as in the presence of human serum and CCL28 since the bacteria might benefit from the added protective measures of L-Ara4N addition under these conditions.

The second objective of this study was to measure the effect that the *pmrI* gene (required for L-Ara4N addition) has on bacterial resistance to antimicrobial chemokine CCL28. In order for *Y. pseudotuberculosis* to establish a successful infection, they must cross through the mucosal layer of the intestinal epithelium. This means they need to be able to avoid host defense peptides, including antimicrobial chemokines, and phagocytic cells. We wanted to see the importance of L-ara4N addition in the bacterial resistance of these various conditions. Since the *pmrI* gene is responsible for multiple steps in the production of L-ara4N [63], it seemed a clear target to knock-out in an attempt to remove L-ara4N addition to the bacteria. Since CCL28 is produced in

mucosal surfaces, it is reasonable to assume that this is one antimicrobial chemokine that *Y. pseudotuberculosis* would encounter during an infection. This first required the creation of a Δ*pmrI* mutant and complemented mutant in the *Y. pseudotuberculosis* IP32953 background. Using the Δ*pmrI* and wild-type strains of *Y. pseudotuberculsosis*, we then used flow cytometry to detect levels of CCL28 binding to bacteria, as well as bacterial susceptibility to killing by the peptide. We hypothesized that the cationic CCL28 would be repelled by the positively charged L-Ara4N, resulting in the Δ*pmrI* mutant showing higher binding and reduced survival compared to the wild-type strain.

The third objective of these studies was to intended to help us better understand the impact that the L-Ara4N addition and CCL28 binding may have on the outcome of interactions with human neutrophils. Since neutrophils are the most common white blood cell and are very important first responders to bacterial infection, we decided to use them as a model for observing the effects of L-Ara4N addition on phagocytosis. In addition, the consequences of CCL28 binding to the surface of a bacterium have not been studied. Pathogens binding to host proteins often result in different consequences. For example, when a bacterium is coated in the complement protein they are marked for phagocytosis by immune cells [12, 13]. However, there are some pathogens that can coat themselves in human proteins to avoid detection by the immune system [82, 83]. We wanted to see if CCL28 binding to the surface of *Y. pseudotuberculosis* would result in either of these effects. We developed a phagocytosis assay in which GFP expressing strains of *Y. pseudotuberculosis* were used to measure the proportion of neutrophils that are able to phagocytose bacteria under different conditions. We compared the efficiency of the neutrophils to engulf wild-type, Δ*pmrI* mutant, and Δ*pmrI* mutant complemented strains using flow cytometry. We also measured the effect of CCL28 binding to

the bacterial surface had on neutrophil phagocytosis. We hypothesized that a lack of L-Ara4N on the surface of the Δ*pmrI* mutant, would increase the ability of neutrophils to phagocytose bacteria. We also hypothesized that CCL28 bound bacteria would increase phagocytosis, acting as an opsonin.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Yersinia pseudotuberculosis IP32953, a virulent clinical isolate, was used in these studies. The *Y. pseudotuberculosis hldD*::Tn5 mutant was made previously in a non-virulent *Y. pseudotuberculosis* via random transposon mutagenesis and identified as having a truncated LPS down to the heptose group in the core region [84, 85]. The *hldD*::Tn5 mutant contains kanamycin resistance. GFP fluorescent versions of wild-type (wt) *Y. pseudotuberculosis*, *Y. pseudotuberculosis* Δ*pmrI*, and *Y. pseudotuberculosis* Δ*pmrI* + pACYCpmrI (see below) were created by electroporating pAKgfp1 plasmid (Addgene plasmid # 14076) into each strain [86]. The MFDpir strain of *E. coli* has been previously infected with Mu bacteriophage, contains genes that allow replication of Mu-dependent suicide plasmids, and requires diaminopimelic acid (DAP) addition to media to replicate [87]. Overnight cultures were inoculated in 3mL Terrific Broth (TB) (Gentrox, Worcester, MA) shaking at 37°C unless otherwise stated. Antibiotics were added when appropriate; chloramphenicol (10μg/mL), ampicillin (100 μg/mL), and kanamycin (30μg/mL).

Electroporation

Electrocompetent cells were made by growing 5mL of the strain overnight, pelleting the cells into 4 microcentrifuge tubes, and washing the cells four times in molecular grade water on ice. The electroporation took place in a 1mm cuvette on setting EC1. The transformed cells were immediately suspended in 800μL of Recovery Media (Lucigen) and shaken at the appropriate temperature for 1-2 hours before plated.

Mutant Generation

The Δ*pmrI* mutant of *Y. pseudotuberculosis* was created using a technique previously described [88]. Briefly, by amplifying the upstream region and downstream region (with 20bp out of gene either side and 40 bp within the gene) of the *pmrI* gene using primers that would create products with areas of homology with each other. The primers used were the *pmrI* upstream F, *pmrI* upstream R, *pmrI* downstream F, and *pmrI* downstream R (Table 1). Once the two pieces were amplified, 1µL of each product was combined together with 8µL of taq polymerase and 10µL of molecular grade water. To "stitch" the upstream and downstream regions together, a PCR was run with the following conditions: 98°C for 1 minute and 30 seconds, then 15 cycles of 98°C for 15 seconds, 55°C for 20 seconds, and 72°C for 1 minute and 30 seconds. Then 1µL of this new product is used as a template for a new PCR reaction using 2 nested primers, one of which is upstream of the gene and one downstream, pmrI nested F and pmrI nested R (Table 1). The resulting product, as well as suicide plasmid pRE112 (Addgene plasmid # 43828) was digested with XbaI and SacI and then ligated together. The pRE112 plasmid contains a SacB gene for sucrose sensitivity [89]. This new plasmid was then heat shocked into chemically competent *MFDpir* and the cells were grown on Luria Broth (LB) plates that had be supplemented with chloramphenicol and diaminopimelic acid (DAP). *MFDpir* cells require the addition of DAP to grow. Once the *MFDpir* cells with the plasmid were grown up, they were mated with *Y. pseudotuberculosis* by combining 500µL of each culture and pelleting the cells together, resuspending them in 30µL TB and spotting them on a TB + DAP plate for 24 hours. The cells were then collected and grown overnight in TB with chloramphenicol. The resulting *Y. pseudotuberculosis* cells contained single crossover event with the plasmid, since the plasmid cannot replicate in *Y. pseudotuberculosis* without crossing over. Cells were then grown in

antibiotic free media to allow a double-crossover event to take place. The cells were then plated on TB + sucrose plates and a colony PCR was performed to check the length of the *pmrI* gene, using the pmrI upstream F and pmrI downstream R primers, several colonies were positive for deleted *pmrI* gene.

Chemokine Preparation

Lyophilized human CCL28 and CCL20 were purchased from PeproTech, Rockyhill, NJ and prepared by diluting each vial of 100µg into 2mL of filtered PBS containing 0.1% BSA. The mixture was left at room temperature to allow the chemokine to properly dissolve. The chemokine was then put into single dose aliquots of 12µL to avoid multiple freeze/ thaw cycles. The final concentration of the chemokine preparation was 0.5µg/10µL.

pmrHFIJKLM Reporter Strain Generation

The *pmrF* operon promoter was amplified using the "*pmrH* pro F" and "*pmrH* pro R" primers (Table 1), resulting in the addition of XbaI and HindIII restriction sites to the 5' and 3' end respectively. The product was then digested with XbaI and HindIII restriction enzymes. The gfp gene was amplified from the pAKgfp plasmid using the gfp F HindIII and gfp R SalI primers (Table 1), resulting in the addition of HindIII and SalI restriction sites to the 5' and 3' end respectively. The gfp gene product was then digested with HindIII and SalI restriction enzymes. The digested promoter and gfp products were then ligated together. The pACYC184 plasmid (GenBank Accession #: X06403) was digested with XbaI and SalI restriction enzymes. This plasmid contains a chloramphenicol resistance gene [90]. The *pmrF* promoter-gfp construct was then ligated into the digested pACYC184. The plasmid was then heat shocked into chemically competent *E. coli* (Lucigen, Middleton, WI). Once the *E. coli* were grown overnight, the plasmid

was isolated using the QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA). The plasmid was then electroporated into electrocompetent wild-type *Y. pseudotuberculosis* and grown overnight in TB media with chloramphenicol. A colony PCR was then performed to confirm plasmid presence.

pmrF Expression Assay

The *pmrF* expression reporter strain of *Y. pseudotuberculosis* was grown overnight at 30ºC in M9 and TB media. The bacteria were subcultured by adding 50µL of culture to 1mL of TB or M9 with a negative control and the appropriate test condition; either 10% human serum or 0.4nM CCL28 or CCL20. After 6 hours of incubation the samples were run on the flow cytometer to detect GFP levels. Flow cytometry data was obtained using a BD Accuri C6 Flow Cytometer and analyzed using the BD Accuri software. The mean fluorescence was compared between the varying conditions to determine gene expression.

Growth Rate in Polymyxin

After the wild-type, Δ*pmrI,* and Δ*pmrI* complemented strains of *Y. pseudotuberculosis* were grown overnight, each strain was diluted to an OD600 of 1.0 +/- 0.1. The cultures were then centrifuged and resuspended in 1mL of PBS. In triplicate, 20µL of bacteria and 10µL of BSA were added to individual microcentrifuge tubes and incubated at 37ºC for 30 minutes. A 96 well plate was prepared with an LB/ rezasurin/ polymyxin mixture at 0.135mg/mL concentration rezasurin. 50µL of this initial mixture was added to columns 1,2, and 4-12 of the 96 well plate. Polymyxin was added to the wells in column 3 in LB at a concentration of 100µg/mL and 1:2 dilutions of polymyxin were created across the plate. The highest level of polymyxin is 100µg/mL and the dilutions continue until 0.2µg/mL. Column 1 and 2 have 50µL of PBS added.

After the 30 minute bacteria incubation is complete, the cells are washed and resuspended in 2mL of PBS. 50µL of this culture is then added to columns 2-12. The plate is then read on a plate reader measuring rezasurin fluorescence to obtain a time 0 reading and then incubated at 37ºC for the duration of the experiment, with plate readings being taken every 30 minutes. Resazurin becomes fluorescent when metabolized by the bacteria, this allows resazurin to be used to achieve a very accurate minimum inhibitory concentration.

Chemokine Binding Assay

After the wild-type, Δ*pmrI,* and Δ*pmrI* complemented strains of *Y. pseudotuberculosis* were grown overnight, each strain was subcultured and grown for approximately 2 hours, until the OD600 read 0.6. 1mL of each culture was then centrifuged and resuspended in 100µL 0.01% BSA in PBS (PBSA). In triplicate, 5µL of culture was added with 100uL BSA and either 10µL 0.1% BSA in PBS or 10µL of CCL28 with a final concentration of 0.5µg/15µL. The samples were then incubated on ice for 30-60 minutes. The samples were all then centrifuged and washed three times with PBSA. Then 10µL of streptavidin-labeled anti-CCL28 antibody (R&D Systems) was added to each tube for 30 minutes to allow the antibody to bind. They were then washed in PBSA, and 5µL fluorescent APC (which binds to streptavidin) (BD Biosciences) is added to 1mL PBSA and then 100µL of this was added to each sample. The samples incubated for another hour, when they were then washed in PBSA again. The samples were then run on a flow cytometer to see how many of the bacterial cells were APC positive, meaning the cells had bound CCL28. Flow cytometry data was obtained using a BD Accuri C6 Flow Cytometer and analyzed using the BD Accuri software.

