Polysaccharides of *Burkholderia*: Virulence and Vaccine Development

Sara Kathryn Bondi
Sarasota, FL

B. A., New College of Florida, 1998

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Department of Microbiology

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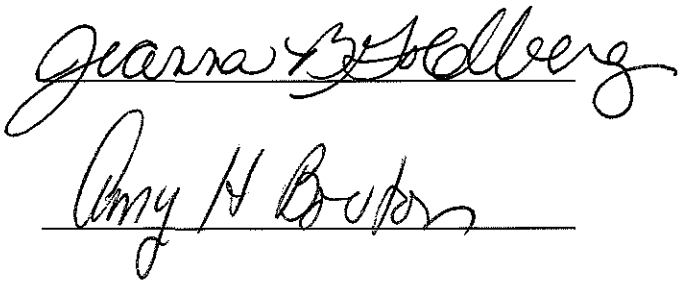

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Dedication

This body of work is dedicated to Gene Cassidy. If not for him, this thesis would not exist.

Vaccine Development Targeting *Burkholderia mallei*

Abstract

Burkholderia mallei and *Burkholderia pseudomallei* are Gram-negative, rod-shaped bacteria and are the causative agents of the diseases glanders and melioidosis, respectively. These bacteria have been recognized for over 100 years as important and potentially fatal pathogens, yet there has been a relative dearth of available information regarding the virulence determinants and immunopathology of either microorganism. However, classification of both bacteria by the NIAID and CDC as category B priority pathogens has spurred a dramatic increase in interest in these bacteria. There is no vaccine available against either infectious agent. In this study we have tested the O antigen portion of the lipopolysaccharide (LPS) of *B. mallei* as a vaccine candidate. The O antigen locus of *B. mallei*, expressed on a plasmid (pIC3), was transduced into the attenuated *Salmonella* enterica sp. Typhimurium strain SL3261. We found that SL3261 was able to express *B. mallei* O antigen on its surface. Mice that were intranasally immunized with this construct generated a robust IgG Th1-like antibody response against *B. mallei* LPS. Following vaccination, mice were intranasally infected with 1000 CFU (5 x LD_{50}) of *B. mallei*. Two weeks post challenge infection, we found that 100% of vaccinated mice survived, while only 25% and 50% of the mice given PBS or *Salmonella*, respectively, survived. Long term vaccinated survivors were sacrificed and assessed for CFU in the lungs,

liver and spleen. Overall, 50% of vaccinated mice successfully cleared the challenge dose, indicating that this vaccination strategy may provide sterile, protective immunity in the context of *B. mallei* infection in BALB/c mice.

Introduction

Infections caused by *Burkholderia mallei* and *Burkholderia pseudomallei*

Burkholderia mallei and *Burkholderia pseudomallei* cause the disease glanders and melioidosis, respectively. While these bacteria are phylogenetically similar, their lifestyles and epidemiology are quite divergent. Specifically, *B. mallei* is an obligate mammalian pathogen that typically infects solipeds, such as horses, mules, and donkeys, and only occasionally infects humans. Individuals most at risk of contracting the disease are animal handlers in close contact with infected creatures and those who ingest contaminated meat. Glanders was effectively eradicated in North America and Western Europe in the 1950's by mass culling of infected animals but remains in the equine population in Africa, Asia, the Middle East, Central and South America. There have been no reported natural cases of glanders in the US in over 60 years.

Conversely, *B. pseudomallei* is recognized as an important human pathogen endemic in Southeast Asia and Northern Australia, although not limited to these areas, as cases of melioidosis have been reported elsewhere (6; 91; 134). The bacterium is an environmental saprophyte and can be cultured from wet soil and ground water. Humans most likely to contract the disease are those who have prolonged contact with contaminated water and soil, such as farmers and others exposed to the natural environment. Interestingly, outbreaks of melioidosis are subsequent to typhoon season and flooding in several endemic areas (28; 73; 101). Farmers in this part of the world rarely wear protective

footwear when harvesting rice and other crops that are dependent on these wet seasons; the feet of these farmers often show signs of repeated trauma and injury, which likely represents the route of infection (138). Aerosols created by heavy rains can also increase the likelihood of inhalation of this pathogen. Additionally, epidemiological studies suggest an inverse correlation between physical well-being and susceptibility to infection, as individuals with diabetes, compromised liver or decreased renal function appear to have increased risk for infection (27).

Both glanders and melioidosis may present as either acute or chronic diseases and there are no pathognomonic signs of infection, which may hinder prompt diagnosis. In an acute infection, general symptoms include fever, malaise, abscess formation, pneumonia and sepsis. Even with aggressive antibiotic therapy, septicemia caused by *B. pseudomallei* has a mortality rate of approximately 40% (137). Since there has only been one documented case of human glanders in North America since 1949 (120), less is known about survival rates in individuals with *B. mallei* infections. Chronic melioidosis is often characterized by similar, albeit milder, symptoms than the acute disease and may last for months or even years (136). Reactivation of chronic *B. pseudomallei* infections have occurred in Vietnam veterans up to 18 years after their last exposure to the bacteria, a condition nicknamed “the Vietnamese time bomb” (74). Reactivation is often correlated with the onset of other illnesses, such as influenza infection, type 2 diabetes, and even cancer (90). *B. mallei* and *B. pseudomallei* can be contracted via abrasions in the skin and/or inhalation; the

dose and route of infection likely determines the severity of symptoms that develop.

B. mallei and *B. pseudomallei* are facultative intracellular bacteria, capable of infecting a wide range of cell types (54). This fact may help explain the long periods of latency observed in some infections. It is likely that intracellular replication and survival may also provide the bacteria with a means for evading the humoral immune system. Such factors should be taken into account when developing possible vaccine strategies.

The high rate of infectivity via aerosols of these bacteria, their resistance to many common antibiotics, and the absence of a vaccine for either infection make them of great concern as modern bioterror agents. In fact, *B. mallei* is a proven bioweapon that was used in both WWI and WWII and more recently by the former Soviet military in Afghanistan. It is believed that USSR also weaponized *B. pseudomallei.* With respect to natural infection with *B. pseudomallei*, even when the infection is treated early and aggressively with antibiotics, melioidosis has a high rate of relapse (136). However, a more recent study suggests that approximately one-quarter of those relapses may actually be re-infection with another strain (84) or that the initial infection was caused by multiple strains (105). An effective vaccine against these pathogens would not only protect those at high risk for natural infection, such as those in countries where the microorganisms are endemic, but could also decrease the desire to weaponize and intentionally release these bacteria.

Genomic analysis of *B. mallei* and *B. pseudomallei*

Although these bacteria have been relatively understudied, the recently sequenced genomes of *B. mallei* and *B. pseudomallei* have fostered the development of genetic tools for the determination of virulence factors, and allowed for the comparison of these two pathogens on a genetic level. Sequence analyses have revealed remarkable similarities and differences between the two species. Notably, the genome of the sequenced *B. mallei* strain ATCC 23344 (95) is smaller than that of the sequenced *B. pseudomallei* strain K96243 (58) (5.8 Mb compared to 7.25 Mb, respectively). When these genomes are compared to one another, more than 1400 genes are either absent or variant in *B. mallei*. However, genes common to both species are highly homologous and organized similarly along the genome. Amino acid identities of predicted homologs between these two organisms are greater than 96%. Full alignment of the two genomes reveal over 80% identity, and predicted proteins have high mean values of identity (98.8%) and length match (99.7%) (69). The majority of genes absent in the *B. mallei* genome are clustered on the *B. pseudomallei* genome and the deletion of these genes is consistent with insertion element-mediated mechanisms. As such, *B. mallei* is widely regarded as a niche-specific deletion-derivative of *B. pseudomallei*.

Strikingly, there are relatively few *B. mallei* specific genes (69), which suggests that a vaccine effective against *B. mallei* may be effective against *B. pseudomallei* as well. More recently, the genomes of *B. mallei* and *B. pseudomallei* were compared to five non-pathogenic but closely related

Burkholderia sp. *In silico* genomic subtraction identified 650 genes common to both pathogens but absent in the non-pathogenic strains, indicating that the products of these genes are putative virulence determinants (115) and possible cross-protective vaccine targets. Interestingly, even within the type strain of *B. mallei* (ATCC 23344) genetic variation can be observed. This strain was investigated for genomic stability when passaged through a variety of mammalian hosts (112). The majority of observed changes occurred within intergenic regions; however some mutated coding sequences resulted in altered protein expression. The observed genome instability was often due to changes in the number of repeating units within "simple sequence repeats". Another report recently described the attenuation of a previously virulent strain of *B. mallei*, SAVP1, upon passage of the bacterium in a cohort of equids (116). Sequencing of this attenuated strain revealed the loss of a number of virulence-associated genes, notably those encoding the type III secretion system required for virulence in both hamsters and mice. As such, the authors make the argument that this strain should be considered a candidate for exclusion from Select Agent regulations (116). If accepted, this may encourage increased research on this pathogen in the US, as experiments would not need to be performed in Biosafety Level (BSL)-3 facilities.

While similar passage experiments have yet to be published for *B. pseudomallei*, it has been documented that morphotypic variation exists among clinical clones in *B. pseudomallei* infection (16). In this study, particular morphotypes were associated with extremely high mortality when transferred to

BALB/c mice, while other morphotypes were much less fatal, and appeared to display chronic infection phenotypes. This implies that morphotype switching may be correlated with an increase in the production of factors associated with *in vivo* concealment (16). The genomic plasticity of these two pathogens suggests that host adaptation and/or immune evasion may alter gene expression, and should be considered during the development of therapeutics against these two pathogens.

Animal models of glanders and melioidosis

Identifying a relevant infection model is critical for defining the virulence of a particular pathogen and key in the advance of efficacious vaccines against that microorganism. **Table 1** summarizes the animal models that have been described for *B. mallei* and/or *B. pseudomallei* as well as their significance for vaccine development. Those anticipated to be of greatest clinical relevance will be discussed in more detail.

One of the most common models used for the study of *B. pseudomallei*, and more recently *B. mallei*, pathogenesis is the Syrian golden hamster (9; 41). These animals are extremely susceptible to virulent *B. pseudomallei* and *B. mallei* infection, while less virulent strains require a 4- to 5-log increase in inoculum to achieve equivalent mortality. Thus the hamster provides an elegant model by which the virulence of particular strains may be assessed. Where this model falls short however, is in the exquisite sensitivity of these animals to the pathogens, which does not accurately mimic human infection. Therefore, a

model slightly more resistant to infection with these organisms may provide a more physiologically relevant system for vaccine development.

As with other infectious agents, inbred mice represent powerful tools for the study of both virulence and immunity, and both BALB/c and C57BL/6 mice have been characterized as relevant infection models for melioidosis. Experiments performed by Leakey *et al.* (77) described in detail the differential infection outcomes of *B. pseudomallei* in these two mouse strains. Various bacterial inoculae were administered intravenously to assess the LD_{50} values. A 4-log difference in LD_{50} was measured between these two strains of mice, with BALB/c mice more sensitive than C57BL/6 mice. Additionally, BALB/c mice appeared to mirror acute melioidosis unlike infection in C57BL/6 mice, which mimicked the chronic course of infection. While only BALB/c mice presented with bacteremia, both BALB/c and C57BL/6 mice showed significant bacterial colonization in the liver and spleen. Recently it was discovered that BALB/c and C57BL/6 mice develop differential inflammatory responses as a result of infection with aerosolized *B. pseudomallei* (122). Specifically, the production of pro-inflammatory cytokines was markedly higher in BALB/c compared to C57BL/6 mice. It was concluded that hyperproduction of pro-inflammatory cytokines, specifically IFN-γ, in the BALB/c mice was not protective and may even lead to septic shock in this model. In contrast, the C57BL/6 mice reacted to infection with a moderate and transient induction of pro-inflammatory cytokines, which enabled them to clear the infection at the given dosage (122). The finding that these two mouse models respond in a dissimilar immunological fashion to *B.*

pseudomallei infection may account for their differences in susceptibility and may reflect similar differences between susceptible vs. non-susceptible individuals.

The BALB/c mouse model of infection has also been used for the study of glanders pathogenesis. The organotropism present in melioidosis infection is also present in glanders infection in this model system, as bacteria localize specifically to the liver and spleen. Of particular biodefense interest, these mice appear to be more susceptible to *B. mallei* by the aerosol route than the intraperitoneal route (129). This route of infection is also clinically relevant, since inhalation of *B. mallei* is one of the major routes of human disease, making it an attractive model in which to study the efficacy of potential vaccine strategies.

Until very recently, little was documented concerning experimental equine glanders. For *B. mallei* infection, solipeds are certainly the most physiologically appropriate model, since they represent the natural reservoir for this bacterium, although other animals have been noted as harboring the disease as well (40). In 2003, Lopez *et al.* (82) systematically described the clinical presentation of glanders in horses intratracheally inoculated with *B. mallei*. Infected animals developed inflammatory nodules and ulcers in the nasal cavity, with increased sticky, yellow nasal secretions, and enlarged and firm submaxillary lymph nodes. The horses also exhibited progressive debility, febrile episodes and dyspnea (82). Interestingly, necropsy of these animals revealed that the nasal mucosa was reliably positive for *B. mallei* colonies, but bacterial dissemination to other organs was uncommon. Although working with the equine model of glanders is

extremely challenging and expensive, the use of this biologically relevant model may prove to be valuable for the future of vaccine research against *B. mallei*.