Chemokine Killing Assay

After the wild-type, Δ*pmrI,* Δ*pmrI* complemented strain, and *hldD::*Tn5 strains of *Y. pseudotuberculosis* were grown overnight, each strain was subcultured by adding 50µL overnight culture to 2mL TB and grown for approximately 2 hours, until the OD600 read 0.6. Then 1mL of each culture was centrifuged and resuspended in 1mL of 1mM potassium phosphate buffer (PPB) (a low osmolarity buffer). 40µL of each culture was added to 1mL of 1mM PPB to dilute. From this, in triplicate, 100uL of culture was added individual microcentrifuge tubes. Either 10µL of 0.1% BSA or 10µL of CCL28 was added for a final concentration of 0.5µg/100uL. The tubes were then vortexed thoroughly and incubated at 37°C for 90 minutes. After the incubation, 200µL of dilute 1µm polystyrene beads (1:62500) (Polybead ® Polystyrene 1µm Microspheres, Polysciences Inc., Warrington, Pennsylvania) were added to each sample, followed by 7µL of propidium iodide (PI) (Invitrogen, Carlsbad, California) and vortexed. Flow cytometry was used to count to 30,000 beads and numbers of viable cells were compared to bead count. Flow cytometry data was obtained using a BD Accuri C6 Flow Cytometer and analyzed using the BD Accuri software. The percent survival is then calculated with the following equation (Treated (bacteria total-PI+)) / (Untreated (bacteria total-PI+)) x100.

Neutrophil Isolation

Human neutrophils were isolated from whole human blood using a density gradient according to a protocol previously outlined [91]. Briefly, 5mL whole human blood obtained from volunteer donors was layered onto 5mL of Lympholyte Poly (Cedarlane Laboratories, Burlington, Ontario) and centrifuged at room temperature for 35 minutes. The polymorphonuclear cell layer was

removed and treated with red blood cell lysis buffer multiple times until there were no red blood cells visible. The cells were then washed in Hanks Balanced Salt Solution and are counted using a hemocytometer.

Phagocytosis Assay

After the GFP wild-type, GFP Δ*pmrI,* and GFP Δ*pmrI* complemented strains of *Y. pseudotuberculosis* were grown overnight with ampicillin, each culture was diluted to an OD600 of 1+/- 0.1 and then resuspended in Hanks' Balanced Salt Solution (HBSS)(Gibco by Life Technologies). In triplicate, 1,000,000 bacteria from each culture were added to individual microcentrifuge tubes containing 90µL HBSS. Each tube either had 10uL of human serum (obtained from a volunteer donor), BSA, or CCL28 added. All of the tubes were then vortexed and incubated at 37°C for 30 minutes. The bacteria were then washed in HBSS and 100,000 human neutrophils were added to each tube in a volume of 200uL. These samples were then incubated at 37°C for 30 minutes. The samples were then put on ice and run on the flow cytometer. Flow cytometry data was obtained using a BD Accuri C6 Flow Cytometer and analyzed using the BD Accuri software. The flow cytometer counted to 10,000 neutrophils by gating around the appropriate population and the percent of neutrophils that were positive for GFP were observed.

RESULTS

Expression of *pmrHFIJKLM* operon increases at 21ºC, in rich growth media, and in human serum

It is known that in *Yersinia pestis and Yersinia enterocolitica* the susceptibility to polymyxin is much greater at 37ºC than at lower temperatures [53, 79], and *Y. enterocolitica* has been shown to have decreased *pmrF* expression at 37ºC [79]. Interestingly, *Y. pseudotuberculosis* PB1 strain has shown to follow the opposite trend, in that susceptibility to polymyxin is greater at 21ºC [53]. The effect of temperature on expression of the *pmrF* operon has not been reported in *Y. pseudotuberculosis* strain IP32953. We wanted to determine whether the expression of the *pmrF* operon in *Y. pseudotuberculosis* IP32953 follows the same trend that has been seen in *Y. pseudotuberculosis* strain PB1 and *Y. pestis,* or if it would resemble *Y. enterocolitica*. A reporter plasmid carrying the *pmrF* promoter (including the PhoP binding site) fused to GFP was created. We tested the effect of growth temperature, both in rich growth media Terrific Broth (TB) and minimal media M9, on *pmrF* promoter activity. After the bacteria were grown in their respective media overnight at 30ºC, they were subcultured into the same media at 21ºC or 37ºC for six hours and the fluorescence of the population was determined by flow cytometry. As seen in Fig. 4, the expression of the *pmrF* operon is slightly higher at 21ºC than at 37ºC in both media conditions ($p<0.05$). In addition, there was significantly increased expression of the *pmrF* operon in TB over M9 media at both temperatures.

We then wanted to determine if *pmrF* activity correlated with polymyxin resistance in *Y. pseudotuberculosis* IP32953. We predicted that since we observed higher *pmrF* promoter activity at 21ºC than 37ºC, we would also see higher resistance to polymyxin at this temperature. We grew bacteria in TB media at either 21 or 37ºC and then exposed them to polymyxin for 2

hours in a low-osmolarity buffer. As seen in Fig. 5, the survival of the bacteria grown at 37 ºC was significantly higher than those grown at 21ºC, consistent with the pattern reported for strain PB1. Therefore, resistance to polymyxin does not mirror promoter activity of *pmrF*.

When exposed to sublethal concentrations of antimicrobials, other bacteria increase PhoP-PhoQ signaling and thereby upregulate L-Ara4N addition [92]. This may be a survival mechanism where bacteria detect early signs of membrane disturbance and increase defensive mechanisms. To investigate whether similar response could occur in *Y. pseudotuberculosis*, we tested the effect of membrane-damaging antimicrobial substances that could be encountered during infection on *pmrF* expression. These included the chemokine CCL28, CCL20, and human serum. To test the effect of antimicrobial chemokines, the reporter strain was incubated with CCL28 or CCL20 for 3-4 hours in TB at 37ºC and flow cytometry was used to measure *pmrF* promoter activity. Incubating the bacteria in phosphate-buffered saline with either CCL28 or CCL20 had no effect on *pmrF* expression (Fig. 6). To test the effect of normal human serum on *pmrF* expression, bacteria were cultured in TB broth with 10% serum for 6 hours at both 21ºC and 37 ºC. The presence of serum did result in a significant increase in promoter activity of the operon ($p < 0.05$), but only at 21ºC (Fig. 7).

We reasoned that increased *pmrF* expression caused by serum in the growth media should result in enhanced resistance to polymyxin. To test this, we pretreated the bacteria in the same way as for the serum expression assay above. After the bacteria had incubated with or without serum at 21ºC and 37ºC in TB media, they were washed and their ability to withstand a polymyxin challenge was determined. Surprisingly, growth with serum at both temperatures increased survival of polymyxin-challenged bacteria (Fig. 8) compared to bacteria grown without serum. Therefore, increased expression of the *pmrF* operon when bacteria are exposed to human

serum at 21ºC may enhance bacterial protection against polymyxin. However, since we saw a similar increased resistance of bacteria grown in serum at 37ºC (despite no effect on *pmrF* expression), other changes independent of *pmrF* may be caused by serum that result in increased polymyxin resistance.

The Δ*pmrI* mutant of *Yersinia pseudotuberculosis* is more susceptible to killing by antimicrobial chemokine CCL28

To investigate the role of the *pmrF* operon in resistance to antimicrobial peptides, we created a Δ*pmrI* deletion mutant in the *Y. pseudotuberculosis* IP32953 strain. Since *pmrF* expression was shown to vary with temperature, we wanted to determine whether Δ*pmrI* mutation would affect resistance to polymyxin at 21ºC and 37 ºC. We tested the ability of wild type and the Δ*pmrI* mutant to grow in the presence of varying concentrations of polymyxin. As seen in Fig. 9, the mutant strain was significantly more sensitive to polymyxin at both growth temperatures. Whereas the wild-type strain was uninhibited even at the highest levels of polymyxin, the Δ*pmrI* mutant was no longer able to grow in polymyxin at the concentration of 1.5 μg/mL at 21ºC and 3.1μg/mL at 37ºC. This verified that the polymyxin resistance phenotype was lost with knock-out of the gene. Interestingly we consistently saw slightly increased resistance to polymyxin at 37ºC compared to 21ºC in the Δ*pmrI* mutant.

We then sought to determine the importance of the *pmrF* operon in *Y. pseudotuberculosis* survival against antimicrobial chemokines. Bacterial defenses against antimicrobial chemokines have not been thoroughly studied. However, since parallels have been made between antimicrobial chemokines and cationic antimicrobial peptides [28-30], we predicted that L-Ara4N addition would be important in bacterial protection against these chemokines. We

measured the survival of Δ*pmrI* mutant strain of *Y. pseudotuberculosis* when challenged with CCL28 compared to those of the wild-type strain and to an *hldD*::Tn5 mutant, which has been previously shown to be very sensitive to CCL28 killing [84, 85]. Survival was calculated by dividing viable bacteria of the treated sample by viable bacteria in the BSA control. As shown in Fig. 10, the Δ*pmrI* mutant strain had decreased survival compared to the wild-type, yet was able to survive better than the *hldD*::Tn5 mutant. The difference between survival of the wild type and *pmrI* knock-out strain was significant ($p < 0.05$).

CCL28 binding is reduced in the Δ*pmrI* mutant compared to wild type *Y. pseudotuberculosis*

In previous studies, it has been shown that *Y. pseudotuberculosis* mutations that increase binding to CCL28 also result in reduced survival in the presence of the chemokine [84, 85]. To determine if this relationship is also true for the Δ*pmrI* mutant, binding levels of CCL28 to wild-type, Δ*pmrI*, and Δ*pmrI* complemented strains of *Y. pseudotuberculosis* were measured using a flow cytometry assay. While the wild-type and the complemented mutant had similarly high levels of binding, surprisingly the Δ*pmrI* strain bound far less ($p < 0.05$) (Fig. 11).

Since these results conflict with previous studies using other mutants that have shown killing correlates with binding, we wanted to investigate this result further. One possible explanation is that the CCL28 is internalizing into the bacteria or falling off in solution. In an attempt to determine if either of these situations are occurring, a binding assay with 5, 15, and 30 minute incubations with the chemokine was performed once. The results show that at each time point the Δ*pmrI* mutant has lower binding levels compared to the wild type and *pmrI* complement strains (data not shown).

Neutrophils less efficiently phagocytize Δ*pmrI* mutant *Y. pseudotuberculosis*

Y. pseudotuberculosis depends on being able to resist the antimicrobial activities of phagocytic cells, including neutrophils. While colonizing mucosal surfaces, high *pmrF* expression levels and the presence of antimicrobial chemokines may influence the ability of neutrophils to phagocytose the bacteria. To investigate these possibilities, a neutrophil phagocytosis assay was developed wherein bacteria constitutively expressing GFP were incubated with human neutrophils and flow cytometry was used to observe the percent of neutrophils that had engulfed bacteria. The Δ*pmrI* strain of *Y. pseudotuberculosis* showed decreased phagocytosis efficiencies compared to wild-type, even when opsonized with human serum ($p < 0.05$) (Fig. 12). To determine if CCL28 binding has an effect on phagocytosis, wild-type *Y. pseudotuberculosis* constitutively expressing GFP was pretreated with either human serum (positive opsonization control), CCL28, or BSA (negative control) and then incubated with human neutrophils. Flow cytometry analysis revealed that the serum-opsonized bacteria were better engulfed than non-opsonized bacteria. There was no change in neutrophil phagocytosis ability when CCL28 was bound to the bacteria (Fig. 13).