Table 1. Animal models described for *B. mallei* and *B. pseudomallei* infections

Animal model	Pros	Cons	Reference
Syrian golden hamster	Well characterized, cost effective	Extremely sensitive (LD_{50} < 10 CFU)	(9; 41)
C57BL/6 mouse	Isogenic, well characterized, multiple routes of infection	Chronic infection specific	(77)
BALB/c mouse	Isogenic, well characterized, multiple routes of infection	Acute infection specific	(40; 77; 123)
Horses and other equines	Clinically relevant	*B. mallei* specific, cost prohibitive	(82)
Pigs	Clinically relevant	Only *B. pseudomallei* tested, chronic infection specific, high infectious dose	(93)
Diabetic rat	Clinically relevant	*B. pseudomallei* specific, only infant rats are susceptible	(140)
Galleria mellonella (wax moth)	Cost effective, rapid screening of virulence factors	Physiological revelance to clinical infection unknown	(115)
Caenorhabditis elegans	Cost effective, rapid screening of virulence factors	Limited sensitivity due to high infectious dose, may be *B. pseudomallei* specific, not physiologically relevant	(42; 96)

Vaccine approaches for *B. mallei* and *B. pseudomallei*

As mentioned, these facultative intracellular bacteria are capable of residing in vacuoles of eukaryotic cells (67; 129), which can complicate both antibiotic treatment and vaccine development. It is believed that intracellular survival is a key virulence determinant for both of these microorganisms. Therefore, it is likely that a cell-mediated immune response, perhaps in addition to a humoral response, may be critical for protection.

A number of virulence factors have been recognized in *B. mallei* and, in most cases, have also been identified as relevant in *B. pseudomallei* virulence (139). **Table 2** outlines some of the antigens of these two pathogenic agents that have been exploited for the development of vaccines as well as immunotherapy treatments used in an attempt to thwart the diseases caused by these bacteria. While this table is not meant to be an exhaustive list, it does highlight some of the more promising vaccine candidates against these two pathogens detailing the routes of administration and challenge. For reasons described previously, the overwhelming majority of these vaccines were tested in the BALB/c mouse model of infection. A notable exception to this is a protein polysaccharide conjugate vaccine mentioned in the study describing the equine model of glanders (82). To our knowledge, none of the vaccine procedures outlined have progressed to clinical trials. Antigens and therapies of particular interest will be discussed further.

Killed whole cell vaccines

Non-viable whole cell bacterial preparations represent a general starting point for the development of many vaccines and have proven to successfully immunize hosts against a variety of infections. This vaccine approach was employed by Amemiya *et al.* (3) in 2002 against *B. mallei* in BALB/c mice using three separate preparations of the bacteria: heat-killed, irradiated, and an irradiated capsule negative mutant. It was reported that each vaccine preparation yielded similar immune responses when splenocyte activation and sera immunoglobulins were analyzed. Splenocytes expressed a variety of cytokines, such as IFN-γ, IL-4 and IL-10, which indicated that there was a mixed reaction to the vaccine and that mice were unable to generate a directed Th1- or Th2-like response. Additionally, antibody titers in the sera of these mice suggested a Th2 skew, as the relative level of IgG1 was markedly higher than the level of IgG2a. The authors saw no protection at very high doses (>300x LD_{50}) when mice were challenged intraperitoneally (3). However, a more recent paper from the same laboratory showed that the addition of IL-12 to a vaccine preparation of irradiated *B. mallei* was able to preferentially enhance the amount of IgG2a generated in the sera, thereby inducing a more Th1-like antibody response (4). IL-12 also induced increased proliferation of splenocytes as well as the amount of IFN-γ produced by these cells when compared to mice vaccinated with killed *B. mallei* alone. Ultimately, mice vaccinated with IL-12 and *B. mallei* were better protected against a high challenge dose (>100x LD_{50}) 21 days after infection when compared to mice that received either killed *B. mallei* or

IL-12 alone. However, the spleens of the vaccinated survivors were greatly enlarged and heavily infected with *B. mallei*. These results suggest that the Th1-like response, induced by the addition of IL-12 to the preparation, improved the efficacy of this vaccine strategy in the context of BALB/c infection.

Live attenuated vaccines

The efficacy of attenuated live bacteria as a protective vaccine has been tested for both *B. mallei* and *B. pseudomallei*. In 2005, Ulrich *et al.* (124) studied the ability of two differently attenuated strains of *B. mallei* (a capsule-negative mutant and a branched-chain amino acid auxotroph), delivered aerogenically, to protect against aerosolized *B. mallei* challenge. Serum samples from vaccinated mice revealed that animals generated a Th2-like antibody response to the capsule negative mutant, with substantially higher titers of IgG1 compared to IgG2a. It was determined that this immune response was not protective, as no mice challenged with aerosolized *B. mallei* survived 5 days after infection. Conversely, the immune response to the auxotrophic mutant was skewed toward a Th1-like antibody response with a high IgG2a to IgG1 ratio. As a result 50% of these mice were able to survive high (>300x LD_{50}) aerosolized challenge. When the spleens of these mice were analyzed 30 days later, the authors found $>10^5$ *B. mallei* cells in every mouse, indicating that while the mice were able to survive the challenge dose, they were not able to completely clear the infection (124).

Similar results were obtained when a homologous *B. pseudomallei* auxotroph was investigated as a protective vaccine against *B. pseudomallei*

infection (52). However, these investigators delved further into the mechanism of partial protection. Splenocytes from vaccinated mice proliferated *in vitro* in response to whole non-viable *B. pseudomallei*, and $CD4^+$ and $CD8^+$ T cells responded by increasing the production of IFN-γ, consistent with previous reports underlining the importance of IFN-γ in protection. To determine the roles specific T cells play in protection with regards to this vaccine, immunized mice were antibody depleted of either $CD4^+$ or $CD8^+$ T cells before and after *B. pseudomallei* challenge. Immunized $CD4^+$ cell-depleted mice were substantially more susceptible to infection compared with immunized mice given isotype control antibodies and succumbed to infection at the same time as unimmunized control mice, indicating that protection was abrogated with the loss $CD4^+$ T cells. In contrast, $CD8^+$ T cell depletion had no effect on vaccine-mediated protection. Taken together, these data demonstrate that protection generated by this vaccine is mediated by $CD4^+$ T cells, but not by $CD8^+$ T cells (52).

Polysaccharide-based vaccines

Bacterial polysaccharides are often potent stimulators of host immune responses and represent critical components of subunit and conjugate vaccines for clinically relevant diseases, such as *Haemophilus influenzae* type b (Hib), pneumococcal, and meniginococcal infections. Lipopolysaccharide (LPS) has been shown to be an immunodominant antigen recognized in patients infected with *B. pseudomallei.* Importantly, the level of antibody to LPS on admission to the hospital is higher in patients with melioidosis who survive compared with

those who die, and in patients with non-septicemic versus septicemic melioidosis (138), suggesting that these antibodies may protect the host from death. Alternatively, it may be that the presence of high anti-LPS antibody titers is indicative of a more efficient host immune response, including cell-mediated killing (138).

Importantly, the LPS of *B. pseudomallei* and *B. mallei* are remarkably similar. The O antigen portion of LPS from each species is composed of a disaccharide: [3)-β-D-glucose-(1→3)-6-deoxy-α-L-talose-(1→]$_n$. Where these two O antigens differ however, is in the location and level of *O*-acetyl substitutions on the talose residue. None of the O antigen polysaccharide structures from *B. mallei* LPS are acetylated at *O*-4 and are variably acetylated or methylated at *O*-2, while *B. pseudomallei* produces two structures; one that is acetylated at *O*-4 and partially methylated (33%) at *O*-2, and a second that is not acetylated at *O*-4 and is partially acetylated (67%) at *O*-2 (11; 71).

B. mallei and *B. pseudomallei* are encapsulated bacteria whose capsular polysaccharides have been shown to be important virulence determinants in Syrian golden hamsters and BALB/c mice (102; 108). The structure of the major capsular polysaccharide of *B. pseudomallei* was determined as [-3)-2-*O*-acetyl-6-deoxy-β-D-*manno*-heptopyranose-(1-] by Perry *et al.*, but it was characterized at the time as type I O-polysaccharide. However Reckseilder *et al.* later identified this structure as the capsular polysaccharide (102; 108). The chemical structure of *B. mallei* capsular polysaccharide has yet to be determined, but based on gene homology and organization DeShazer *et al.* suggests that the

polysaccharide is similar, if not identical, to the major capsule structure of *B. pseudomallei* (30). Both *B. mallei* and *B. pseudomallei* have additional surface polysaccharides that have been recently described in another review (47). Taking into account the genetic and structural similarity that exists between characterized polysaccharides of these species, it is possible that a vaccine specific for either the LPS and/or the capsule of *B. mallei* or *B. pseudomallei* may be able to protect against both pathogens.

Both the LPS and capsular polysaccharides have been evaluated as subunit vaccine candidates against *B. pseudomallei* by Nelson *et al.* in 2004 (94). In this study, it was discovered that BALB/c mice responded differently to the two surface polysaccharides. Specifically, mice intraperitoneally immunized with LPS generated high titers of IgM and IgG3, which were augmented with the addition of adjuvant to the vaccine. Mice immunized with capsular polysaccharide, on the other hand, presented with an IgG2b response. When adjuvant was added to the capsule preparation, an increase in IgM was measured but no increase in any other antibody subtype was reported (94). Vaccination with either subunit was able to increase the mean time to death in mice when compared to unimmunized controls (2.6 days for unimmunized, 10.5 days for capsule and 17.6 days for LPS) when challenged intraperitoneally at 250 x LD_{50}. The authors saw a negligible increase in protection when mice were challenged with lower doses (2.5 x LD_{50}) via the aerosol route (94). Unfortunately, as with all other *B. mallei* and *B. pseudomallei* vaccination protocols that we have seen in the literature to date, protection engendered by this procedure was not complete.

Recognizing the importance of LPS-specific antibodies in patients with melioidosis, passive immunization with monoclonal antibodies for *B. mallei* LPS has been evaluated for vaccine efficacy against glanders infection (123). In this study 3 different LPS-specific monoclonal antibodies were able to significantly protect BALB/c mice from death up to 14 days when challenged with a high aerosolized dose of *B. mallei* (20 x LD_{50}). These results represent the first successful immunotheraepeutic protection against the bioweaponization relevant administration of *B. mallei*. This suggests that these antibodies provide relevant protection against this pathogen in the initial stage of infection. The anti-LPS antibodies were not, however, able to provide protection when administered 18 hours after *B. mallei* infection (123), suggesting that the antibodies are cleared by this time or the bacteria have been internalized and therefore are resistant to the circulating antibodies. Alternatively, during this later stage of the infectious process, *B. mallei* may not be expressing the specific epitope to which the antibodies are directed. In any case, lack of effective antibodies after infection allowed the bacteria to spread and cause disease.

Cell-mediated immunity

The results of the various vaccination strategies outlined in this review signify that a cell-mediated immune response, in conjunction with a humoral response, is likely required to successfully clear infections caused by *B. mallei* and *B. pseudomallei*. However, the cellular immune response needed to clear these infections is proving to be intricate and complex. Perhaps surprisingly,

Haque e*t al*. demonstrated that $CD8^+$ cells were dispensable in protection when mice were vaccinated with an auxotrophic mutant of *B. pseudomallei*. However, the precise level of attenuation of this mutant has yet to be defined. It is possible that these bacteria are less able to invade host cells to cause disease, which would lessen the requirement for a cytotoxic T cell response in this particular model of protection. Additionally, since vaccinated mice were unable to successfully clear challenge infection in this study, even in the presence of both $CD4^+$ and $CD8^+$ cells, the potential remains for the necessity of cytotoxic T lymphocytes in the clearance of host cells infected with *B. pseudomallei*. Amemiya *et al.* were able to highlight the importance of both IL-12 and IFN-γ in the promotion of an effective immune response against *B. mallei*. Similarly, melioidosis patients routinely present with elevated serum levels of IFN-γ and IL-12 (76). It is widely accepted that IFN-γ production is the hallmark of Th1 cells; NK and $CD8^+$ T cells are also known to produce this cytokine. Several of the studies previously outlined in this review also indicate that a skewed Th1-like antibody response increases survival in mice infected with either *B. mallei* or *B. psueodmallei.* However, Tan *et al.* (122) demonstrated that brute force expression of IFN-γ is likely to contribute to the immunopathogenesis of *B. pseudomallei* in BALB/c mice, underlining the importance of a balanced and regulated immune response in the clearance of this pathogen. Ultimately, exploitation of the differential immune responses generated by BALB/c and C57BL/6 mice during experimental infection may provide additional clues into the proper immune response needed to effectively eradicate these infections, and

may provide the nidus for the next generation of vaccine development against these fatal pathogens.