DISCUSSION

In these studies, we investigated how the bacterial environment influences expression of the *Y. pseudotuberculosis* strain IP32953 *pmrF* operon, and how its activity influences bacterial phenotypes relevant to virulence. We found that similar to other *Yersinia* species [53, 81], *pmrF* expression in strain IP32953 at 37ºC is decreased as compared to 21ºC. However, it appears that the media has a stronger influence on expression than growth temperature. There was a significant decrease in expression when grown in M9 minimal media, compared to rich growth media TB (Fig. 4).

The effects of nutrient availability may be relevant to the infectious strategy of *Y. pseudotuberculosis.* Previous studies have used rich growth media when measuring *pmrF* activity [53, 60], and we have determined here that nutrient availability may be a greater influence on *pmrF* expression than temperature. As *Y. pseudotuberculosis* move through the intestinal environment they would be exposed to high levels of nutrients, resulting in an increase in *pmrF* expression. This could prepare them to resist antimicrobial peptides that are present in the mucosal layer and are produced by epithelial cells when crossing the epithelial membrane. After the bacteria cross the epithelial barrier and migrate through the lymph system they would have decreased nutrient availability, possibly resulting in lower levels of *pmrF* expression.

According to our results in nutrient-reduced media, potentially reduced *pmrF* expression beyond the intestinal mucosa could then increase survival against immune cells. The fact that neutrophils were better able to phagocytose wild-type (*pmrF* expressing) bacteria than *pmrI* mutants (Fig. 12) could indicate that downregulation of these genes might prevent clearance by phagocytes. Further, others have shown that lipopolysaccharides from *Burkholderia* that have L-Ara4N induce stronger inflammatory responses than LPS without L-Ara4N [76, 77]. If the same

is true for *Y. pseudotuberculosis* LPS then downregulation of the *pmrF* operon in this environment might reduce cytokine production and activation of immune cells, thereby promoting colonization in deeper tissues. To test this, *pmrF* transcript levels could be measured using qPCR after *Y. pseudotuberculosis* mouse infection. It has previously been shown that L-Ara4N modified LPS can strongly activate the TLR4 complex on immune cells, even when lipid A has a lower acylation pattern [77]. To test if the change in phagocytosis that is seen is purely a TLR4 response to L-Ara4N modified bacteria, an anti-TLR4 antibody could be added to the neutrophils to inhibit TLR4 activity before the bacteria are introduced. If the decrease in phagocytosis was absent in the Δ*pmrI* strain, then it could be assumed that the decrease is due to lower TLR4 activation on the neutrophils.

After determining that *pmrF* promoter activity was increased at 21ºC compared to 37 ºC, we decided to test whether *pmrF* promotor activity correlated with polymyxin resistance. As seen in Fig. 5, the survival of the bacteria grown at 37 ºC was significantly higher than those grown at 21ºC, consistent with the pattern reported for strain PB1 [53]. Therefore, resistance to polymyxin does not mirror promoter activity of *pmrF.* This may suggest that there are additional regulatory mechanisms that act downstream of *pmrF* promoter activity that could modulate L-Ara4N addition, or that bacterial factors unrelated to the *pmrF* operon could influence survival in polymyxin, especially at 37 ºC. The fact that a slight increase in resistance to polymyxin was observed in the Δ*pmrI* mutant at 37ºC compared to 21ºC (Fig. 9) may indicate another mechanism contributing to polymyxin resistance. To test this, we could create random transposon mutants of the Δ*pmrI* mutant and perform the resazurin growth curve in various levels of polymyxin to look for a mutant that results in no difference in survival against polymyxin at either temperature.

An important caveat of our results is that we measured expression only by *pmrF* reporter expression. To verify these results, it will be necessary to further test *pmrF* expression by qPCR transcriptional analysis of genes in this operon. Others have reported that transcripts of *pmrF* virtually disappear at temperatures below 37ºC [60], but those studies used a different strain and they did not show this data. If the qPCR analysis showed increased transcripts compared to promoter activity, then we could assume that there is possibly another promoter site and alternate regulator for the genes. However, if the transcript levels were comparative to that of the promoter activity, then it's possible that there is a downstream regulator possibly cleaving the RNAs and preventing them from being translated. It would also be reasonable to assume that there is another mechanism contributing to polymyxin resistance at 37ºC. Our finding that the Δ*pmrI* mutant strain is hyper-susceptible to polymyxin both at 21ºC and at 37ºC strongly suggests that L-Ara4N is added at both temperatures. Further, others have detected the presence of L-Ara4N by mass spectrometry analysis of purified lipid A from *Y. pseudotuberculosis* at both temperatures [60]. It is unknown the exact amount of L-Ara4N that is added at either temperature. It will also be important to determine whether the PhoP-PhoQ system in *Y. pseudotuberculosis* controls the temperature-dependent *pmrF* expression and polymyxin resistance.

We decided to test *pmrF* expression in other conditions that *Y. pseudotuberculosis* might encounter during infection that could cause membrane disruption, such as human serum and antimicrobial chemokines. We found that in the presence of normal human serum there was an increase in *pmrF* expression at 21ºC, but no significant effect at 37ºC (Fig. 7). *Y. pseudotuberculosis* has the ability to resist killing by human serum at 37ºC due to expression of YadA and Ail proteins at this temperature that prevent complement related killing [93]. Thus it is

possible that complement activation does not progress sufficiently at 37ºC to activate similar transcriptional changes seen at 21ºC. Clearly, it would be rare for *Y. pseudotuberculosis* to be exposed to serum at lower temperatures. Thus the physiological relevance of the enhanced *pmrF* expression in the presence of serum may be questioned. However, our results showing that growth in serum at both 21ºC and 37ºC enhances bacterial survival on subsequent exposure to polymyxin suggests an additional adaptive response independent of *pmrF* and L-Ara4N addition that may contribute to resistance to host defense mechanisms (Fig. 8). In order to test if *pmrF* has an effect on *Y. pseudotuberculosis* resistance against human serum, we could perform a normal human serum killing assay with the wild-type and Δ*pmrI* strains of *Y. pseudotuberculosis*. To test if the effects of the *pmrF* expression changes were due to complement binding, the same expression assay could be repeated with heat killed serum. If there was still an increase in *pmrF* expression, it could not be due to complement.

The addition of L-Ara4N in *Y. pseudotuberculosis* has benefits to the survival of the bacteria when challenged against cationic antimicrobial peptides. This is well characterized in several bacterial strains and the *pmrF* operon genes responsible for the addition of L-Ara4N have appropriately been named for their effect on resistance to polymyxin (pmr stands for polymyxin resistence). Antimicrobial chemokines can be constitutively produced by the intestinal lumen and increased when bacteria invade. It is reasonable to assume that *Y. pseudotuberculosis* would encounter CCL28 while entering a host. To determine the role that the *pmrF* operon plays in bacterial protection against antimicrobial chemokines, two different antimicrobial chemokines, CCL28 and CCL20 (a non-mucosal chemokine produced most highly in the lymph nodes, liver and appendix [94]), were incubated with the bacteria for 3-4 hours and *pmrF* expression was measured using flow cytometry. As seen in Fig. 6, there was no difference in *pmrF* expression

when incubated with antimicrobial chemokine. This result is surprising, since when we tested survival of the Δ*pmrI* mutant strain of *Y. pseudotuberculosis* against CCL28, we identified a decreased survival rate compared to the wild-type (Fig. 10). However, survival of the bacteria, while significant, is only marginally decreased, and other bacterial mutations have shown greater reduction of survival [85]. It is possible that the addition of L-Ara4N is not one of the most important methods to avoid killing by antimicrobial chemokines.

Previous research has shown that strains of *Y. pseudotuberculosis* that have high binding to CCL28 also have low resistance to killing by CCL28 [84, 85]. Since we were seeing decreased survival against CCL28 in the Δ*pmrI* mutant, we predicted that we would see increased binding of the mutant compared to the wild-type bacteria. The levels of CCL28 binding to wild-type, Δ*pmrI,* and Δ*pmrI* complemented strains of *Y. pseudotuberculosis* were not what we predicted. As seen in Fig. 11, CCL28 appears to have high levels of binding to the wild-type and Δ*pmrI* complemented strains, while the Δ*pmrI* strain had very low levels of binding. It is hard to say what exactly is happening, but it is possible that the chemokine may be quickly internalizing into the bacteria, or not stable enough on the surface of the bacteria to stay attached to the Δ*pmrI* mutant. In an attempt to clarify the binding result, CCL28 levels on the surface were measured 5, 15, and 30 minutes after addition to the bacteria. If internalized, we expected to see high binding of the Δ*pmrI* strain at the 5-minute mark, with increasingly lower binding at the other time points. The Δ*pmrI* strain still had consistently lower binding than the wild-type and complemented strains at every time point (data not shown). This would suggest that if the chemokine is internalizing into the bacteria or falling off, then it is happening before we are able to measure it using flow cytometry. It may be possible to detect internalized CCL28 by Western

blot of bacterial lysates. It is also possible that the Δ*pmrI* mutation causes changes that interfere with detection of bound CCL28.

One key difference in these experiments is that we used a virulent (pYV+) strain of *Y. pseudotuberculosis* IP32953. CCL28 binding and killing patterns were previously determined in a non-virulent strain of IP32953 [85]. Some preliminary data comparing the non-virulent and virulent strains of IP32953 showed that CCL28 binding was very low in the pYV- strain and high binding was seen in the pYV+ (data not shown). To follow up on this result, we created a new pYV- strain from the pYV+ strain to see if the low binding phenotype would be observed, and it was not. We chose to use the virulent strain for these experiments to maximize applicability to strains able to cause human infection. CCL28 binding differences between virulent and non-virulent strains of *Y. pseudotuberculosis* should be further explored.

Since the effects of CCL28 binding to the surface of bacteria have not been studied, we wanted to know if there were any consequences to neutrophil phagocytosis when encountering a bacterium that has been coated with CCL28. While it was hypothesized that binding of antimicrobial chemokine to the surface of a bacterium would have an effect on neutrophil phagocytosis, there appeared to be none (Fig. 13). It is possible that if the experiment were modified to use a phagocyte that had receptors for CCL28, then perhaps the opsonization effect would be seen. Eosinophils have chemokine receptor CCR10 that is one of the receptors for CCL28 [33]. While neutrophils have not been shown to have CCR10, it has been seen in people with chronic inflammatory diseases that neutrophils are able to produce the other CCL28 receptor, CCR3 [95].

Overall, these results indicate that additional investigation of polymyxin resistance mechanisms in *Y. pseudotuberculosis*, as well as regulatory mechanisms for the *pmrF* operon in

Yersinia species are warranted. They also suggest the intriguing possibility that while the *pmrF* operon could be important for resistance against antimicrobial peptides, it may also increase neutrophil phagocytosis (Fig. 14). This could help explain why previous research has shown no effect of *pmrF* deletion on *Y. pseudotuberculosis* virulence in mice [60]. However, in a highly virulent species like *Y. pestis*, removal of L-Ara4N at higher temperatures may be important for escaping immune detection and phagocytosis. Thus, the regulatory differences in *pmrF* expression that we observe may be adaptive and help explain the pathogenic differences between these species.