Table 2. Antigens, immunotherapies, and other treatments used as vaccines against *B. mallei* and *B. pseudomallei*

Antigen	Bacteria targeted	Route of administration	Challenge method	Reference
Killed whole cell	*B. mallei*	SC	IP	(3)
Attenuated live whole cell	*B. mallei and B. pseudomallei*	Aerogenic IP IN	Aerosol IP IP	(52; 121; 124)
Lipopolysaccharide (LPS)	*B. pseudomallei*	IP	IP or IN	(94; 114)
Capsule polysaccharide (CP)	*B. pseudomallei*	IP	IP or IN	(94; 114)
Pili	*B. mallei*	SC	Aerosol	(37)
Outer membrane proteins	*B. pseudomallei*	IP	IP	(53)
Type 3 secretion subunits	*B. pseudomallei*	IP IP	IP IP	(34)
Immunotherapy and other treatments	**Bacteria targeted**	**Route of administration**	**Challenge method**	**Reference**
Interleukin-12 with killed whole cells	*B. mallei*	SC	IP	(4)
Monoclonal antibodies to LPS	*B. mallei*	IP	Aerosol	(68; 123)
Monoclonal antibody cocktail to LPS, CP and proteins	*B. pseudomallei*	IV	IP	(68)
CpG oligodeoxynucleotide	*B. mallei*	IP	Aerosol	(130)
Primed dentritic cells with CpG	*B. pseudomallei*	ID	IP	(35)
Flagella DNA	*B. pseudomallei*	IM	IV	(18)

Table 2 abbreviations: IP = intraperitoneal, IV = intraveneous, IN = intranasal, IM = intramuscular, SC = subcutaneous, and ID = intradermal

On the fringe of vaccine development

The vaccine approaches discussed in the previous section represent strategies that have proven efficacy against important pathogens, but unfortunately, none have yet provided sterile immunity against either *B. mallei* or *B. pseudomallei* in the mouse model of infection. However the merits of these approaches have yet to be fully determined for these microorganisms and the definition of new virulence factors and immunogens may improve the host response and promote full clearance in the context of classical vaccination schemes.

More recently however, researchers have been exploring the benefits of using attenuated bacteria as heterologous antigen delivery systems. Facultative intracellular, attenuated bacteria are considered strong candidates for passenger antigen carriage, as they are known to generate humoral and cell-mediated immune responses. *Salmonella* sp. in particular have been recognized as antigen delivery systems with many advantageous characteristics. They are amendable to genetic manipulation and can be administered orally or intranasally. Additionally, there is a vast body of literature regarding the genomes, life cycles, immunology and molecular pathogenesis of *Salmonella*, providing a solid foundation for the elucidation of protection mechanisms. A major advantage to using *Salmonella* sp. is the selective targeting of mucosa. The ability to generate mucosal immunity is highly desirable; mucosal immunity provides an extra layer of host protection that prevents infection from occurring, where as systemic immunity resolves the infection before disease can occur.

A strain of *Salmonella* commonly used for the expression of heterologous antigens is *Salmonella typhimurium* (SL3261 aroA$^-$), a murine-specific pathogen. Deletion of the *aroA* gene metabolically cripples the bacterium; it retains the capacity to invade epithelial cells but requires aromatic amino acids for replication, abrogating its ability to cause disease in mice. Immune responses against heterologous antigens have been demonstrated in *S. typhimurium* expressing a variety of proteins including, but not limited to, tetanus toxin (36), streptococcal M protein (106), anthrax protective antigen (25; 39), and HIV gp120 (39). Previous work in our laboratory illuminated the ability of *S. typhimurium* to successfully express polysaccharide antigens as well. Mice vaccinated with *Salmonella* constructs expressing the O antigen of *Pseudomonas aeruginosa* were protected from *Pseudomonas* challenge administered in a variety of clinically relevant ways. DiGiandomenico *et al.* also discovered that mucosal delivery of this vaccine was more efficacious than systemic delivery (32; 33). Drawing from this work, we set out to determine if *S. typhimurium* expressing the O antigen portion of LPS from *B. mallei* could similarly protect mice from subsequent challenge infection. We found that 62% of mice vaccinated survived beyond 3 months post exposure. Importantly, 50% of vaccinated mice had no detectable CFU in the lungs, liver or spleen. To our knowledge sterile immunity has not yet been documented for any vaccination scheme attempted to combat this pathogen. Our results suggest that mice vaccinated with the attenuated *Salmonella* construct have the ability to generate the proper immune response required to completely clear this infection.

Materials and Methods

Transduction of *B. mallei* O antigen biosynthetic locus into *S. typhimurium aroA.* The identification of the biosynthetic gene cluster responsible of the addition of O antigen onto the lipid A core of *B. mallei* was originally described by Burtnick *et al.* (see **figure 1**) (11) . This locus, on plasmid pIC3, was generously supplied to us by Dr. David Waag USAMRIID Fort Detrick, MD. The plasmid was introduced into *Salmonella typhimurium* strain SL3261 via transduction as previously described (103), and this protocol was performed by Antonio DiGiandomenico.

Transfer of plasmid pCPoacA into SL3261 and SL3261/pIC3. The plasmid, pCPoacA (containing the O acetylation gene of *B. pseudomallei*), and the empty vector control plasmid, pUCP31T (117), were generously provided by Dr. Paul J. Brett, Rocky Mountain Laboratories, Hamilton, MT. Plasmids were introduced into MAX Efficiency® DH5α chemically competent *E. coli* according the manufacturer's instructions (Invitogen™). Positive clones were selected for on 20 µg/ml gentamycin LB agar plates, and grown overnight at 37°C with rotation in LB with antibiotic. Plasmid was harvested from these cells using a QIAprep® Spin Miniprep Kit (Qiagen Sciences, MD) per the manufacturer's instructions. Plasmid concentration was estimated by UV spectroscopy and sequenced for confirmation of the insert. Competent cells were made using overnight culture of SL3261 was diluted 1:100 in 25 ml of LB and grown at 37°C with shaking until an

optical density at 600nm (OD_{600}) of 0.35 was reached. The culture was then poured into 250 ml centrifuge bottles and incubated on ice for 1 hour, after which the cells were spun at 4°C for 15 min at 2500 X *g*. Supernatant was discarded and pellets were washed in 2 ml of ice cold 10% glycerol by shaking. Once the pellet was dissolved, 25 ml of cold 10% glycerol was added to the mixture and spun at 4°C for 15 min at 2500 X *g*. This wash process was repeated three more times. After the fourth spin, 1ml of cold 10% glycerol was used to dissolve the cell pellet and spun again using the same parameters. The final pellet was completely dissolved in 300 µl of cold 10% glycerol and transferred to 100 µl aliquots for electroporation.

One aliquot of competent SL3261 or SL3261/pIC3 cells and 2 µl of freshly purified plasmid were added to an ice cold electroporation cuvette. The cuvette was incubated on ice for 5 min and mixed with a pipette tip. Cells were then electroporated at 2.5 kV, 25 µFd capacitance and 200 Ω resistance using an Eppendorf Electroporator 2510 (Eppendorf North America, Westbury, NY). Cells were brought up to a volume of 1 ml with SOC medium (Invitrogen, Corp. Carlsbad, CA), which was then poured into a 1.5 ml tube and allowed to recover at 37°C for 1 h. Recovered cells were briefly spun in a microcentrifuge (30 sec), the supernatant was discarded and then replaced with 200 µl LB without antibiotic. The resulting culture was split and spread onto LB plates containing the appropriate antibiotic (100 µg/ml ampicillin + 20 µg/ml gentamycin SL3261/pIC3/pCPoacA and 20 µg/ml gentamycin for SL3261/pCPoacA) for selection and allowed to grow overnight at 37°C. Single positive transformants

were picked, grown overnight with the appropriate antibiotic concentration, and stored at -80°C until needed.

LPS extraction and analysis. LPS from SL3261, SL3261/pIC3, SL3261/pCPoacA and SL3261/pIC3/pCPoacA was isolated from whole cells using a hot phenol extraction method (135). Overnight LB cultures (SL3261/pIC3 cultures were supplemented with 100 µg/ml ampicillin, SL3261/pCPoacA with 20 µg/ml gentamycin and SL3261/pIC3/pCPoacA with 100 µg/ml ampicillin and 20 µg/ml gentamycin) were diluted to an OD_{600} of 0.5 followed by pelleting of 4 ml. Pellets were resuspended in 200 µl of 1X sodium dodecyl sulfate (SDS) buffer (0.1M Tris-HCl pH 6.8, 2% beta-mercaptoethanol, 2% SDS, 10% glycerol) and boiled for 15 min. Once samples were cooled, 10 µg/ml concentrations of each RNase and DNase were added and incubated for 30min at 37°C. Ten µg/ml proteinase K was added and samples were incubated at 59°C for 3 h. Two hundred µl of cold Tris-saturated phenol was then added and the samples were vortexed and incubated at 65°C for 15 min. To these samples 1 ml of diethyl ether was added, vortexed, and then centrifuged at 14,000 rpm for 10 min. The bottom layer of each sample was transferred to a new microcentrifuge tube and extracted using phenol-ether method once more. A 200 µl volume of 2X SDS buffer was added to the samples after the final extraction.

LPS samples were separated on 12% SDS-polyacrylamide gels and visualized using the Pro-Q® Emerald 300 Lipopolysaccharide Gel Stain Kit (Invitrogen, Corp. Carlsbad, CA) or transferred directly to nitrocellulose for

immunoblotting. Nitrocellulose blots were blocked in “killer filler” (10% 0.1M NaOH, 5% casein, 5% BSA fraction V, 0.01% Phenol red and 0.02% sodium azide in PBS, pH 7.4) for 1 hour, rinsed with PBS-T (PBS + 0.05% Tween-20) three times, and then incubated in primary antibody overnight with shaking at 4°C. The blots were analyzed using either a *B. mallei*-specific monoclonal LPS antibody (5C8-IC3) or a *B. pseudomallei* monoclonal LPS antibody (3B3-5), both of which were generously provided by Dr. David Waag, USAMRIID Fort Detrick, MD, and diluted 1:1000 in “killer filler”. Primary antibody dilutions were retained after use and stored at 4°C until needed further. The nitrocellulose was then rinsed three times in PBS-T. The secondary antibody used was anti-mouse immunoglobulin G3 (1:5000 in “killer filler”) coupled to alkaline phosphatase (Southern Biotechnology Associates, Inc., Birmingham, AL), and was allowed to bind to the blot for 1 hour. Secondary was then drained; blots were rinsed three times with PBS-T and twice with PBS. Alkaline phosphatase conjugation was visualized using Sigma *Fast* NCIP/NBT tablets (Sigma-Aldrich, Inc., St. Louis, MO), diluted in 10ml of sterile de-ionized water.

***B. mallei* LPS extraction and ELISA plate coating.** The following procedures were performed by members of Dr. Thomas J. Inzana’s laboratory, Virginia Tech, Blacksburg, VA. LB broth inoculated with *B. mallei* (ATCC 23344) was incubated overnight with vigorous shaking. Cell pellets were obtained by centrifugation and LPS was extracted using a modified hot aqueous-phenol procedure (102). Following extraction, the resulting phenol and aqueous phases

were combined and dialyzed in distilled water to remove the phenol. The dialysates were then clarified by centrifugation and concentrated by lyophilization. The crude preparations were solubilized to a concentration of 20 mg/ml in RD buffer (10 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 50 mg/ml RNase A and 50 mg ml-L DNase I) and incubated for 3 h with shaking at 37°C. Proteinase K was then added to a final concentration of 50 mg/ml and the digests were incubated for 3 h at 60°C. The samples were clarified by centrifugation and the supernatants were filter sterilized. LPS was isolated from the supernatants as precipitated gels following three rounds of ultracentrifugation at 100,000 X *g* and 4°C. After the final spin, the gelatinous pellets were resuspended in pyrogen-free water and lyophilized. To remove contaminating phospholipids, lyophilized LPS samples were repeatedly extracted with 90% ethanol.

Immulon IV microtiter plates were coated with 100 µl of 10 µg/ml *B. mallei* LPS (as extracted above) in PBS (pH 7.2) supplemented with 20 mM $MgCl_2$ and incubated for 1 h at 37°C. The wells were then washed three times with PBS + 0.05% Tween-20 (PBS-T) and subsequently blocked with 200 µl 5% skim milk/ PBS-T for 1 h at 37°C. The plates were then washed three times with PBS-T and air-dried. Dried plates were shipped overnight to the University of Virginia for serum analysis, where they were stored at 4°C until needed.

Intranasal vaccination of mice. The University of Virginia Animal Care and Use Committee approved all procedures concerning the use of mice for this study. Female 6-8 week old BALB/c mice (Harlan Sprague-Dawley Farms,

Chicago, IL) were housed under climate and pathogen controlled conditions and fed autoclaved rodent feed and acid-free water. Mice were anesthetized intraperitoneally with 200 µl of ketamine (6.7 mg/ml) and xylazine (1.3 mg/ml) in a 0.9% saline solution prior to vaccination. Mice were intranasally instilled (10 µl per nostril) with 20 µl of PBS, 1 x 10^7 CFU SL3261, or 1 x 10^7 CFU SL3261/pIC3. Boosters were performed using the same protocol as the initial vaccination approximately 14 days later.

Serum collection. Blood samples were collected from the tail vein of each mouse after warming with a heat lamp approximately 1 week before initial vaccination (preimmune sera) and approximately 2 weeks after the boost vaccination. The samples were allowed to rest at room temperature for 4 h and then incubated overnight at 4°C. Serum was collected by centrifugation at 1,700 X *g* for 10 min and then stored at -80°C until needed. All serum samples were diluted in PBS supplemented with 1% bovine serum albumin (PBS-B) prior to ELISA analysis.

Serum titer analysis using ELISA. Serum samples were serially diluted 1:100 up to 1:102,400 in PBS-B and 100 µl was placed into each well on *B. mallei* LPS-coated plates in duplicate. After overnight incubation at 4°C, the plates were washed three times with PBS-T and air-dried. Secondary antibodies (anti-mouse total IgG, IgG1, IgG2a, IgG2b, IgG3, IgA or IgM conjugated to alkaline phophatase [Southern Biotechnology Associates, Inc., Birmingham, AL])

were added to individual plates, diluted 1:5000 in PBS-B and incubated at 37°C for 1 hr. The plates were then washed three times with PBS-T and air-dried. For analysis, 200 µl of substrate containing 4-nitrophenol phosphate disodium salt hexahydrate (PNPP) (Sigma Chemical Co., St. Louis, MO) diluted to 1 mg/ml in PNPP substrate solution (10% diethanolamine, 25 µM $MgCl_2$) was added to each well and incubated in the dark at room temperature for 30 min. Hydrolysis of PNPP was stopped by the addition of 3 M NaOH to each well. The plates were examined using a Molecular Devices Thermo microplate reader at OD_{405} and the data was displayed using SOFTmax Pro version 1.1 software. IgG subtype quantification of serum samples was based on interpolated values derived from standard curves as described previously (32).

Survival studies. These studies were performed by members of Dr. Inzana's laboratory, Virginia Tech, Blacksburg, VA. *B. mallei* (ATCC 23344) cultures were started at a Klett reading of 40 in 20 ml LB + 4% glycerol broth and grown with 200 rpm at 37°C to a Klett value of 200 (approximately 2 x 10^9 CFU/ml). The culture was then serially diluted to 5 x 10^4 CFU/ml in PBS and held at room temperature during the challenge procedure. Mice were anesthetized with isofluorane gas and subsequently challenged intranasally with 20 µl of diluted culture (1000 CFU or 5 x LD_{50}). For each round of experiments, 2-4 mice were challenged with PBS to ensure that the method was not detrimental to the health of the mice.

A wellness check was performed on all challenged mice every 24 h until signs of illness occurred, after which they were checked three times daily and euthanized if extremely moribund.

Mice that survived at various times post challenge were euthanized, and the lungs, liver and spleen were harvested. Organs were weighed and then macerated in 500 µl PBS. Macerated tissue was either plated neat onto LB + 4% glycerol agar plates or serially diluted and then plated based on the appearance of the organ. CFU were determined after 3 day incubation at 37°C in 5% CO_2.

Statistical analysis. All analyses were performed using GraphPad Prism version 4 software. ELISA endpoint titers were calculated using the linear regression of duplicate measurements of adjusted OD_{405}s and were expressed as the reciprocal dilution. The *x* intercept served as the endpoint titer. Antibody titers were compared using the Kruskal-Wallis U test for comparison of three groups or the Mann-Whitney U test for two group analysis. IgG subtype values were analyzed using a mixed-linear-model methodology. The results of survival studies were represented using Kaplan-Meier survival curves and were analyzed by the log-rank test.

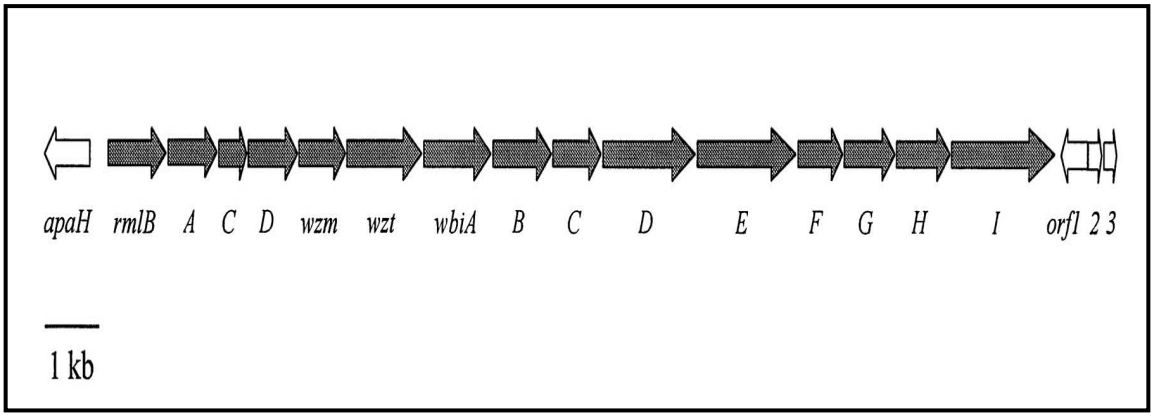

Figure 1. Genetic map of the *B. mallei* (ATCC 23344) region required for O antigen synthesis. The genes names are based on homology to those from *B. pseudomallei*. The direction of transcription is shown by arrows; the dark gray arrows represent those genes involved in O antigen biosynthesis (11).

Results

Expression and acetylation of *B. mallei* O-antigen on the surface of *S. typhimurium*. In order to determine whether *S. typhimurium* was able to express *B. mallei* O antigen on its surface, LPS was extracted from strains SL3261 and SL3261/pIC3 and analyzed by immunoblotting with a monoclonal, *B. mallei*-specific LPS antibody (**Fig. 2b**). **Figure 2a** depicts the same SDS-PAGE gels used for immuno-detection, stained for LPS. These blots confirm that the genes contained on plasmid pIC3 can add the O antigen subunit of *B. mallei* onto the lipid A of *S. typhimurium*. The acetylation gene of *B. pseudomallei* (*oacA*) was added on a plasmid to strain SL3261/pIC3 to make strain SL3261/pIC3/oacA in order to determine if this construct could cross-react with *B. pseudomallei* LPS-specific antibodies. However, conclusive results have not yet been obtained from these experiments.

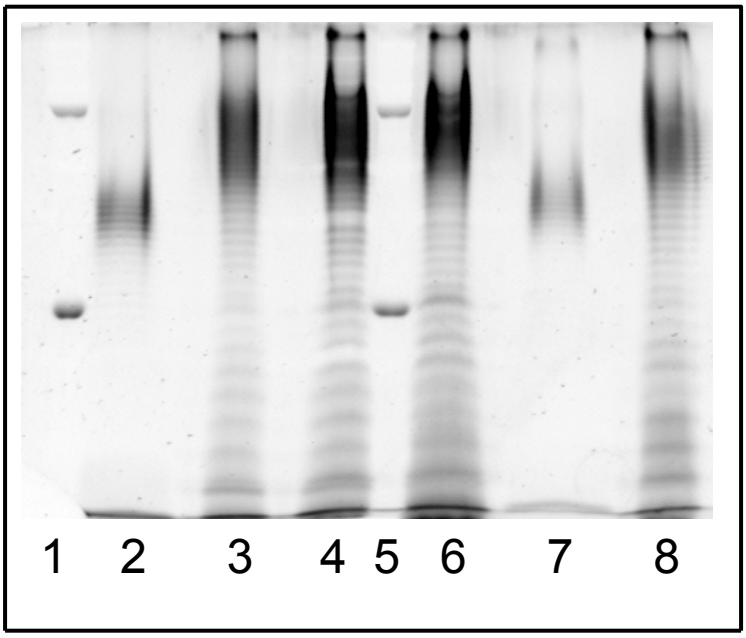

Figure 2a. SDS-PAGE LPS gel stained with Pro-Q® Emerald 300 Lipopolysaccharide Gel Stain Kit from Invitrogen™. The lanes correspond to LPS extracted from the following bacterial samples: 1 =MW marker, 2 = *B. mallei*, 3 = SL3261, 4 = SL3261/pIC3 5 = molecular weight marker, 6 = SL3261/pIC3/oacA, 7 = *B. pseudomallei*, 8 = SL3261/oacA

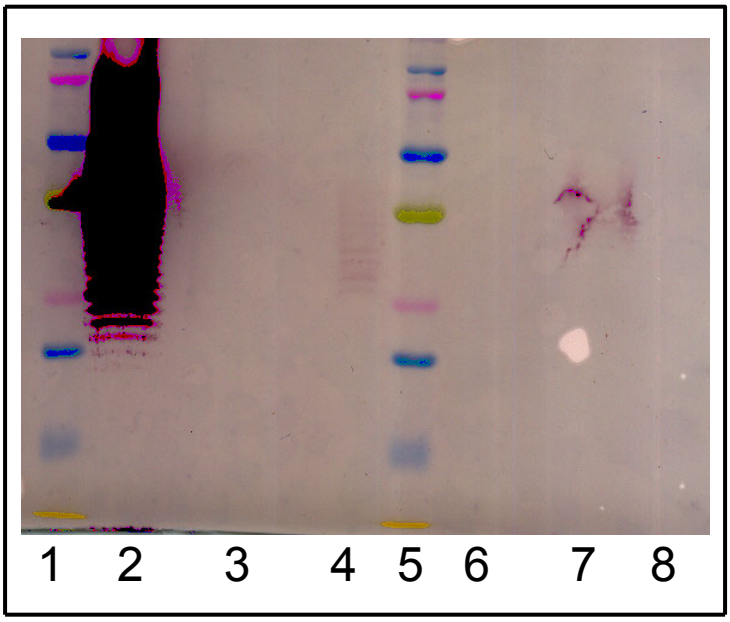

Figure 2b. Western immunoblot with the monoclonal *B. mallei* LPS antibody, 5C8-IC3. The lanes correspond to LPS extracted from the following bacterial samples: 1 =MW marker, 2 = *B. mallei*, 3 = SL3261, 4 = SL3261/pIC3 5 = molecular weight marker, 6 = SL3261/pIC3/oacA, 7 = *B. pseudomallei*, 8 = SL3261/oacA.

The immunogenicity of SL3261/pIC3. Groups of 8, 8-10 week old, female BALB/c mice were intranasally vaccinated with PBS, 1 x 10^7 CFU SL3261, or 1 x 10^7 CFU SL3261/pIC3 and boosted with the same dosage approximately 2 weeks later. Fourteen days after the boost, sera was collected and analyzed for reactivity to *B. mallei* LPS coated microtiter plates using ELISA. Endpoint titers for total IgG, IgA and IgM antibodies specific for *B. mallei* LPS were measured and values are depicted in **figure 3**. Mice vaccinated with the SL3261/pIC3 generated a strong IgG response compared to mice receiving either SL3261 or PBS. Vaccinated mice also generated a secretory IgA response that was not seen in either control group. IgG subtype analysis revealed that IgG2a production was predominant, with lesser amounts of IgG2b and IgG3 (**fig. 4**). The least abundant immunoglobulin G subtype was IgG1. We also examined the levels of IgG1 and IgG2a subclass response to *B. mallei* as representing a Th2- or Th1-like immune response, respectively. The mean ratio of IgG2a to IgG1 for these mice was 53.72, indicating that mice vaccinated with this strain generated a Th-1 like antibody skew.

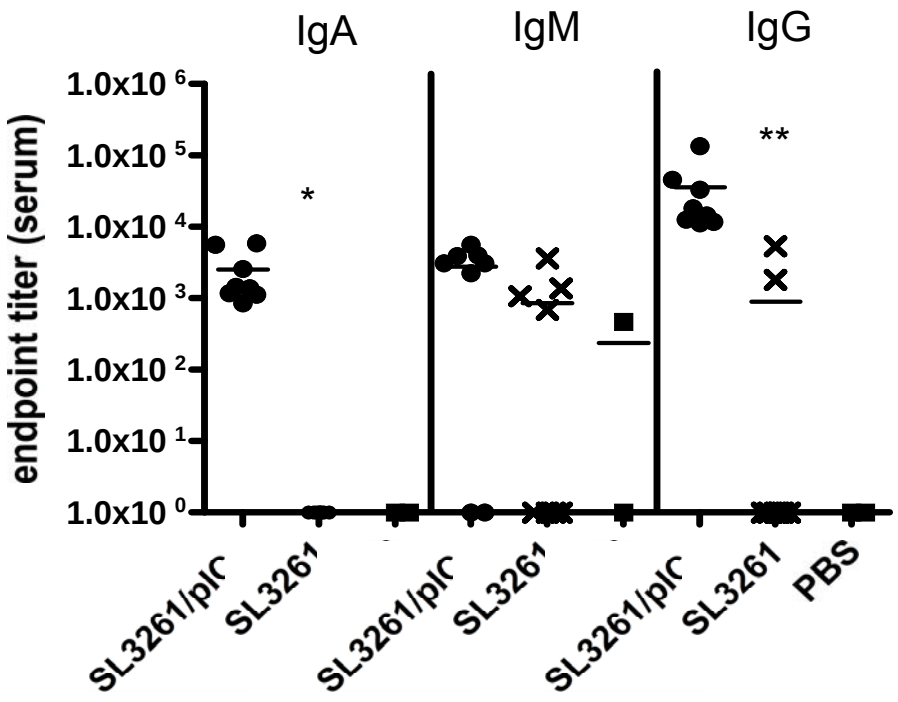

Figure 3. Serum *B. mallei* LPS specific antibody titers of isotype total IgG, IgM and IgA from BALB/c mice, as determined via ELISA using plates coated with purified *B. mallei* LPS. Statistically significant differences in antibody titers of mice receiving the vaccine strain were measured for secretory IgA (single asterisk, p value < 0.001 using Kruskal Wallis analysis of all three groups) and total IgG (double asterisk, p value < 0.001 using Mann Whitney pairwise analysis of vector vs. vaccine, p value < 0.001 using Kruskal Wallis analysis of all three groups).