TABLES

Table 1. Primers used

Listed are all primers used for mutant and reporter generation.

pmrI upstream F	CTA CAG GGA AAT CAA TGG GTG ACT
pmrI upstream R	ACT CAT CTT TAG GGG CTG CAG TTT TCC CAC GCA ACC AAT ATC ATG ATA GG
pmrI downstream F	TGC CTA TCA TGA TAT TGG TTG CGT GGC CCT AAA GAT GAG TTG AAC GCA TG
pmrI downstream R	GTA CAC TCC CTA AGC TGC CAT TAG
pmrI nested F	GGC CGA TCT AGA GGT GAC TAC GGC TGC ATG CT
pmrI nested R	GCG ACG TCG ACC CTA AGC TGC CAT TAG GCA AC
pmrH pro F	GCG TGT CTA GAG GCG TTT AGT TTT CGT TAA CTT ATC TGG GC
pmrH pro R	GCG CCG AAG CTT ACC TAT TGC TGG CCT AGA AAA AGG CAA
Gfp F HindIII	GCG CCG AAG CTT ATG AGT AAA GGA GAA GAA CTT TTC ACT G
Gfp R SalI	GCG AGC GTC GAC TTA TTT GTA TAG TTC ATC CAT GCC ATG

FIGURES

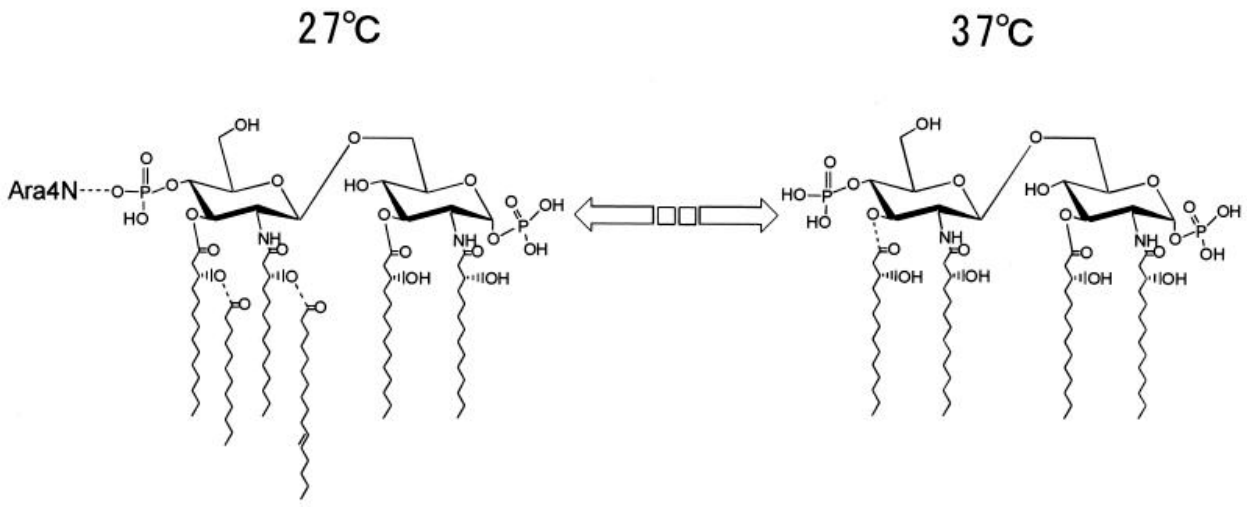

Figure 1. Lipid A modifications

The addition of L-Ara4N and acyl chain differences in the lipid A is shown here. These are modifications made to *Y. pestis* as a model. The addition of L-Ara4N reduces the net-negative charge of the bacteria to decrease detectability by cationic AMPs. *Y. pseudotuberculosis* has been shown to add L-Ara4N at both temperatures, but follows the same trend for acylation pattern. Decreasing to a tetra-acylated pattern results in lower immunostimulation, and increased chances of a successful infection.

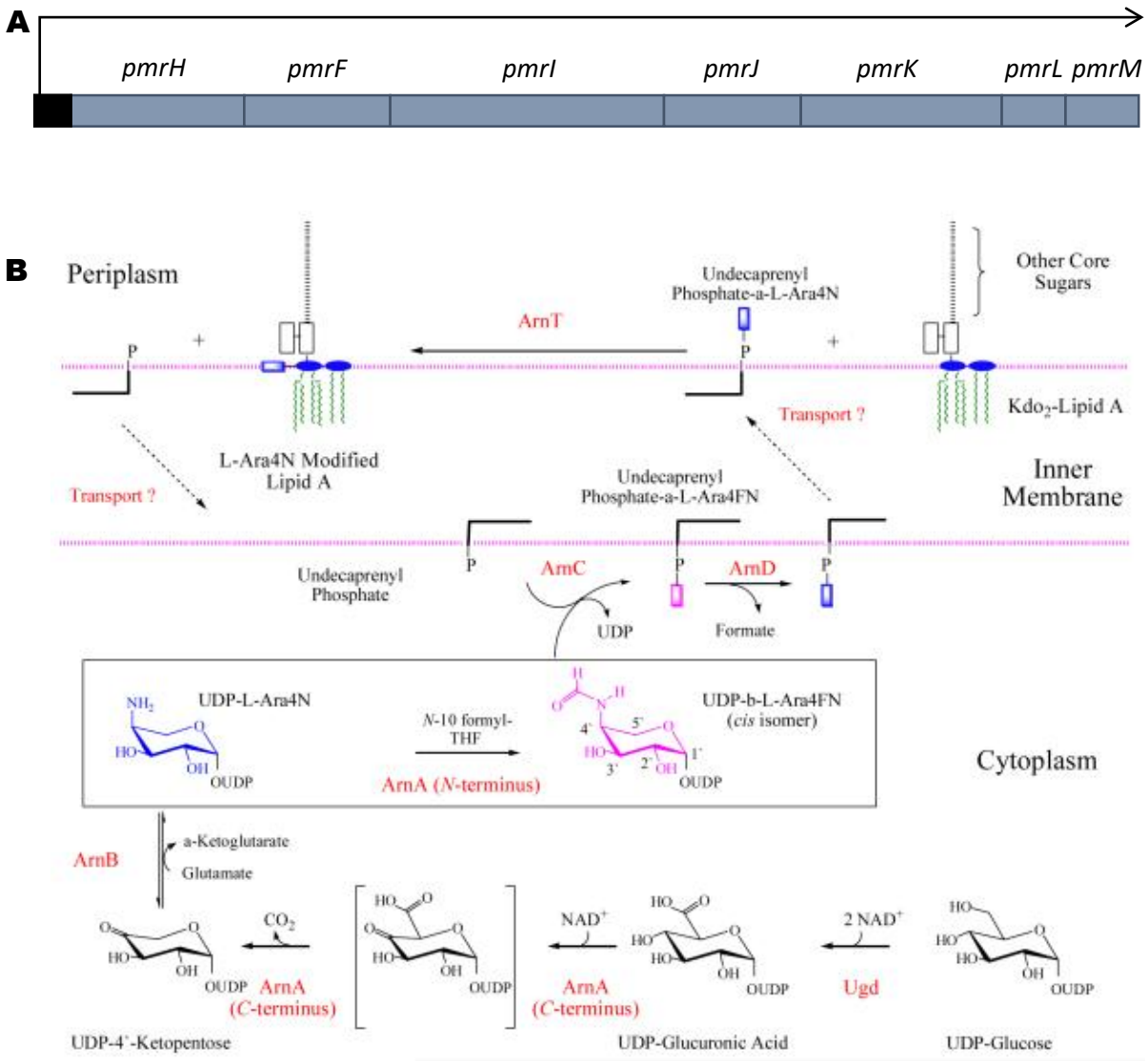

Figure 2. The role of individual genes in the addition of L-Ara4N

Figure 2a. The *pmrHFIJKLM* (*pmrF*) operon in *Y. pseudotuberculosis* is responsible for the production and addition of L-ara4N to the lipid A.

Figure 2b. The functions that each gene in the *pmrF* operon carry out for the addition of L-ara4N. The *pmrE* gene (not a part of the *pmrF* operon) produces the PmrE protein (Ugd) which transforms UDP-Glucose to UDP-Glucuronic Acid. The C-terminal domain of PmrI (ArnA) causes the transition to UDP-ara4O. PmrH (ArnB) makes a reversible change to UDP-L-ara4N. The N-terminal domain of PmrI can then add a formyl group to the molecule and then PmrF (ArnC) transfers the molecule to the inner membrane by losing UDP and attaching to an undecaprenyl phosphate. PmrJ (ArnD) removes the formyl group and then is transferred to the outer membrane by an unknown function. PmrK (ArnT) is then able to transfer the L-ara4N onto the lipid A. It has been shown that a loss of *pmr*L and *pmrM* does not prevent L-Ara4N addition (Yan 2007), but they are able to aid in the transferring of L-Ara4N to the outer membrane. It is assumed that there are other genes that can supplement the loss of *pmrL* and *pmrM* (Marceau 2009).

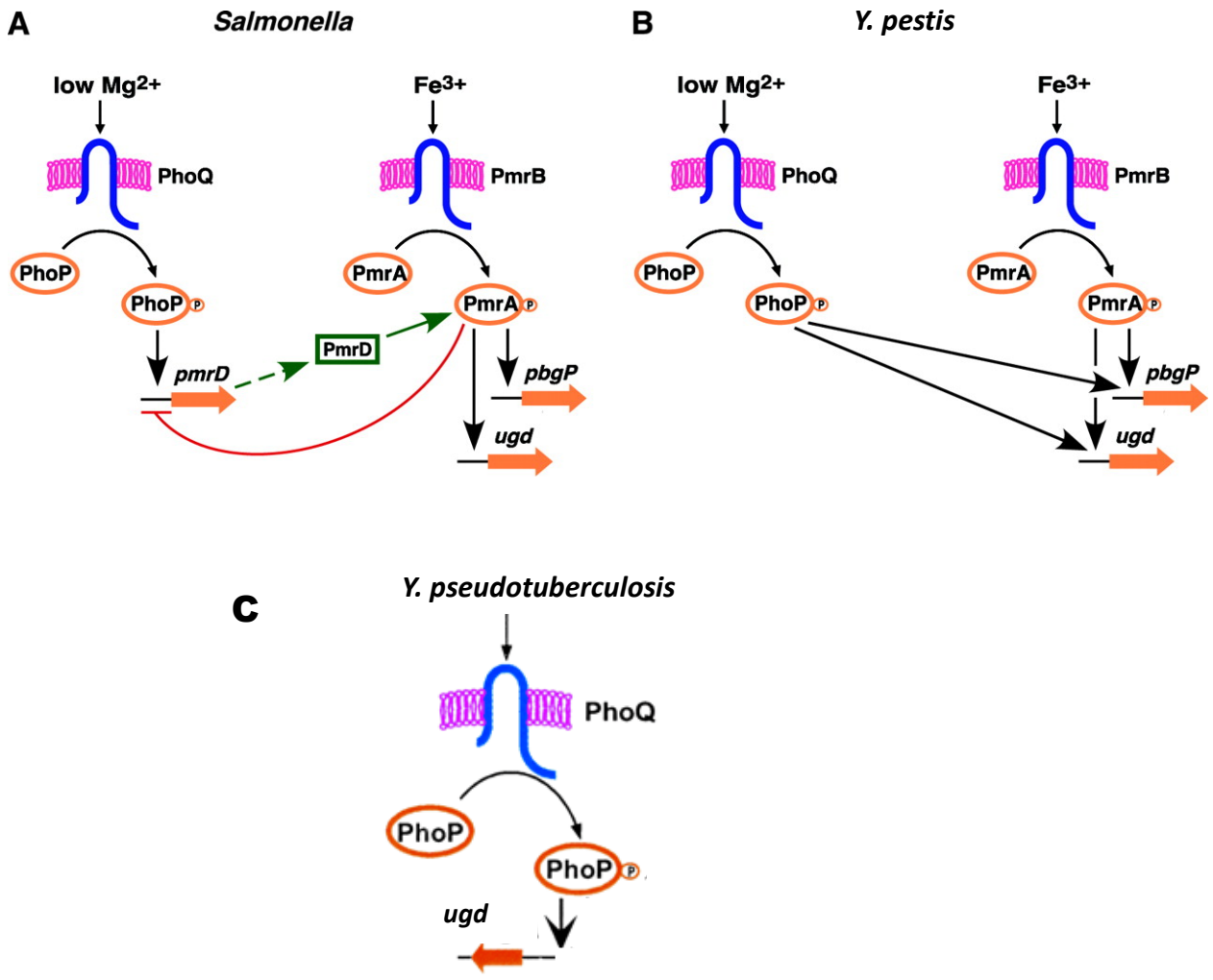

Figure 3. PhoP-PhoQ regulatory mechanisms are compared between *Y. psedotuberculosis*, *Y. pestis*, and *Salmonella typhimurium*

In *Salmonella typhimurium*, as well as *Y. pestis*, both the PhoP-PhoQ and the PmrA-PmrB regulatory mechanisms are required for *pmrF* operon expression (A.). In *Salmonella*, when PhoP-PhoQ is activated by low Mg^{2+} the *pmrD* gene is turned on and produces the PmrD protein which can activate the PmrA-PmrB regulatory system, resulting in activation of the *pmrF* operon. PmrA-PmrB can also be activated by high Fe^{3+} levels. *Y. pestis* lacks the *pmrD* gene and PmrA-PmrB activation is independent of PmrD (B).