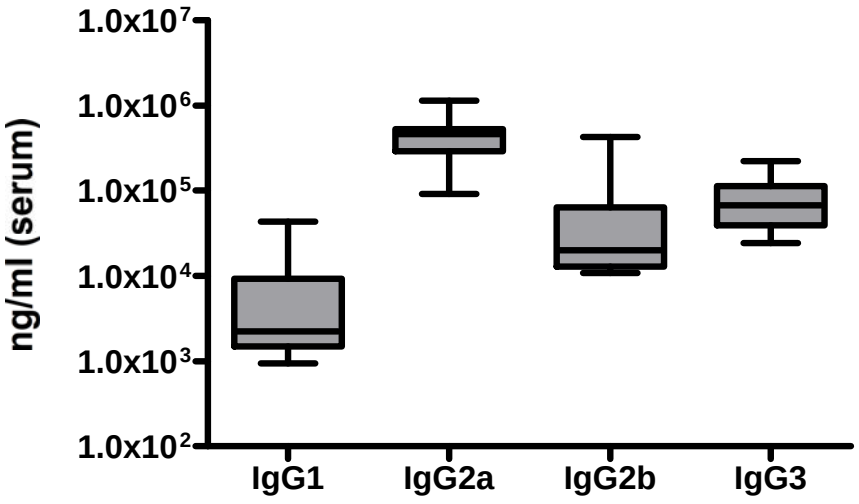

Figure 4. IgG subtype responses in the sera of vaccinated mice, as determined by ELISA on plates coated with *B. mallei* LPS. Box and whisker plots represent analysis of eight vaccinated mice. The box depicts the first and third quartiles, the median is represented as the line within the box, and the whiskers depict the range. The relative amount of each IgG subtype was calculated by extrapolation from standard curves that were generated for secondary antibody reactivity to each IgG subtype.

Intranasal vaccination with SL3261/pIC3 increases survival of challenged BALB/c mice. To test the ability of strain SL3261/pIC3 to confer protection from death upon challenge with *B. mallei* ATCC strain 23344 when compared to control treatments, mice were anesthetized and instilled intranasally with either 1000 CFU *B. mallei* (5 x LD_{50}) or PBS. A significant increase in short-term survival, as a function of time post infection, was observed in mice receiving the recombinant vaccine compared to mice that received vector alone or PBS (**fig. 5**). Death was not observed in vaccinated mice until day 40 post infection, after which a total of three mice succumbed to infection (**fig. 6**).

By day 73 post infection, all control mice had succumb and surviving vaccinated mice were sacrificed and analyzed for colonization in the liver, lungs and spleen. Of the five survivors, one was visibly ailing. Autopsy of this mouse revealed splenomegaly with pustulent abscesses. The lungs and liver of this mouse were pale compared to healthy tissue. CFU determination revealed that the spleen contained 3.55×10^5 bacteria, the liver was colonized with 1.0×10^3 bacteria, and the lungs were sterile. The four remaining vaccinated survivors, however, appeared healthy prior to sacrifice and harbored no detectable CFU in any organ tested.

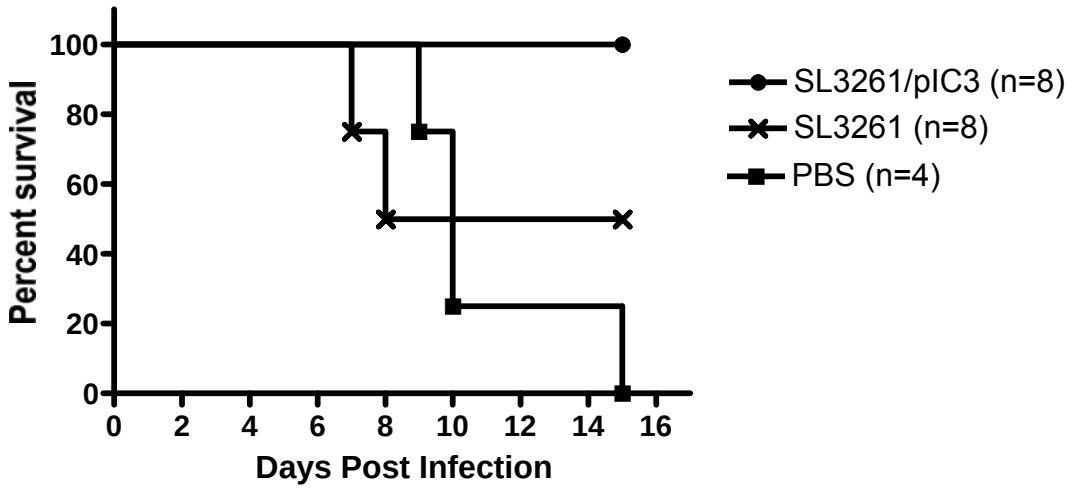

Figure 5. Short term survival of BALB/c mice challenged with 5 x LD_{50} (1000 CFU) *B. mallei* (ATCC 23344). At day 15 there was a statistically significant difference in the survival of vaccinated (SL3261/pIC3) mice compared to vector and PBS treated (p value < 0.02 using log rank analysis).

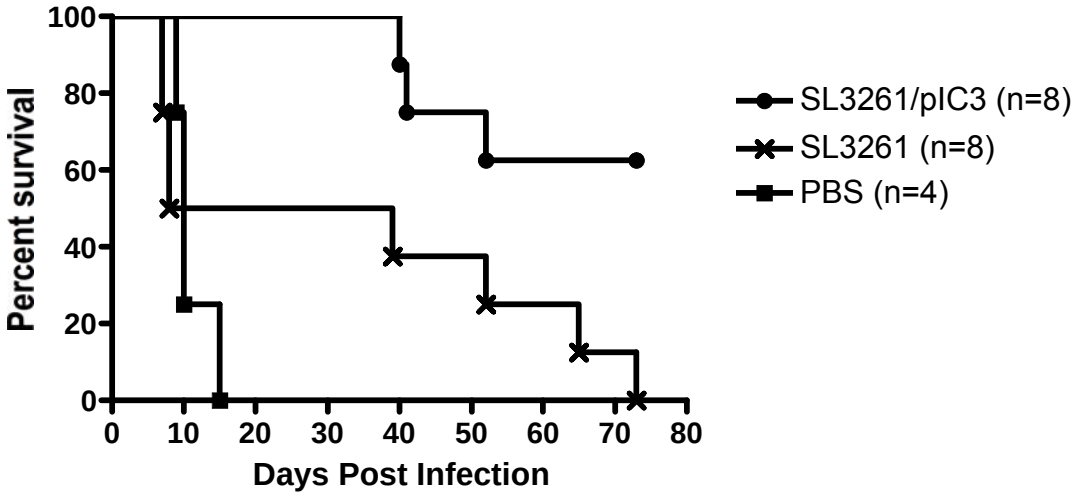

Figure 6. Long term survival of the same cohort of BALB/c mice challenged with 5 x LD_{50} (1000 CFU) *B. mallei* (ATCC 23344). There was a significant increase in survival of vaccinated mice compared to control treated mice 73 days after infection (p value < 0.007 using log rank analysis). At this time point, surviving mice were sacrificed and assessed for *B. mallei* CFU (discussed in the text).

Discussion

B. mallei is a zoonotic pathogen whose natural reservoir is equids, while only occasionally infecting humans. However, the historical use of this bacterium as an agent of biological warfare, its high infectivity via aerosols, antibiotic resistance, the ambiguity of symptoms, and the lack of a vaccine make it an attractive candidate for bioweaponization. The highly related pathogen, *B. pseudomallei*, not only poses similar risks if weaponized, but is also endemic to Southeast Asia and Northern Australia and can cause infections in native inhabitants and tourists with severe consequences. These bacteria can enter the body of the host via abrasions in the skin and by ingestion, but inhalation is widely regarded as the portal of entry leading to the most severe disease. As such, generating mucosal immunity to these pathogens may provide the greatest protection from pneumonic illnesses caused by these bacteria, which have the highest morbidity and mortality compared to cutaneous and GI infections.

Attenuated *Salmonella* strains have been used as heterologous antigen expression systems for a variety of proteins (17; 43; 100) and polysaccharides (103; 111; 131) due in part to their ability to stimulate mucosal immunity and activate systemic immunity after mucosal delivery. For this study, we utilized *Salmonella* as a vehicle for the delivery of a heterologous antigen, *B. mallei* O antigen, and evaluated its potential as a vaccine against *B. mallei* infection. To our knowledge, no other vaccination attempts have been recorded using *Salmonella* as an expression vector against *B. mallei* or *B. pseudomallei*. We

showed that mice intranasally vaccinated with this construct generated a strong IgG and secretory IgA response to *B. mallei* LPS when compared to PBS or vehicle alone. There was a small, but negligible, increase in IgM in the sera of these mice, suggesting that isotype switching is required for efficient protection. Sera from 2 of 8 mice vaccinated with *Salmonella* alone reacted with *B. mallei* LPS (**fig. 3**), although not to a significant degree. It is likely that these mice generated antibodies to a portion of the *Salmonella* LPS that were able to cross-react with purified *B. mallei* LPS. Importantly, both vaccinated and vector control mice generated similar antibody responses to whole *Salmonella* coated ELISA plates (data not shown), indicating that reactivity measured in the sera of vector treated mice to *B. mallei* LPS is not likely due to hyper immune-reactivity of these animals, and that the *Salmonella* vector is an appropriate immunogen in the context of this vaccine.

The relative ratio of IgG2a to IgG1 is commonly used to assess the presence of a Th1-like or Th2-like immune response, in the absence of cytokine profiles. The very high IgG2a:IgG1 ratio (53.72) measured in the sera of vaccinated mice implicates the generation of cell-mediated immunity, and large amounts of IgG2a and IgG2b act as surrogate makers of complement activation (70).

Mice vaccinated with the recombinant *Salmonella* strain were significantly protected from death compared to vector and PBS treated mice when challenged intranasally with 5 x LD_{50} of *B. mallei* (ATCC 23344) and followed for 2 weeks (100% survival, $p = 0.015$) out to 3.5 months (62.5% survival, $p = 0.006$). Surprisingly, mice receiving vector alone survived longer than anticipated and

longer (although not to a statistically significant degree) than mice receiving only PBS. This could be due to discrepancies in the dosage of *B. mallei* administered to these mice, or that the mice were not sufficiently anesthetized prior to challenge. Alternatively, it is possible that the immune response generated to the *Salmonella* provided partial, albeit fleeting, protection against *B. mallei* infection. More challenge experiments will be needed to sufficiently determine the cause for prolonged survival in mice receiving vector alone.

Recently, it was reported that mice administered monoclonal antibodies specific for *B. mallei* LPS via the intraperitoneal route survived aerosol challenge with 20 x LD_{50}. Interestingly, when the spleens from surviving mice were examined, high levels of *B. mallei* were present. The authors concluded that this antibody-mediated protection is likely effective during the initial phase of the infection, but that a specific cell-mediated response would likely be necessary to generate sterile immunity (123). However, an alternate explanation may be that protection requires the generation of high levels of local antibodies at the site of infection or that the surviving bacteria had altered expression of the O antigen. Additionally, higher titers of antibodies specific for *B. pseudomallei* LPS in patients with melioidosis correlate with better clinical outcomes (138). The immunogenicity of LPS from *B. mallei* has been systematically described using polysaccharide microarray technology (99). This microarray was able to detect antibodies specific for capsular polysaccharide of *B. mallei* in the sera of a human glanders patient, whereas pre-infection sera from the same patient was not reactive. These results indicate that polysaccharides are important

immunogens for both infections and may be protective antigens if administered in the proper context.

Ultimately, 5 vaccinated mice survived *B. mallei* challenge 73 days post infection, and only one was harboring bacteria in the liver and spleen. Interestingly, the lungs of this mouse contained no detectable CFU, even though this is the organ to which the bacteria were initially introduced. It is possible that the mouse lung environment is not conducive to colonization by *B. mallei*, and therefore the bacteria migrated away from the lungs to colonize distal sites. Alternatively, it is possible vaccination with *Salmonella* expressing *B. mallei* O antigen generated sufficient mucosal immunity in the lungs to protect against colonization but insufficient systemic immunity, which allowed for the establishment of infection in other organs. One way to tackle these questions would be to monitor the course of infection in naïve, vector inoculated, and vaccinated mice over time using an *in vivo* imaging system (IVIS) and infection with *B. mallei* expressing *lux* genes. The advantage of IVIS is that the exact location of infection can be monitored within each mouse over time, which would discriminate between the absence of and clearance of an established infection. However, no detectable CFU were found in any organs of the other 4 surviving vaccinated mice, indicating that LPS may be a protective antigen and complete immunity may be achievable using this vaccination scheme.

We have shown that vaccination of BALB/c mice with *S. typhimurium* expressing the O antigen of *B. mallei* on its surface significantly increases survival rates upon challenge infection when compared to controls. The recovery

of sterile organs from vaccinated mice post challenge suggests that this vaccination protocol may provide protective immunity within the context of this animal model. Further work is needed to determine the exact mechanisms conferring, and the extent of, protection, and whether or not the delayed time to death for mice vaccinated with vector alone is significant.

Future Directions

The experiments outlined here describe recent advances toward a vaccine against the potential bioterror pathogen, *B. mallei*. We hope to exploit the recent surge in information regarding *B. mallei* and *B. pseudomallei* in order to find an antigen common to both bacteria that can be harnessed for the generation of a cross-protective vaccine. LPS is immunodominant in *B. pseudomallei* infections and, given the similarities between these two pathogens, LPS is likely a key immunogen in human *B. mallei* infections as well. Specifically, genomic and structural comparisons of the O antigens of *B. mallei* and *B. pseudomallei* revealed remarkably similar structures, with variances in acetylation and methylation. While monoclonal antibodies designed specifically for the detection of either *B. mallei* or *B. pseudomallei*, are excellent tools for the enumeration of these pathogens, it's highly likely that infection with either bacteria will generate a polyclonal response *in vivo*, with some specificity to the O antigen backbone. To our knowledge, passive polyclonal administration of LPS antibodies against *B. mallei* or *B. pseudomallei* has yet to be described. It is also possible that, like other characterized pathogens, *B. mallei* and *B. pseudomallei* are able to modify their LPS during the course of infection. The overwhelming majority of *B. pseudomallei* isolates exhibit the same LPS banding pattern on silver stained SDS-PAGE, and Anuntagool *et al*. recently analyzed *B. pseudomallei* LPS extracted from 1,327 different clinical and geographical sources (5). They determined that approximately 2% of analyzed isolates had an LPS banding

pattern divergent from the type-strain, and approximately 1% had no detectable ladder pattern. This may account for the inability of mice treated with monoclonal LPS antibodies to clear *B. mallei* after challenge (123). However, whether there is a correlation between LPS modification and pathogenesis has yet to be determined. Interestingly, in our experiments the monoclonal *B. mallei* LPS antibody consistently reacted with purified *B. pseudomallei* LPS (**fig. 2a** and data not show) indicating that these two polysaccharides are highly homologous. However, immunodetection of *B. mallei* LPS was not seen using the *B. pseudomallei* monoclonal LPS antibody (data not shown).