In *Y. pseudotuberculosis* the regulation of the *pmrF* operon is independent of the PmrA-PmrB system and only requires PhoP-PhoQ activation (C). *Y. pseudotuberculosis* does not have *pmrD*. It has been shown that ΔpmrA-pmrB mutants of Y. pseudotuberculosis do not have any *pmrF* defect. The same group has also shown that *pmrF* expression can be regulated by a LysR-type regulator that increases expression in low Fe^{3+} conditions (Marceau et al. 2004,2009).

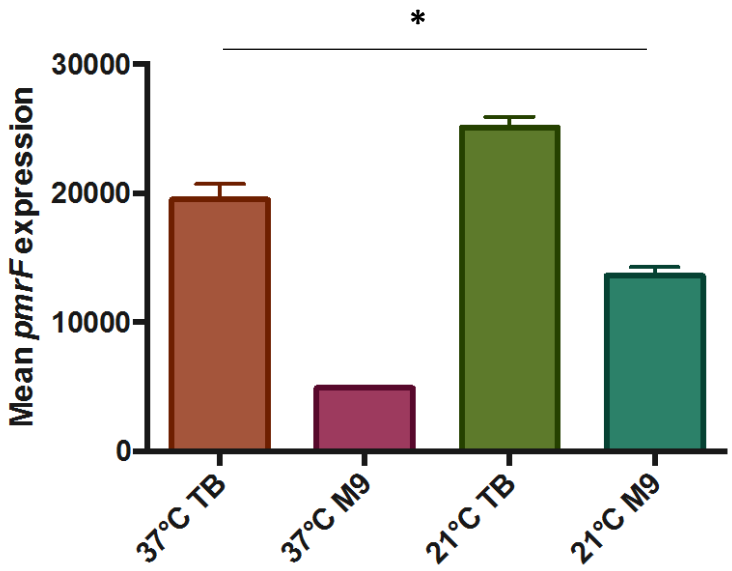

Figure 4. *pmrF* expression depends more on nutrient availability than temperature

A *pmrF* operon reporter strain of *Y. pseudotuberculosis* was incubated at 37°C and 21°C in either TB or M9 media. It is well known in other bacterial strains that *pmrF* expression is downregulated at 37°C, however it appears that growth in rich media compared to minimal media is more important for expression. Differences in expression are statistically significant between all test conditions ($p > 0.05$) and figure is representative of one experiment done in triplicate. Experiment was performed with the same results three times.

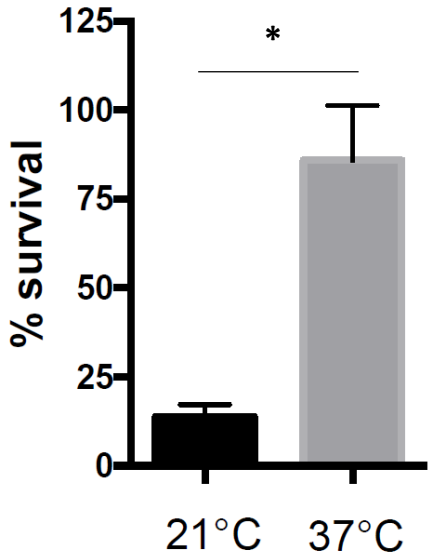

Figure 5. *Y. pseudotuberculosis* is more resistant to polymyxin at 37ºC than 21ºC

Y. pseudotuberculosis was grown in TB media at either 21ºC or 37ºC and then exposed to polymyxin for 2 hours in a low-osmolarity buffer. The survival of the bacteria grown at 37 ºC was significantly higher than those grown at 21ºC ($p > 0.05$), consistent with previous studies.

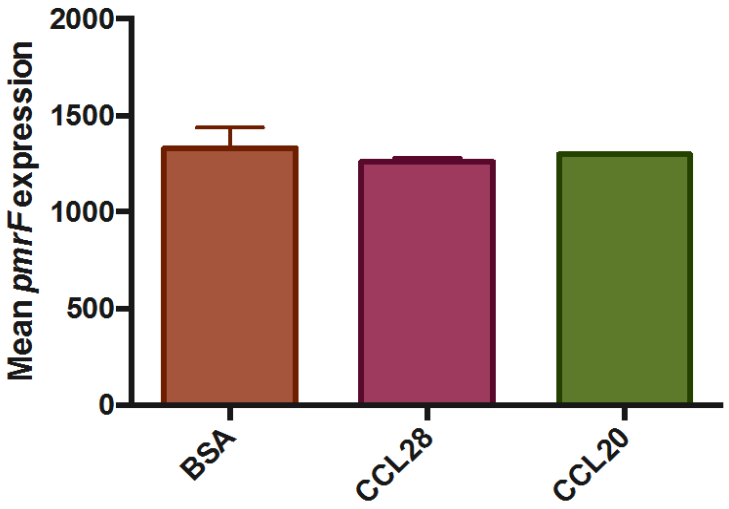

Figure 6. Antimicrobial chemokine presence does not affect *pmrF* expression

A *pmrF* operon reporter strain of *Y. pseudotuberculosis* was incubated at 37°C with and without the presence of antimicrobial chemokines CCL28 and CCL20. There was no difference in *pmrF* expression between the negative control and the addition of chemokine.

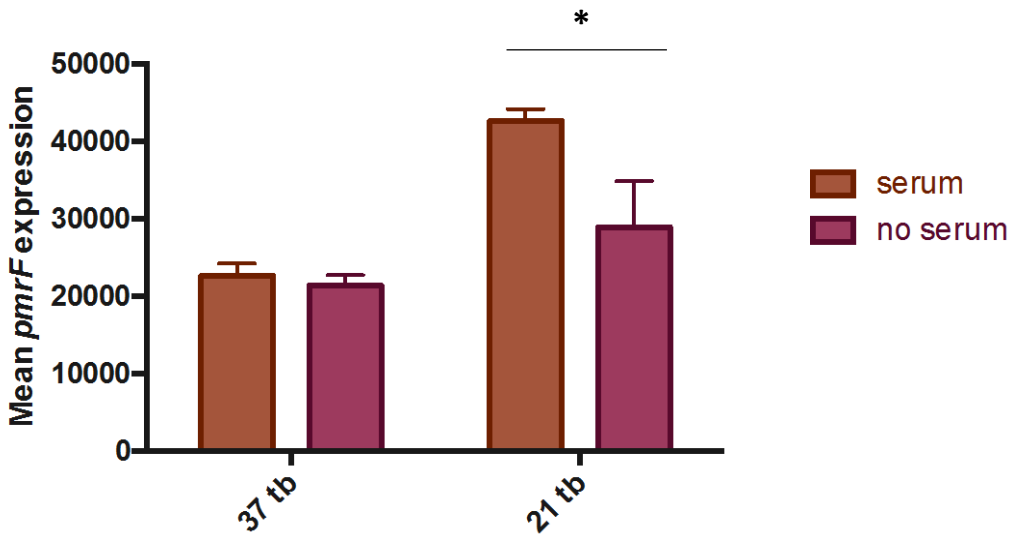

Figure 7. Human serum increases *pmrF* expression at 21°C in TB

A *pmrF* operon reporter strain of *Y. pseudotuberculosis* was incubated at 37°C and 21°C in TB and M9 media with and without being supplemented with 10% human serum. It is known that at 37°C, *Y. pseudotuberculosis* is resistant to killing by human serum, and it appears that at this temperature serum has no effect on *pmrF* expression. However, when incubated at 21°C, there appears to be an increase in expression when grown in TB. Expression of pmrF at 37ºC is higher with serum than without ($p > 0.05$) and figure is representative of one experiment done in triplicate. Experiment was performed with the same results three times.

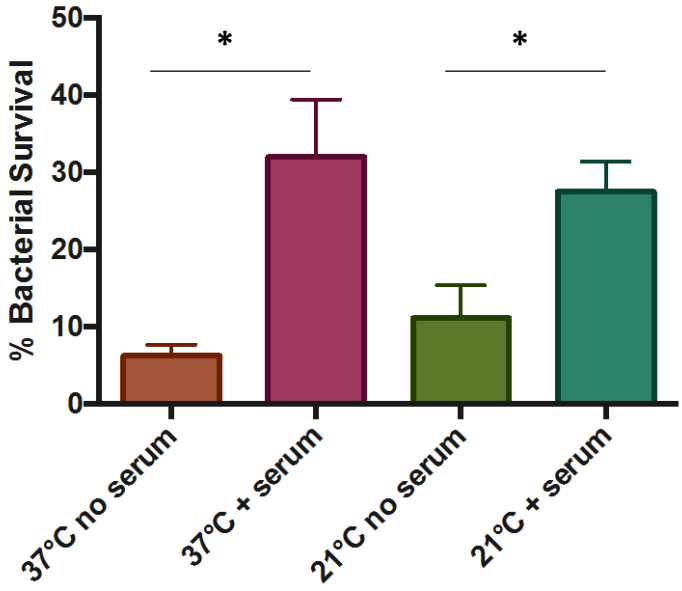

Figure 8. Human serum helps protect *Y. pseudotuberculosis* from death by polymyxin

Y. pseudotuberculosis was pretreated in TB at 37°C and 21°C with and without 10% human serum for three hours. A killing assay was then performed with polymyxin to see if the apparent increase in *pmrF* expression would increase survival of the bacteria. The survival rate of the bacteria was significantly higher ($p<0.05$) when pretreated with human serum at both 37°C and 21°C. Figure is representative of one experiment done in triplicate. Experiment was performed with the same results three times.

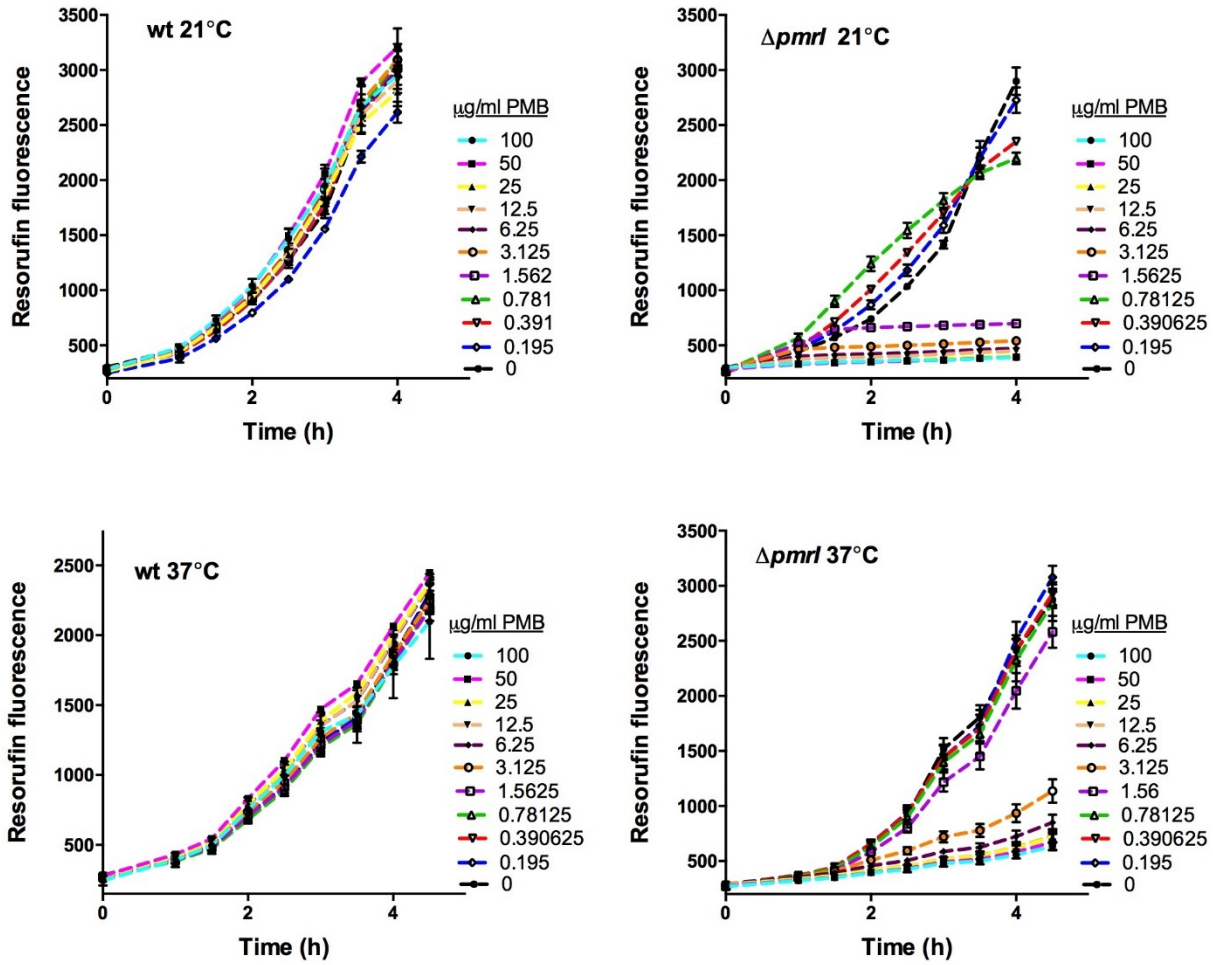

Figure 9. The Δ*pmrI* mutant has decreased survival in polymyxin

To confirm that the created Δ*pmrI* mutant of *Y. pseudotuberculosis* had indeed lost the ability to survive in polymyxin, and that *pmrF* expression related to polymyxin resistance, a growth curve was performed with wild-type and Δ*pmrI* over 4.5 hours in varying levels of polymyxin at 21ºC and 37ºC. Growth was measured by rezasurin metabolism. The wild-type bacteria were uninhibited by even the highest level of polymyxin (left), while the Δ*pmrI* mutant was unable to grow in the higher concentrations (right). Suggesting that L-ara4N is not being added in the Δ*pmrI* strain. This also shows that *pmrF* promoter activity does not necessarily correlate with polymyxin resistance.

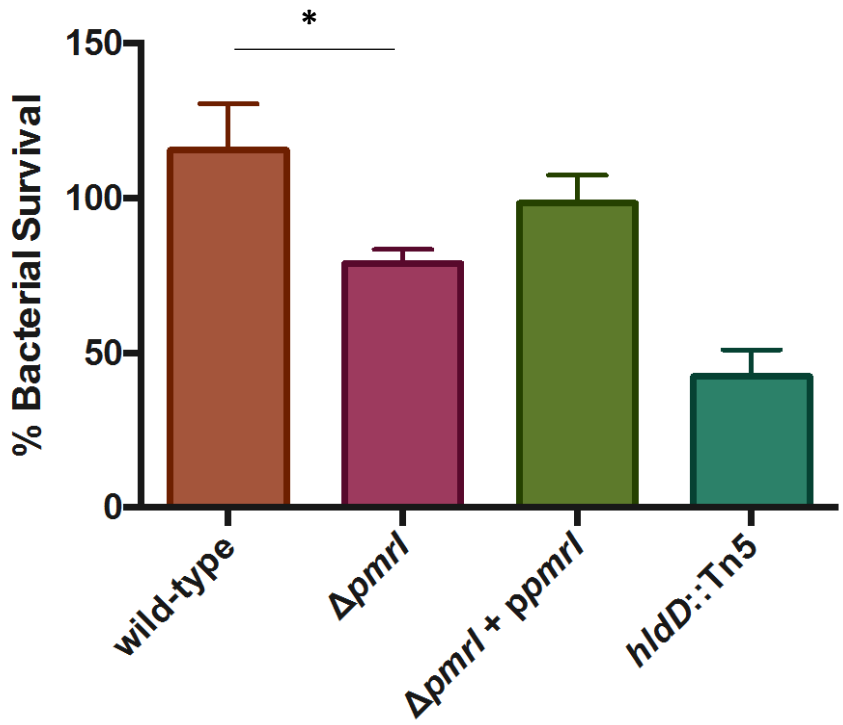

Figure 10. Addition of L-ara4N helps protect *Y. pseudotuberculosis* against killing by antimicrobial chemokine CCL28

The wild-type, Δ*pmrI*, Δ*pmrI* complement, and *hldD*::Tn5 strains of *Y. pseudotuberculosis* were incubated with CCL28 in a low osmolarity media to determine how well they can survive against CCL28 killing. The wild-type and Δ*pmrI* + p*pmrI* strains were unaffected by CCL28, while the Δ*pmrI* strain had about a 30% decrease in survival. *HldD*::Tn5 was used as a positive control for killing, with about a 50% survival rate. These results show that L-ara4N addition is important as a bacterial defense against antimicrobial chemokines. The ΔpmrI mutant has decreased survival compared to wild-type survival ($p > 0.05$) and figure is representative of one experiment done in triplicate. Experiment was performed with the same results three times.

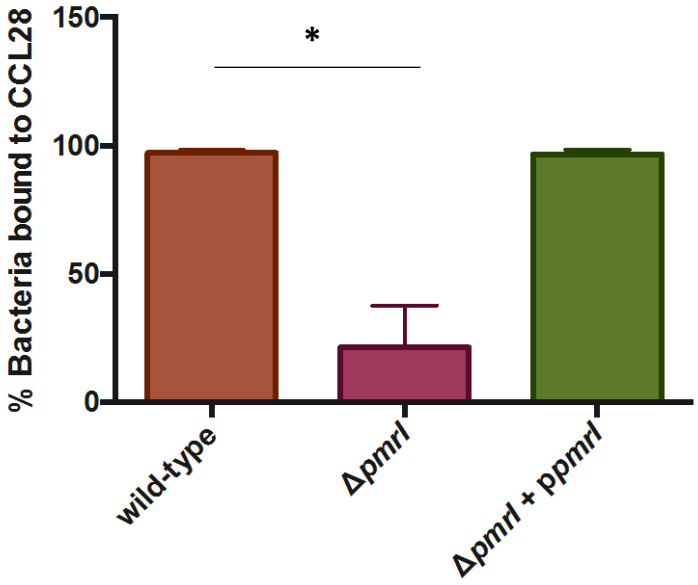

Figure 11. The ΔpmrI mutant has decreased CCL28 binding

Wild-type, Δ*pmrI*, and Δ*pmrI* complemented strains of *Y. pseudotuberculosis* were incubated with CCL28 followed by anti-CCL28 flurecently labeled antibody, to observe the amount of bacteria that were binding to CCL28. These results show high binding for the wild-type and Δ*pmrI* + p*pmrI* strains of *Y. pseudotuberculosis,* with very low binding to the Δ*pmrI* strain ($p < 0.05$). This conflicts what has been seen previously (Erickson 2016).

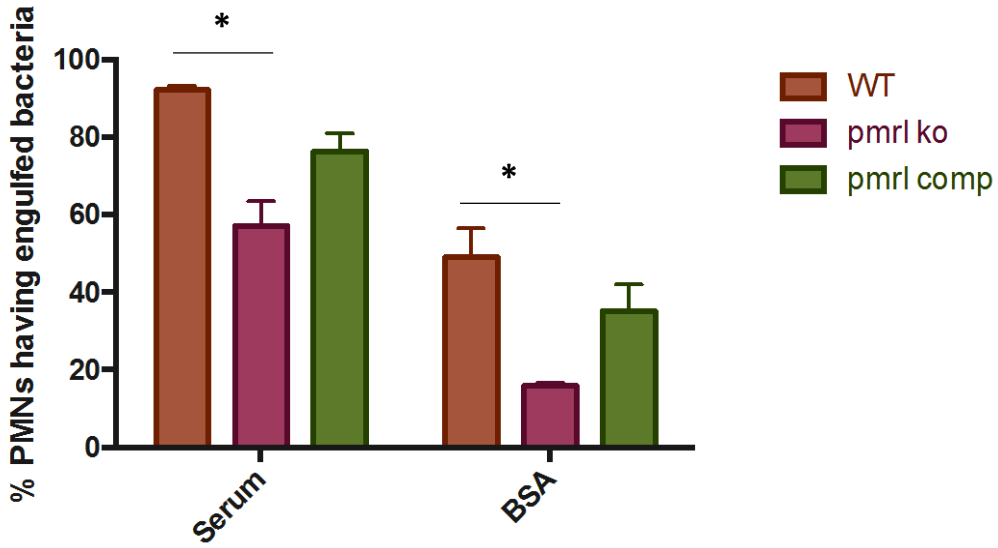

Figure 12. L-ara4N addition increases neutrophil phagocytosis

The neutrophil phagocytosis assay was performed with fluorescent wild-type, Δ*pmrI*, and Δ*pmrI* complemented strains. They were pretreated with or without serum opsonization. The results show that there is a decrease in neutrophil phagocytosis when the *pmrI* gene is knocked out ($p < 0.05$). This is consistent with other recent research that has been done showing that L-ara4N modified lipid A can be a greater stimulator of the innate immune system. This can help to explain why the *pmrF* operon is down-regulated at 37°C. Figure is representative of one experiment done in triplicate. Experiment was performed with the similar results three times.