The reports of many vaccine attempts against these microorganisms suggest that a cell-mediated immune response, in addition to a humoral response, is necessary for complete clearance of the pathogen. We believe that the cell-mediated response mounted against one of these bacteria may be aptly suited for clearance of the other. It is possible that the expression of *B. mallei* O antigen on the surface of attenuated *Salmonella* will provide the immune response needed to protect against *B. mallei* (which we have tested) and *B. pseudomallei* (which will be tested in the future). Future work may also include vaccination of mice with the recombinant *Salmonella* containing O antigen locus from *B. mallei* and the O antigen acetylation gene from *B. pseudomallei*, and evaluating its ability to protect against either or both pathogens. Other immunogenic antigens, as defined by protein microarrays probed with sera from animals treated with killed *B. mallei* or *B. pseudomallei*, will be evaluated for their potential as protective vaccine candidates as well.

Goldberg *et al*. previously showed that *Pseudomonas aeruginosa* O antigen constructs can be expressed in the attenuated human *Salmonella* serovar Typhi vaccine strain Ty21a (48), suggesting that our vaccine construct has the potential to be clinically useful. While *B. mallei* is an uncommon infection in most parts of the world, a vaccine may be beneficial for animal handlers at high risk for contracting the bacterium. Because *B. pseudomallei* is endemic to certain parts of the world, we envision that a vaccine would be most beneficial to those who live in, or travel to, those areas. A vaccine against either or both of these bacteria would be significant for military personnel and emergency medical workers, who would be the first to respond in the event of an intentional release. But importantly, the generation of an effective vaccine against *B. mallei* and *B. pseudomallei* would significantly decrease their effectiveness as bioweapons and would likely diminish the desire to weaponize these potentially fatal pathogens.

Poly-*N*-acetyl-glucosamine of *Burkholderia cenocepacia*

Abstract

Burkholderia cenocepacia is an emerging, opportunistic pathogen of cystic fibrosis patients, implicated in patient-to-patient transmission and poor clinical prognosis. To better understand the contribution of polysaccharides in the virulence of this bacterium, the genome of the sequenced strain *B. cenocepacia* J2315 was scanned for homology with a known polysaccharide synthesis locus, called PGA in *E. coli* and PIA in *Staphylococcus* sp. The PGA locus is an operon that encodes the proteins necessary for the synthesis of poly-β-1,6-*N*-acetyl-glucosamine (PNAG), an important virulence determinant in many biofilm producing bacteria. A strain mutated in the putative glycosyltransferase gene of this locus (*pgaC*) was constructed in *B. cenocepacia* K56-2. We were able to reliably detect a decrease in crystal violet staining (a surrogate marker of polysaccharide secretion) of 48 h mutant cultures compared to the parental strain. We also tested these strains for virulence in the *Caenorhabditis elegans* model system of infection. We found that the mutant strain was significantly attenuated in worm killing compared to the parental strain, implicating that PNAG is an important virulence factor of this bacterium in the worm model of infection.

Introduction

Burkholderia cepacia complex genomics and taxonomy

The *Burkholderia cepacia* complex (Bcc) is a cohort of highly related, but genetically distinct bacteria, made up of at least ten species. Bcc species have very large genomes (around 8 Mb) which are typically composed of three replicons and a plasmid (110). The genome of *B. cenocepacia* strain J2315, sequenced by the Wellcome Trust Sanger Institute, has 8.056 Mb of total DNA, which is divided between three chromosomes of 3.87, 3.217, and 0.876 Mb, and a plasmid of 92.7 kb (http://www.sanger.ac.uk). Bcc genomes are also rich in insertion elements, lending to genomic plasticity which is suggested to promote survival and growth in a variety of environments (79). It is likely that the large size of the Bcc genome and this inherent plasticity have played an important role in the adaptability of this group of bacteria.

Bcc bacteria were characterized as the genus *Pseudomonas* until 1992, when seven species (of note, *B. cepacia*, *B. mallei* and *B. pseudomallei*) were considered distinct enough from the Pseudomonads to warrant reclassification. The transfer of these seven species to the genus *Burkholderia* was based on 16S rRNA sequences, DNA-DNA homology values, cellular lipid and fatty-acid composition and phenotypic characteristics (141). In 1997 Vandamme *et al.* (127) determined that the species previously defined as *B. cepacia* was actually composed of five distinct genetic species (genomovars) that were phenotypically indistinguishable. Since that time, at least five more genomovars have been

characterized (126) and are collective termed the *Burkholderia cepacia* complex (Bcc). **Table 3** outlines the Bcc genomovars currently recognized. Some of the members of this group are well-characterized bacteria, such as *B. vietnamiensis* and *B. ambifaria*, which were first identified by environmental microbiologist due to their important relationship with commercial agriculture (see below). However, all ten genomovars have been isolated from cystic fibrosis (CF) patients. It is important to note, that while all genomovars can be found in the CF community, some are more prevalent than others. Specifically, most epidemic strains, which are believed to be clonal and spread from patient-to-patient, are *B. cenocepacia* (genomovar III) (81).

Table 3. The genomovars of Bcc circa 2008

Genomovar	Species designation	Reference
I	*cepacia*	(127)
II	*multivorans*	(127)
III	*cenocepacia*	(127)
IV	*stabilis*	(127)
V	*vietnamiensis*	(127)
VI	*dolosa*	(23)
VII	*ambifaria*	(24)
VIII	*anthina*	(125)
IX	*pyrrochina*	(125)
X	*ubonensis*	(128)

Bcc and the environment

Bcc bacteria are Gram-negative rod-shaped microorganisms that are ubiquitous in the environment and can be found in soil, water and the rhizosphere of plants, where they can exert both detrimental and beneficial effects on crops. Indeed, *B. cepacia* (named *Pseudomonas cepacia* at the time) was first identified by Walter H. Burkholder as a phytopathgen and the causative agent of soft onion rot in 1950 (10). However several members of the Bcc complex are considered to be environmentally and agriculturally advantageous. For example, *B. cenocepacia* protects corn crops from the fungal pathogens *Aphanomyces auteiches* and *Rhizoctonia solani*, and the nitrogen-fixing *B. vietnamiensis* is highly effective at increasing the yield of rice crops in SE Asia (19). At least three strains of Bcc bacteria were approved by the EPA for use in biopesticides in 1992 and 1996 (98). The duality of relationship between Bcc bacteria and plant ecosystems highlights the complexity of the pathogenic mechanisms of these microbes, and this relationship with their natural environment may help provide insight into the connection between the ability of Bcc bacteria to cause opportunistic infections in humans.

Some members of the Bcc can also degrade natural and man-made pollutants, making them of interest for bioremediation applications. They have been shown to degrade phthalates, herbicides, chlorinated hydrocarbons, and tricholorethylene, one of the most widespread and persistent environmental toxins in the groundwater of the United States (78; 98). However, the ability of these bacteria to infect CF as well as chronic granulomatous disease (CGD)

patients has severely restricted their intentional use as biocontrol and bioremediation agents (19). A better understanding of the basic biology, metabolic mechanisms, pathogenesis and genomics of Bcc bacteria may reveal completely avirulent strains within this group that could warrant the reassessment of some members for biotechnical applications.

CF and chronic lung infection

CF is an autosomal recessive disease caused by the mutation of a single gene, called the cystic fibrosis transmembrane conductance regulator (CFTR). CF is the most common genetic disorder among the Caucasian population, with an estimated incidence of 1 in 2500 live, Caucasian births in the United States (72). Several different mutations in the CFTR can result in clinical presentation of disease, but the most common (~70%) mutation seen in CF patients is a 3 bp deletion that results in an absence of phenylalanine at amino acid position 508 (ΔF_{508}) of the CFTR protein (8).

The CFTR protein is a single polypeptide chain of 1480 amino acids that functions as a regulator of several ions, but most importantly in CF, it is a cyclic AMP-regulated chloride ion channel. The CFTR is found in the plasma membrane in normal epithelia. However, biochemical studies indicate that the ΔF_{508} mutation leads to improper processing and intracellular degradation of the CFTR protein (8). The absence of this protein results in disregulation of Na^+ and Cl^- across the epithelium, which alters the hydration state of the cell surface. As such, CF patients have a layer of mucus present on their airway epithelia, thicker

and more adherent then the mucus layer of non-CF patients, which they are unable to clear via ciliary and airway-dependent (cough) mechanisms (8). It is this mucus layer that provides the nidus for opportunistic infection these patients.

Staphylococcus aureus and *Pseudomonas aeruginosa* are the most common bacteria isolated from the lungs of CF patients. Interestingly, *S. aureus* is typically cultured from the lungs of young patients and becomes less prominent as the patient ages and acquires *P. aeruginosa*, likely from environmental sources. It remains unclear whether this shift occurs as a result of effective antibiotic treatment against *S. aureus* or whether there is direct competition between these species for CF lung colonization. Interestingly, a recent paper found that the inhibition of *S. aureus* respiration by a protein secreted by *P. aeruginosa* selects for small-colony variants of *S. aureus* that are resistant to the antibiotics typically used against them in the CF lung, implicating cooperation between these species (57).

P. aeruginosa is a Gram-negative rod-shaped bacterium capable of causing chronic lung infections in CF patients and is responsible for the majority of morbidity and mortality in this population. As CF patients age, their likelihood of becoming colonized with *P. aeruginosa* increases substantially. According to the Cystic Fibrosis Foundation, approximately 80% of CF patients over the age of 18 are chronically colonized with *P. aeruginosa* (29), and there is a plethora of hypotheses attempting to explain this phenomenon (for an overview of some popular hypotheses, see (46)). While *P. aeruginosa* is the most common opportunistic pathogen found in the CF lung, other bacteria, such as the Bcc, are

increasingly being recognized as important pathogens implicated in the deterioration of health in this population.

Bcc and CF

Bcc are acquired late in the course of CF disease, usually after the patient has already acquired *P. aeruginosa*. Whether Bcc isolation from the CF lung is a surrogate marker for the gradual deterioration of lung function in these patients, or whether Bcc colonization specifically exacerbates the deterioration of lung function is still debated. Also poorly understood is the relationship between the resident *P. aeruginosa* population and the subsequent Bcc population, and whether there is cooperation between these species, as is seen with *P. aeruginosa* and *S. aureus.*

Bcc were first recognized as emerging pathogens of the CF population in the early 1970's, when one particular study recognized a near 50% increase in their cohort of patients positive for Bcc over a 10 year period (60). How CF patients respond to infection with Bcc species is quite variable and ranges from asymptomatic carriage to septicemia resulting in death. The rapid decline of a subset of patients infected with Bcc, which includes fulminating pneumonia and bacteremia, has been termed 'cepacia syndrome' (86). Interestingly, substantial variability of symptoms can be observed between patients infected with the same clonal strain, implicating the importance of the host immune response to the infection (J.J LiPuma, personal communication). It has also been shown that some Bcc species are capable of patient-to-patient transmission (80), which is

not generally seen in *P. aeruginosa* isolates. Of the ten species currently recognized as Bcc bacteria, all can cause infection in CF patients, but *B. cenocepacia* is the most prevalent, accounting for 50-80% of all Bcc infections (87). *B. multivorans* is the second most common isolate (9-37%) while the remaining seven species account for less than 10% of all infections (87). Why the CF lung is particularly suited for Bcc, and *P. aeruginosa*, infections is not known, but it is likely that both microbial virulence factors and host response to infection play a role.

Epidemic Bcc lineages

Genotyping analyses of numerous Bcc isolates from different cohorts of CF patients have revealed that some strains of *Burkholderia* are more common than others. These strains have been named “epidemic strains” and may be more transmissible between patients or better adapted to infection than other Bcc strains (22). However it is also possible that these strains are better represented in the natural environment, and therefore, are better represented in the CF community. In 1994 Johnson *et al.* used multilocus enzyme electrophoresis and ribotyping to identify a particular strain, named electrophoretic type (ET) 12, which was well represented in CF patients in Ontario, Canada (66). It is believed that this strain was spread between CF patients during social contact at two different CF camps in the United Kingdom (50). Epidemiology suggests that this strain is particularly virulent as, by 1996, Pitt *et al.* determined that ET12 was

present in the lungs almost 50% of all CF patients in eight different CF centers in the U.K. (104).