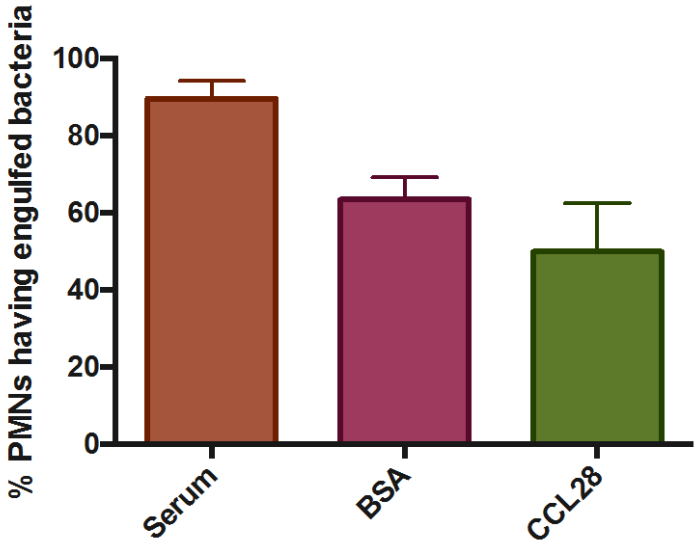

Figure 13. CCL28 binding has no effect on neutrophil phagocytosis

Y. pseudotuberculosis containing the pAKgfp plasmid was pretreated with human serum (positive control), BSA (negative control), and CCL28. The bacteria were then incubated with isolated human neutrophils for 30 minutes. The cells were then observed on a flow cytometer and the percent of neutrophils positive for GFP were counted. The opsonized bacteria show a higher level of neutrophil engulfment. There was no difference in engulfment from the negative control when the bacteria were bound to CCL28. These results suggest that CCL28 does not act as a neutrophil opsonin.

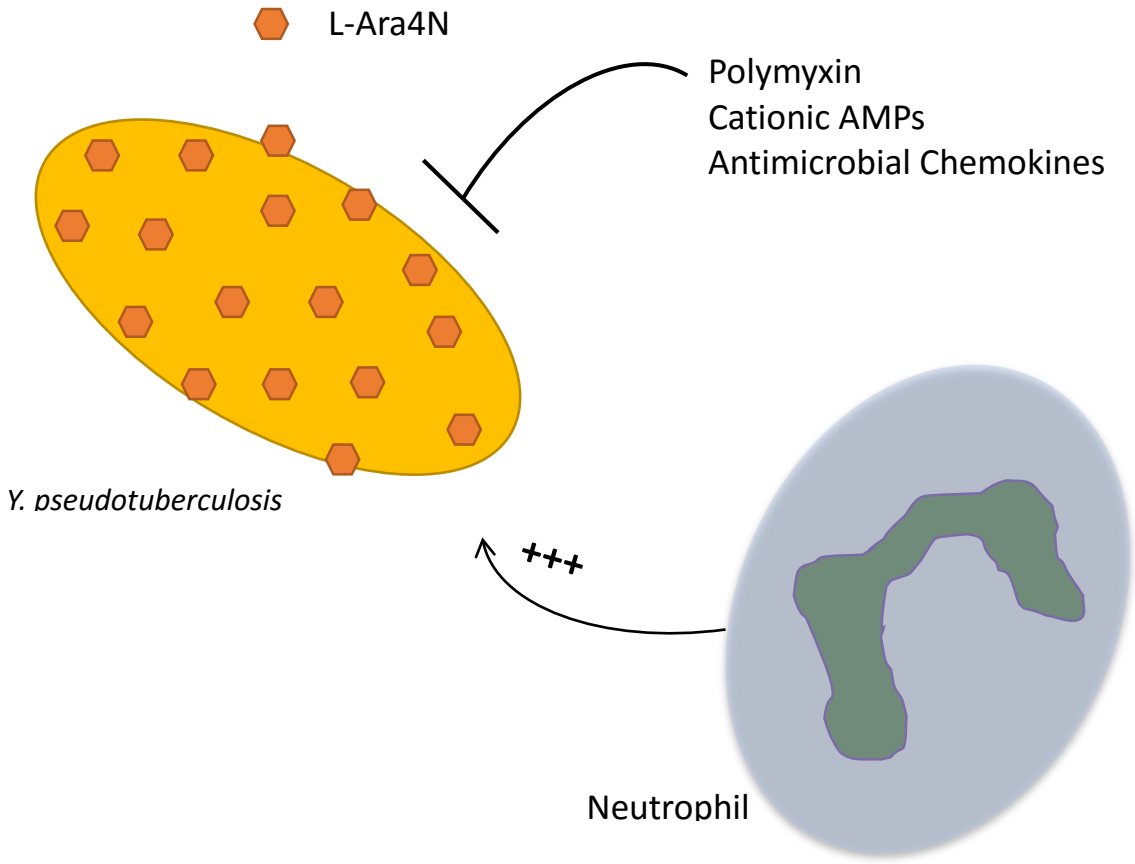

Figure 14. L-Ara4N addition most likely results in no change to net virulence

Although data suggests that the *pmrF* operon is be important for resistance against antimicrobial peptides, data presented here suggests that it may also increase neutrophil phagocytosis. Combined these results help explain why previous research has shown no effect of *pmrF* deletion on *Y. pseudotuberculosis* virulence in mice (Marceau et al 2004).

REFERENCES

1. Shishido, S.N., et al., *Humoral innate immune response and disease.* Clin Immunol, 2012. **144**(2): p. 142-58.
2. Nadif, R., et al., *The role of eosinophils and basophils in allergic diseases considering genetic findings.* Curr Opin Allergy Clin Immunol, 2013. **13**(5): p. 507-13.
3. Cadman, E.T., et al., *Eosinophils are important for protection, immunoregulation and pathology during infection with nematode microfilariae.* PLoS Pathog, 2014. **10**(3): p. e1003988.
4. Huang, L., et al., *Eosinophils mediate protective immunity against secondary nematode infection.* J Immunol, 2015. **194**(1): p. 283-90.
5. Segal, A.W., *How Neutrophils Kill Microbes.* Annu Rev Immunol, 2005. **23**: p. 197-223.
6. Hampton, M.B., A.J. Kettle, and C.C. Winterbourn, *Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing.* Blood, 1998. **92**(9): p. 3007-17.
7. Brinkmann, V., et al., *Neutrophil extracellular traps kill bacteria.* Science, 2004. **303**(5663): p. 1532-5.
8. Prince, L.R., et al., *The role of TLRs in neutrophil activation.* Curr Opin Pharmacol, 2011. **11**(4): p. 397-403.
9. Hayashi, F., T.K. Means, and A.D. Luster, *Toll-like receptors stimulate human neutrophil function.* Blood, 2003. **102**(7): p. 2660-9.
10. Takeuchi, O. and S. Akira, *Pattern recognition receptors and inflammation.* Cell, 2010. **140**(6): p. 805-20.
11. Hayashi, F., et al., *The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5.* Nature, 2001. **410**(6832): p. 1099-103.
12. Rus, H., C. Cudrici, and F. Niculescu, *The role of the complement system in innate immunity.* Immunol Res, 2005. **33**(2): p. 103-12.
13. Sengelov, H., *Complement receptors in neutrophils.* Crit Rev Immunol, 1995. **15**(2): p. 107-31.
14. Sengelov, H., et al., *Secretory vesicles are the intracellular reservoir of complement receptor 1 in human neutrophils.* J Immunol, 1994. **153**(2): p. 804-10.
15. Graves, D.T. and Y. Jiang, *Chemokines, a family of chemotactic cytokines.* Crit Rev Oral Biol Med, 1995. **6**(2): p. 109-18.
16. Berri, M., et al., *CCL28 involvement in mucosal tissues protection as a chemokine and as an antibacterial peptide.* Developmental and comparative immunology, 2014. **44**(2): p. 286-90.
17. Charo, I.F. and R.M. Ransohoff, *The many roles of chemokines and chemokine receptors in inflammation.* N Engl J Med, 2006. **354**(6): p. 610-21.
18. Marchese, A., *Endocytic trafficking of chemokine receptors.* Curr Opin Cell Biol, 2014. **27**: p. 72-7.
19. Izadpanah, A. and R.L. Gallo, *Antimicrobial peptides.* Journal of the American Academy of Dermatology, 2005. **52**(3): p. 381-390.
20. Kosciuczuk, E.M., et al., *Cathelicidins: family of antimicrobial peptides. A review.* Mol Biol Rep, 2012. **39**(12): p. 10957-70.
21. Yang, D., et al., *Multiple roles of antimicrobial defensins, cathelicidins, and eosinophil-derived neurotoxin in host defense.* Annual Review of Immunology, 2004. **22**: p. 181-215.
22. Durr, U.H., U.S. Sudheendra, and A. Ramamoorthy, *LL-37, the only human member of the cathelicidin family of antimicrobial peptides.* Biochim Biophys Acta, 2006. **1758**(9): p. 1408-25.
23. Jarczak, J., et al., *Defensins: natural component of human innate immunity.* Hum Immunol, 2013. **74**(9): p. 1069-79.
24. Kagan, B.L., T. Ganz, and R.I. Lehrer, *Defensins: a family of antimicrobial and cytotoxic peptides.* Toxicology, 1994. **87**(1-3): p. 131-49.

25. Lehrer, R.I., et al., *Interaction of human defensins with Escherichia coli. Mechanism of bactericidal activity.* J Clin Invest, 1989. **84**(2): p. 553-61.
26. Brogden, K.A., *Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?* Nat Rev Microbiol, 2005. **3**(3): p. 238-50.
27. Nguyen, L.T., E.F. Haney, and H.J. Vogel, *The expanding scope of antimicrobial peptide structures and their modes of action.* Trends Biotechnol, 2011. **29**(9): p. 464-72.
28. Yung, S.C. and P.M. Murphy, *Antimicrobial chemokines.* Front Immunol, 2012. **3**: p. 276.
29. Linge, H.M., et al., *The human CXC chemokine granulocyte chemotactic protein 2 (GCP-2)/CXCL6 possesses membrane-disrupting properties and is antibacterial.* Antimicrob Agents Chemother, 2008. **52**(7): p. 2599-607.
30. Liu, B. and E. Wilson, *The antimicrobial activity of CCL28 is dependent on C-terminal positively-charged amino acids.* Eur J Immunol, 2010. **40**(1): p. 186-96.
31. Karlsson, C., et al., *SufA of the opportunistic pathogen finegoldia magna modulates actions of the antibacterial chemokine MIG/CXCL9, promoting bacterial survival during epithelial inflammation.* J Biol Chem, 2009. **284**(43): p. 29499-508.
32. Sobirk, S.K., et al., *Human chemokines as antimicrobial peptides with direct parasiticidal effect on Leishmania mexicana in vitro*, in *PLoS One*. 2013: United States. p. e58129.
33. Pan, J., et al., *A novel chemokine ligand for CCR10 and CCR3 expressed by epithelial cells in mucosal tissues.* J Immunol, 2000. **165**(6): p. 2943-9.
34. 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.
35. Biswas, B.B., P.S. Basu, and M.K. Pal, *Gram staining and its molcecular mechanism.* Int Rev Cytol, 1970. **29**: p. 1-27.
36. Wang, L., Q. Wang, and P.R. Reeves, *The variation of O antigens in gram-negative bacteria.* Subcell Biochem, 2010. **53**: p. 123-52.
37. De Castro, C., et al., *Microbe-associated molecular patterns in innate immunity: Extraction and chemical analysis of gram-negative bacterial lipopolysaccharides.* Methods Enzymol, 2010. **480**: p. 89-115.
38. Beutler, B. and A. Cerami, *Tumor necrosis, cachexia, shock, and inflammation: a common mediator.* Annu Rev Biochem, 1988. **57**: p. 505-18.
39. Dinarello, C.A., *Interleukin-1 and interleukin-1 antagonism.* Blood, 1991. **77**(8): p. 1627-52.
40. Raetz, C.R., et al., *Lipid A modification systems in gram-negative bacteria.* Annu Rev Biochem, 2007. **76**: p. 295-329.
41. Demeure, C.E., A. Derbise, and E. Carniel, *Oral vaccination against plague using Yersinia pseudotuberculosis.* Chem Biol Interact, 2016.
42. Achtman, M., et al., *Microevolution and history of the plague bacillus, Yersinia pestis.* Proc Natl Acad Sci U S A, 2004. **101**(51): p. 17837-42.
43. Aussel, L., et al., *Novel variation of lipid A structures in strains of different Yersinia species.* FEBS Lett, 2000. **465**(1): p. 87-92.
44. Laws, T.R., et al., *Yersinia pseudotuberculosis is resistant to killing by human neutrophils.* Microbes and Infection, 2011. **13**(6): p. 607-611.
45. Trosky, J.E., A.D.B. Liverman, and K. Orth, *Yersinia outer proteins: Yops.* Cellular Microbiology, 2008. **10**(3): p. 557-565.
46. 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.
47. Westermark, L., A. Fahlgren, and M. Fallman, *Yersinia pseudotuberculosis efficiently escapes polymorphonuclear neutrophils during early infection.* Infect Immun, 2014. **82**(3): p. 1181-91.

48. O'Loughlin, J.L., et al., *Yersinia pestis two-component gene regulatory systems promote survival in human neutrophils.* Infect Immun, 2010. **78**(2): p. 773-82.
49. Spinner, J.L., et al., *Neutrophils are resistant to Yersinia YopJ/P-induced apoptosis and are protected from ROS-mediated cell death by the type III secretion system.* PLoS One, 2010. **5**(2): p. e9279.
50. Spinner, J.L., et al., *Yersinia pestis survival and replication within human neutrophil phagosomes and uptake of infected neutrophils by macrophages.* J Leukoc Biol, 2014. **95**(3): p. 389-98.
51. Vagima, Y., et al., *Circumventing Y. pestis Virulence by Early Recruitment of Neutrophils to the Lungs during Pneumonic Plague.* PLoS Pathog, 2015. **11**(5): p. e1004893.
52. Laws, T.R., et al., *Neutrophils are important in early control of lung infection by Yersinia pestis.* Microbes Infect, 2010. **12**(4): p. 331-5.
53. Rebeil, R., et al., *Variation in lipid A structure in the pathogenic yersiniae.* Molecular Microbiology, 2004. **52**(5): p. 1363-1373.
54. Lee, H., et al., *The PmrA-regulated pmrC gene mediates phosphoethanolamine modification of lipid A and polymyxin resistance in Salmonella enterica.* J Bacteriol, 2004. **186**(13): p. 4124-33.
55. Reynolds, C.M., et al., *A phosphoethanolamine transferase specific for the outer 3-deoxy-D-manno-octulosonic acid residue of Escherichia coli lipopolysaccharide. Identification of the eptB gene and Ca2+ hypersensitivity of an eptB deletion mutant.* J Biol Chem, 2005. **280**(22): p. 21202-11.
56. Murray, S.R., et al., *pmrA(Con) confers pmrHFIJKL-dependent EGTA and polymyxin resistance on msbB Salmonella by decorating lipid A with phosphoethanolamine.* J Bacteriol, 2007. **189**(14): p. 5161-9.
57. Knirel, Y.A., et al., *Cold temperature-induced modifications to the composition and structure of the lipopolysaccharide of Yersinia pestis.* Carbohydr Res, 2005. **340**(9): p. 1625-30.
58. Chen, H.D. and E.A. Groisman, *The Biology of the PmrA/PmrB Two-Component System: The Major Regulator of Lipopolysaccharide Modifications*, in *Annual Review of Microbiology, Vol 67*, S. Gottesman, Editor. 2013, Annual Reviews: Palo Alto. p. 83-112.
59. Bhagya Lakshmi, S.K., et al., *Temperature-dependent incorporation of 4-amino-L-arabinose in lipid A of distinct gram-negative bacteria.* FEMS microbiology letters, 1989. **51**(3): p. 317-22.
60. Marceau, M., et al., *The pmrF polymyxin-resistance operon of Yersinia pseudotuberculosis is upregulated by the PhoP-PhoQ two-component system but not by PmrA-PmrB, and is not required for virulence.* Microbiology-Sgm, 2004. **150**: p. 3947-3957.
61. Winfield, M.D., T. Latifi, and E.A. Groisman, *Transcriptional regulation of the 4-amino-4-deoxy-L-arabinose biosynthetic genes in Yersinia pestis.* Journal of Biological Chemistry, 2005. **280**(15): p. 14765-14772.
62. Zhou, Z., et al., *Lipid A modifications characteristic of Salmonella typhimurium are induced by NH4VO3 in Escherichia coli K12. Detection of 4-amino-4-deoxy-L-arabinose, phosphoethanolamine and palmitate.* J Biol Chem, 1999. **274**(26): p. 18503-14.
63. Williams, G.J., et al., *Structure and function of both domains of ArnA, a dual function decarboxylase and a formyltransferase, involved in 4-amino-4-deoxy-L-arabinose biosynthesis.* J Biol Chem, 2005. **280**(24): p. 23000-8.
64. Breazeale, S.D., A.A. Ribeiro, and C.R. Raetz, *Oxidative decarboxylation of UDP-glucuronic acid in extracts of polymyxin-resistant Escherichia coli. Origin of lipid a species modified with 4-amino-4-deoxy-L-arabinose.* J Biol Chem, 2002. **277**(4): p. 2886-96.
65. Breazeale, S.D., A.A. Ribeiro, and C.R. Raetz, *Origin of lipid A species modified with 4-amino-4-deoxy-L-arabinose in polymyxin-resistant mutants of Escherichia coli. An aminotransferase (ArnB) that generates UDP-4-deoxyl-L-arabinose.* J Biol Chem, 2003. **278**(27): p. 24731-9.

66. Gunn, J.S., et al., *PmrA-PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance.* Mol Microbiol, 1998. **27**(6): p. 1171-82.
67. Breazeale, S.D., et al., *A formyltransferase required for polymyxin resistance in Escherichia coli and the modification of lipid A with 4-Amino-4-deoxy-L-arabinose. Identification and function oF UDP-4-deoxy-4-formamido-L-arabinose.* J Biol Chem, 2005. **280**(14): p. 14154-67.
68. Yan, A., Z. Guan, and C.R. Raetz, *An undecaprenyl phosphate-aminoarabinose flippase required for polymyxin resistance in Escherichia coli.* J Biol Chem, 2007. **282**(49): p. 36077-89.
69. Arafah, S., et al., *An iron-regulated LysR-type element mediates antimicrobial peptide resistance and virulence in Yersinia pseudotuberculosis*, in *Microbiology*. 2009: England. p. 2168-81.
70. Trent, M.S., et al., *An inner membrane enzyme in Salmonella and Escherichia coli that transfers 4-amino-4-deoxy-L-arabinose to lipid A: induction on polymyxin-resistant mutants and role of a novel lipid-linked donor.* J Biol Chem, 2001. **276**(46): p. 43122-31.
71. Petrou, V.I., et al., *Structures of aminoarabinose transferase ArnT suggest a molecular basis for lipid A glycosylation.* Science, 2016. **351**(6273): p. 608-12.
72. Aoyagi, K., et al., *Lipopolysaccharide Modification Promotes Maintenance of Yersinia pestis in Fleas.* Microbiology, 2014.
73. Kuhn, H.M., U. Meier-Dieter, and H. Mayer, *ECA, the enterobacterial common antigen.* FEMS Microbiol Rev, 1988. **4**(3): p. 195-222.
74. Chain, P.S., et al., *Insights into the evolution of Yersinia pestis through whole-genome comparison with Yersinia pseudotuberculosis.* Proc Natl Acad Sci U S A, 2004. **101**(38): p. 13826-31.
75. Gunn, J.S., et al., *Genetic and functional analysis of a PmrA-PmrB-regulated locus necessary for lipopolysaccharide modification, antimicrobial peptide resistance, and oral virulence of Salmonella enterica serovar typhimurium.* Infect Immun, 2000. **68**(11): p. 6139-46.
76. Hollaus, R., et al., *Chemical synthesis of Burkholderia Lipid A modified with glycosyl phosphodiester-linked 4-amino-4-deoxy-beta-L-arabinose and its immunomodulatory potential.* Chemistry, 2015. **21**(10): p. 4102-14.
77. Di Lorenzo, F., et al., *Activation of Human Toll-like Receptor 4 (TLR4).Myeloid Differentiation Factor 2 (MD-2) by Hypoacylated Lipopolysaccharide from a Clinical Isolate of Burkholderia cenocepacia.* J Biol Chem, 2015. **290**(35): p. 21305-19.
78. Kawahara, K., et al., *Modification of the structure and activity of lipid A in Yersinia pestis lipopolysaccharide by growth temperature.* Infect Immun, 2002. **70**(8): p. 4092-8.
79. Reines, M., et al., *Molecular Basis of Yersinia enterocolitica Temperature-Dependent Resistance to Antimicrobial Peptides.* Journal of Bacteriology, 2012. **194**(12): p. 3173-3188.
80. Kato, A., T. Latifi, and E.A. Groisman, *Closing the loop: The PmrA/PmrB two-component system negatively controls expression of its posttranscriptional activator PmrD.* Proceedings of the National Academy of Sciences, 2003. **100**(8): p. 4706-4711.
81. Bengoechea, J.A., et al., *Yersinia pseudotuberculosis and Yersinia pestis are more resistant to bactericidal cationic peptides than Yersinia enterocolitica.* Microbiology, 1998. **144 (Pt 6)**: p. 1509-15.
82. White, J.M., et al., *Structures and mechanisms of viral membrane fusion proteins: multiple variations on a common theme.* Crit Rev Biochem Mol Biol, 2008. **43**(3): p. 189-219.
83. Schmid-Hempel, P., *Immune defence, parasite evasion strategies and their relevance for 'macroscopic phenomena' such as virulence.* Philos Trans R Soc Lond B Biol Sci, 2009. **364**(1513): p. 85-98.
84. 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. 2012.
85. Erickson, D.L., et al., *Lipopolysaccharide biosynthesis genes of Yersinia pseudotuberculosis promote resistance to antimicrobial chemokines*. 2016: (submitted) PLoS One.
86. Karsi, A. and M.L. Lawrence, *Broad host range fluorescence and bioluminescence expression vectors for Gram-negative bacteria.* Plasmid, 2007. **57**(3): p. 286-95.
87. 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.* J Bacteriol, 2010. **192**(24): p. 6418-27.
88. Bryksin, A.V. and I. Matsumura, *Overlap extension PCR cloning: a simple and reliable way to create recombinant plasmids.* Biotechniques, 2010. **48**(6): p. 463-5.
89. 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. 149-57.
90. Sambrook, J., E.F. Fritsch, and T. Maniatis, *Molecular Cloning. A Laboratory Manual*. 2nd ed. 1989, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
91. Oh, H., B. Siano, and S. Diamond, *Neutrophil isolation protocol.* J Vis Exp, 2008(17).
92. Reines, M., et al., *Molecular basis of Yersinia enterocolitica temperature-dependent resistance to antimicrobial peptides.* J Bacteriol, 2012. **194**(12): p. 3173-88.
93. Ho, D.K., et al., *The Yersinia pseudotuberculosis outer membrane protein Ail recruits the human complement regulatory protein factor H.* J Immunol, 2012. **189**(7): p. 3593-9.
94. Hieshima, K., et al., *Molecular cloning of a novel human CC chemokine liver and activation-regulated chemokine (LARC) expressed in liver. Chemotactic activity for lymphocytes and gene localization on chromosome 2.* J Biol Chem, 1997. **272**(9): p. 5846-53.
95. Hartl, D., et al., *Infiltrated neutrophils acquire novel chemokine receptor expression and chemokine responsiveness in chronic inflammatory lung diseases.* J Immunol, 2008. **181**(11): p. 8053-67.