ET12 is a *B. cenocepacia* strain (genomovar III) with distinct characteristics. This strain expresses “cable pili”, which are very long (2 to 4 μm), peritrichous appendages, implicated in pathogenesis primarily through their adherence to aggregates of host cells. Attached to the cable pili, is a 22 kDa adhesin molecule, whose ability to bind cytokeratin 13 is believed to exacerbate invasion, penetration, and cellular damage to bronchial epithelial cells (113). ET12 bacteria also harbor a 1.4 kb fragment of DNA that is absent in non-epidemic strains, and rarely found in environmental isolates, called the *Burkholderia cepacia* epidemic strain marker (BCESM) (85). This fragment of DNA contains an open reading frame with homology to negative transcriptional regulators in other bacteria (85) , however the role of this open reading frame in virulence and transmission remains unclear. With the advent of genome sequencing of members of the Bcc, it is likely that new virulence factors of epidemic strains will be uncovered. Using high throughput screening of these factors in simple model systems, such as *C. elegans,* may help explain the overrepresentation of some Bcc strains in the CF community.

Caenorhabditis elegans as a model system for infection

The nematode *Caenorhabditis elegans* is fast becoming a popular, economical, and easy-to-use model system in which to study pathogenic mechanisms of infection (118) and host innate immunity to invasion (51). Many

mammalian pathogens are able to injure or kill nematodes, and often, the genes involved in mammalian pathogenesis are similarly important in the pathogenesis of *C. elegans*. Advantages to using the *C. elegans* model system include a fully sequenced genome, anatomical simplicity, and susceptibility to RNA interference for targeted knock down of host genes. *C. elegans* is a terrestrial microbivore and can therefore be maintained in the laboratory on agar plates supplemented with auxotrophic *Escherichia coli* (OP50). Using this simple feeding model, the pathogenicity of any bacteria can be ascertained by replacing the *E. coli* with your microbe of interest. When feeding on a variety of virulent microbes, the nematodes exhibit a range of shorter life spans when compared to survival on OP50 (approximately 2 weeks at room temperature), and this “worm killing” is typically indicative of an active pathogenic mechanism (118).

C. elegans and Burkholderia sp.

The ability of *Burkholderia* sp. to kill nematodes was first reported by O’Quinn *et al.* when they monitored the survival of *C. elegans* on a variety of human pathogens including *B. mallei*, *B. pseudomallei*, and some members of the Bcc (96). They determined that representative bacteria from genomovars I, III, and IV were significantly pathogenic to nematodes, while representatives from genomovars II and V displayed only modest nematode killing (96). Interestingly however, the authors were not able to directly correlate nematode pathogenicity to human pathogenicity, as the environmental bacteria *B. thailandensis* was the most nematocidal strain tested.

A more recent paper by Cardona *et al.* attempted to better define the pathogenic relationship between nematodes and Bcc bacteria (13). For this study, the authors employed a panel of transposon mutants in the *B. cenocepacia* strain K56-2 which had previously displayed reduced survival in the rat lung (59), and tested them for nematocidal activity. They found that these mutants displayed varied pathogenicity against *C. elegans*. Approximately 41% displayed similar virulence compared to the parental strain, suggesting that the mutated genes were dispensable for *C. elegans*, but not mammalian, infection (13). The remaining mutants displayed varied levels of attenuation, and 6 of these strains were completely attenuated in *C. elegans* infection for two different strains of nematodes tested. From this study, the authors were able to conclude that some virulence factors of *B. cenocepacia* are conserved between nematodes and mammals, as has been characterized in other bacteria, but the importance of nematode-specific virulence factors is not understood.

Other Bcc virulence factors shown to be important in *C. elegans* pathogenesis are type III secretion (89) and quorum sensing (75). While it is likely these factors play a role in mammalian pathogenesis, their relevance to the ability of Bcc to colonize CF patients remains unclear. However, given that *C. elegans* is a terrestrial microbivore and Bcc are ubiquitous soil and water microbes, it is likely that these organisms encounter each other in their natural environment. As such, the pathogenic mechanisms of these bacteria against the nematodes, and the resulting immune response generated by *C. elegans*, could be physiologically

relevant and may provide cues to the interplay of these bacteria with the innate immune system.

Biofilm formation and exopolysaccharides

Biofilms are complex aggregations of microorganisms that secrete a protective and adhesive polymeric matrix. Biofilms are clinically relevant; they are able to grow on common hospital implant devices and catheters, and are responsible for many hospital acquired infections. Secreted exopolysaccharides (EPSs) are an important component of biofilm matrices and can confer chemical and antibiotic resistance, as well as shield the microbes inside from the immune system of the host. As such, some biofilm forming bacteria have the ability to cause chronic infections. Indeed, EPS secretion and biofilm formation is a critical component of *P. aeruginosa's* ability to persist in the lungs of CF patients (92). *P. aeruginosa* isolates have been reported to convert to a mucoid phenotype during extended lung colonization. Muciody in *P. aeruginosa* is achieved by the increased production of the EPS alginate, a linear copolymer of partially O-acetylated β-1,4-linked D-mannuronic acid and α-L-guluronic acid (107). Alginate is a critical component of the biofilm formed by *P. aeruginosa* in the CF airway, and is likely to contribute to poorer clinical prognosis in this population. Given the importance of EPS production in the pathogenesis of *P. aeruginosa*, it has been hypothesized that the EPS of Bcc species may also contribute to their ability to chronically colonize CF patients.

Many strains of Bcc secrete EPSs. In fact, one study found that 70% of all Bcc CF isolates tested make EPS (109), suggesting that EPS production may be an important virulence factor in the context of these infections. Chung *et al.* reported that increased EPS production was associated with chronic colonization in the mouse model of infection (21). In this study, intranasal challenge of leukopenic mice with a Bcc isolate from genomovar III resulted in a spontaneous variant that was genetically indistinguishable from the parental strain, but had altered morphology and persisted in the lungs of these animals. The variant had a shinier colony appearance and produced significantly more EPS than the parental strain (21). However, another study analyzed EPS formation from a cohort of clinical CF isolates, and could find no correlate between disease severity and EPS production (26), confounding the role EPS plays in these infections.

Bcc bacteria can produce at least five different types of EPS. The most prominent EPS is a heptasaccharide called cepacian (**fig 7a**), which is specific to and commonly produced by most Bcc bacteria (20; 119). The structures of the EPS species determined for Bcc are listed below and are reviewed in (47) and (20). It is possible, given the complexity of EPS production, that Bcc species secrete other EPS structures yet to be characterized.

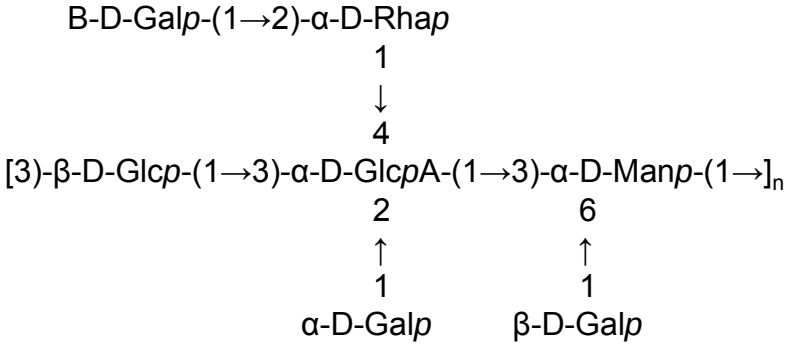

Figure 7a. Cepacian

[3)-β-D-Glc*p*-(1→3)-[4,6-*O*-(1-carboxyethylidene)]-α-D-Gal*p*-(1→]$_n$

Figure 7b.

[5)-β-D-Kdo*p*-(2→3)-β-D-Gal*p*2Ac-(1→4)-α-D-Gal*p*-(1→3)-β-D-Gal*p*-β-D-Gal*p*-(1→]$_n$

Figure 7c.

[6)-β-D-Fru*f*-(2→]$_n$

Figure 7d.

[6)-α-Glc-1→]$_n$

Figure 7e.

Poly-N-acetyl-glucosamine and the pgaABCD locus

Poly-*N*-acetyl-glucosamine was first identified as an important component of biofilm in *Staphylococcus epidermidis* by Mack *et al.* during a transposon mutagenesis screen for biofilm deficient mutants (83), but the structure was characterized at that time as hexamine. Using more stringent purification and biochemical analysis, they isolated a novel structure, a linear β-1,6-linked glucosaminoglycan, implicated in intracellular adhesion and biofilm accumulation, and named this polymer polysaccharide intracellular adhesin (PIA) (a name that is still used to describe this EPS in the *Staphylococcus* literature). Soon after the characterization of PIA, another group discovered the genes involved in its synthesis, a locus they named *icaABC* (55), after which a fourth essential gene was discovered, resulting in a biosynthetic locus called *icaADBC* (45). Around the turn of the century, many groups studying EPS production in a variety of different bacteria were uncovering the EPS generated by this locus. As such, the product of this operon has had many names in the 12 years since its discovery including; PIA, β-1,6-GlucNAc, SAE, PS/A, PGA, and PNAG. However, it is now generally accepted that all these structures are, in fact, the same EPS, β-(1,6)-linked poly-*N*-acetyl-glucosamine. In 2004 Wang *et al.* renamed the biosynthetic locus responsible for production of this polymer *pgaABCD*, likely to better reflect its role in the production of this particular polysaccharide (133). From this point on, for simplicity and uniformity, the locus encoding the genes needed to

synthesize poly-*N*-acetyl-glucosamine will be referred to as *pgaABCD* and the polysaccharide PNAG.

PNAG secretion has now been characterized in a variety of bacteria including; *S. aureus, E. coli*, *Yersina pestis*, *Actinobacillus pleuropneumoniae* and *Aggregatibacter actinomycetemcomitans* (62; 63; 65; 88; 133). In general, there is a high degree of homology between the operons responsible for making this polysaccharide, and these genes appears to be part of a horizontally transferred locus (133). Indeed, it has been shown that antibodies raised against PNAG generated by *S. aureus* were able to protect mice from infection with *E. coli*, suggesting a high degree of similarity between these structures (14; 15). In addition to mediating cell-cell and cell-surface adhesion in biofilms, PNAG is also implicated in the ordered architectural arrangement of *E. coli* biofilms and the switch from temporary to permanent attachment during the initial stages of *E. coli* biofilm development (1; 2). PNAG is an integral part of the infectious biofilm that transmits *Y. pestis* from the flea vector to mammalian hosts (65). Recent work also suggests that PNAG may have a role in innate immune evasion in the *C. elegans* model of infection. Using a collection of mutant *S. epidermidis* and nematodes, Begun *et al.* found that PNAG promotes infection in the intestine of *C. elegans* by working against protective innate immune factors controlled by the nematode SEK-1 PMK-1 p38 mitogen-activated protein kinase pathway (7). As more research is published on PNAG, it becomes increasingly clear that EPS production adds more complexity to the interaction between infecting microbe and host than was previously appreciated.

Much of the genetic regulation and function of the *pgaABCD* genes has been uncovered in *E. coli* by work in the laboratory of Tony Romeo. In 2002 Jackson *et al.* found that a global transcriptional regulator protein, CsrA, regulates biofilm formation in this bacterium (64). Later work determined that the CsrA protein directly interacts with *pgaABCD* transcripts and represses PNAG production by binding to multiple sites within the untranslated leader and proximal coding region of *pgaA* mRNA (132). CsrA binding obstructs ribosomal access to the Shine-Delgarno sequence of *pgaA* and destabilizes the *pgaABCD* transcript (61). Positive regulation of PNAG production in *E. coli* is controlled by NhaR, a LysR family DNA-binding protein. NhaR activates *pgaABCD* in response to high pH and increased concentration of sodium ions (49), pointing to the importance of environmental influence on biofilm production in *E. coli*.

As the name suggests, *pgaABCD* is composed four open reading frames whose specific functions in the production of PNAG have been eloquently characterized by Wang *et al.* (133) and Itoh *et al.* (61). Using a search for conserved protein domains, Wang *et al*. attempted to predict the function for each gene in 2004. PgaA was predicted to be a large (807 amino acid) outer membrane protein, whose function was unclear at the time, since they could find no homologues, and *Staphylococci* (which were well studied for PNAG secretion) lack outer membranes. PgaB was predicted to be a 672 amino acid lipoprotein with a 20 amino acid signal sequence and was homologous to the *S. epidermidis* protein, IcaB. PgaC was predicted as a 441 amino acid *N*-glycosyltransferase inner membrane protein with homology to IcaA. This study also showed that *E.*

coli mutated in the *pgaC* gene were severely deficient in PNAG excretion (133). The final gene in the locus, PgaD, was predicted to be a small (137 amino acid) inner membrane protein. It was known that IcaD was a cytoplasmic membrane protein that functioned to enhance PNAG synthesis by IcaA, but the lack of sequence homology between PgaD and IcaD confounded prediction (133). Four years later Itoh *et al.* used molecular genetic techniques to show that PgaC and PgaD are necessary for the biosynthesis of PNAG, and that PgaB is an *N*-deacetylase whose catalytic activity, along with PgaA, function in PNAG export from the periplasmic space (61). **Figure 8** represents the model proposed for PNAG synthesis and export.

Identification of pgaABCD in B. cenocepacia K56-2

The recent advent of whole genomic sequencing has provided the scientific community with a wealth of information that has eased the identification of both novel and homologous genetic sequences, and provided insight into the conservation of genes among bacterial genera. Knowing that PNAG is an important virulence factor in a variety of microbes, and that polysaccharide secretion is paramount to the chronicity of *P. aeruginosa*, and possibly Bcc bacteria, in the context of CF lung infections, we set out to determine if the *pga* locus was present in the *B. cenocepacia*. The subsequent sequence analyses were performed by Mike Davis in the laboratory of Joanna Goldberg, University of Virginia. The genome of *B. cenocepacia* J2315

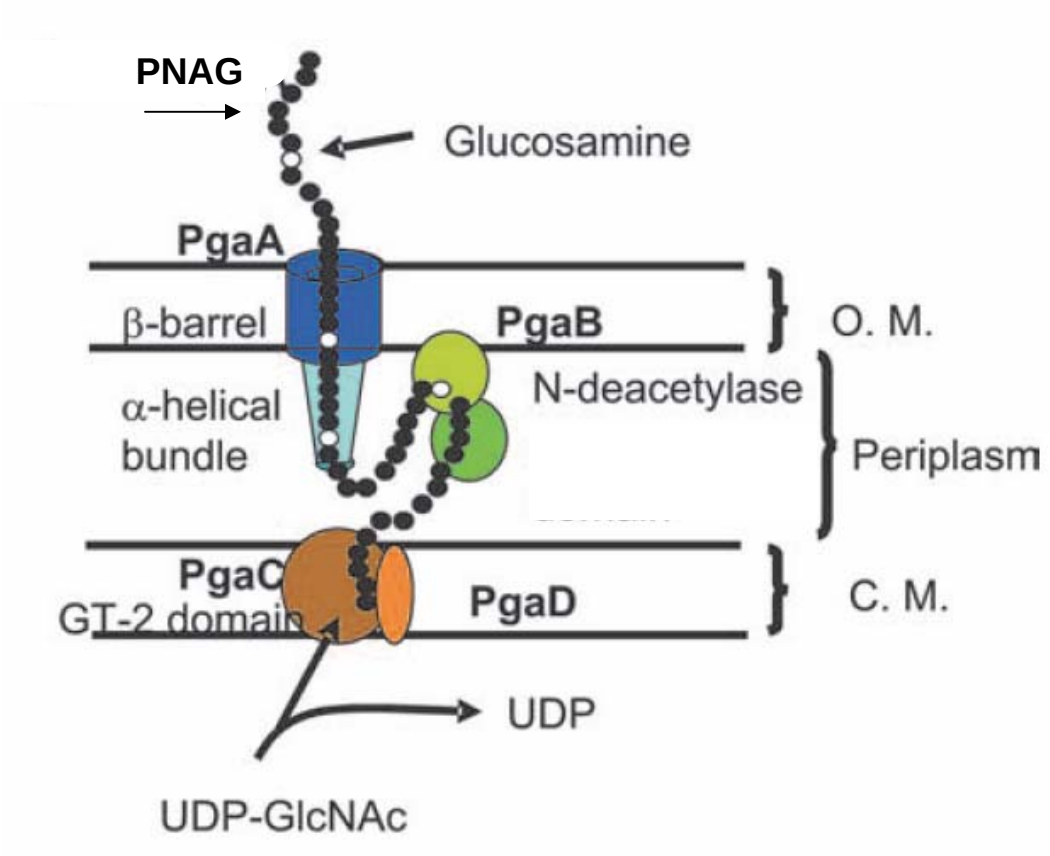

Figure 8. A model for the export and synthesis of PNAG, modified from (61).

(http://www.sanger.ac.uk/Projects/B_cenocepacia/), an epidemic strain from genomovar III, was scanned for homology against the DNA sequences of *pgaABCD* from *E. coli* K12 using BLASTn (http://www.ncbi.nih.gov/BLAST). The protein sequences for the *E. coli* K12 PgaA-D proteins were compared to the predicted *B. cenocepacia* PgaA-D proteins using BLASTp (http://www.ncbi.nih.gov/BLAST). The similarity scores are reported in **table 4** and are all supported by significant E() values (< 0.0001). The deduced amino acid sequence of each gene from J2315 and K12 was used as the input for the ExPasy "Get MW" program (http://ca.expasy.org/tools/pi_tool.html) to determine the predicted molecular weight for each protein. The proposed functions are based on that of the highest scoring homologues in the NCBI database, as found by a BLASTp search, and reported in **table 5**. Similar to the results found by Wang *et al.* (133), no homology was found within the predicted PgaD protein of J2315. However, comparison of hydropathy plots from J2315 and K12 PgaD reveal comparable membrane topology (**fig. 9**), suggesting they may function similarly.

To determine what role, if any, PNAG plays in the pathogenesis of *B. cenocepacia*, a chromosomal disruption was made in the predicted *pgaC* gene of *B. cenocepacia* K56-2, which is highly related to the sequenced *B. cenocepacia* strain J2315. The mutant and parental strains were tested for differences in EPS production and in the *C. elegans* model of infection and monitored for differences in their ability to kill nematodes. Overall, these results suggest that PNAG is an important virulence factor of *B. cenocepacia* in the context of worm infection, and

may have implications in this bacteria's ability to chronically colonize the airways of CF patients.

Table 4. Amino acid similarities of PgaA-D of *E. coli* and *B. cenocepacia*

Predicted proteins from J2315	Similarity to K12
BCAM1228 (PgaA)	37.0%
BCAM1227 (PgaB)	60.0%
BCAM1226 (PgaC)	67.0%
BCAM1225 (PgaD)	0.0%

Amino acid similarity between PgaA-D of *E. coli* K12 and the predicted proteins from *B. cenocepacia* J2315, determined as described in the text.

Table 5. Characteristics of Pga proteins

K12	Size (aa)	Weight	Function	J2315	Size (aa)	Weight
PgaA	807	92 KDa	Outer membrane protein	PgaA	871	84 KDa
PgaB	672	77 KDa	Carbohydrate esterase	PgaB	698	78 KDa
PgaC	441	51 KDa	Glycosyltransferase	PgaC	423	48 KDa
PgaD	137	16 KDa	Inner membrane protein	PgaD	162	18 KDa

The predicted size, weight, and function of proteins from the *pgaABCD* loci of *E. coli* K12 and *B. cenocepacia* J2315, determined as described in the text. AA = amino acid and KDa = kilodaltons.

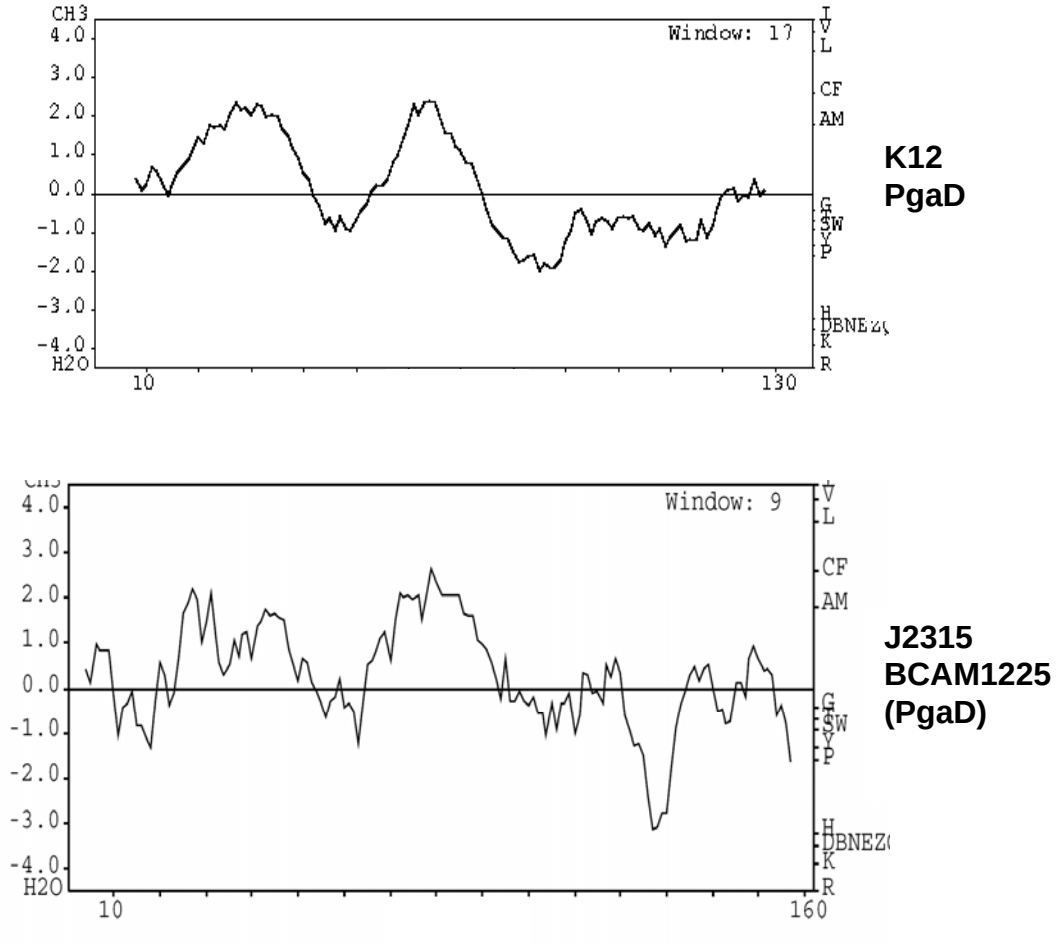

Figure 9. Kyte-Doolittle hydropathy plots of *E. coli* K12 PgaD (top plot) and *B. cenocepacia* J2315 PgaD (bottom plot).

Materials and Methods

Generation of *pgaC::tp* in *B. cenocepacia* strain K56-2. A trimethoprim resistance cassette was inserted into the *pgaC* gene sequence using splicing by overlap extension (SOE) cloning and was performed by Mike Davis in the laboratory of Joanna Goldberg. The 5' end of the *pgaC* was cloned using primers **A** and **G** and the 3' end was cloned using primers **B** and **H** (see **table 6**). Primers **G** and **H** have 17 base-pair tails that corresponded to the 5' and 3' end of the trimethoprim resistance cassette, respectively. The trimethoprim resistance cassette was cloned from plasmid p34ETp (31) using primers **C** and **D**. A standard PCR cycle was used to clone the three aforementioned segments (95°C for 3 mins, followed by 25 cycles of: 95°C for 30 s, 55°C for 30 s, and 72°C for 2 mins). These three products were spliced together in a second PCR reaction using equimolar amounts of the cloned segments and primers **A** and **B**. All PCR was performed on a Perkin Elmer GenAmp System 2400 Thermocycler. The SOE cloned trimethoprim interrupted *pgaC* (*pgaC::tp*) fragment was then cloned into the TOPO pCR2.1 vector (Invitrogen™). The *pgaC::tp* was digested from TOPO pCR2.1 using the restriction enzyme EcoRI (New England Biolabs, Ipswich, MA), and ligated into a similarly digested plasmid, pEX18Tc (56).

Tri-parental matings were performed using *E. coli* DH5α containing the helper plasmid pRK2013 (38), the pEX18Tc plasmid containing *pgaC::tp* and K56-2. After mating, positive transformants were selected on 150 μg/ml trimethoprim (tp). Then, positive clones were patched onto LB-tp plates supplemented with 5%

sucrose, to select against merodiploids. The pEX18Tc plasmid contains the gene *sacB*, which imparts sucrose sensitivity to strains harboring the plasmid backbone. Chromosomal insertion of *pgaC::tp* into K56-2 was confirmed by PCR (see **figs 10** and **11**), sequencing of the PCR product (University of Virginia Biomolecular Research Facility, Charlottesville, VA), and Southern blotting.

Southern blot confirmation of chromosomal insertion. The following experiments were performed by Dr. Karen Brassinga in the laboratory of Dr. Costi Sifri, University of Virginia. Cultures grown overnight at 37°C on LB plates were resuspended in 500 µl of DI water. Chromosomal DNA was extracted using the Promega Maxwell® 16 System (Promega U.S., Madison, WI), per the manufacturers instructions. These extractions yielded approximately 250 µl of purified DNA at a concentration of 0.4 µg/µl. The Southern procedure was performed once using ^{32}P-end labeling of a probe PCR amplified using primers external to the *pgaC* gene and once using electrochemical luminescence (ECL) of a PCR amplicon using primers internal to *pgaC* (see **table 6** for primers used). Chromosomal DNA was digested using the restriction enzyme, Pst1 (New England Biolabs, Ipswich, MA) per the manufacturers instructions. The amount of chromosomal DNA digested for the radioactive probe was doubled compared to ECL. These digests were allowed to incubate for 3 hours, after which 3 µl of 10x loading dye was added to each and the samples were run on 1.2% agarose gels supplemented with 0.2 µg/ml of ethidium bromide (EtBr). An equivalent amount of EtBr was added to the gel running buffer. The gel was run at 120V for about

4.5 h. The Southern protocol was followed according to GE Healthcare Amersham ECL Direct™ Nucleic Acid Labelling and Detection system for both radiolabeled and ECL blots. Both blots were crosslinked using a Stratagene Stratalinker® UV crosslinker on automatic settings. Blots were exposed to film for 2-4 days, depending on signal strength.

Crystal violet biofilm assay. Bacterial cultures were grown overnight at 37°C on TSA +1% glucose (TSBG) agar plates (plates were supplemented with 150 µg/ml of tp for *pgaC::tp*). Strains were then diluted to an OD_{600} of 0.5 in TSBG and 100 µl of this culture was added to polystyrene, round-bottom, 96-well microtiter plates (Pro-Bind™ assay plates, Becton Dickinson Labware, Franklin, NJ) in quadruplicate. Microtiter plates were incubated at either 30°C or 37°C for 48 h. After incubation, plates were rinsed thoroughly with DI water and allowed to air dry. Then, 100 µl of 0.1% w/v crystal violet was added to each well and incubated at room temperature for 30 mins. Wells were rinsed three times with DI water and air dried. After the plates were dry, 100 µl of 95% ethanol + 0.05% Triton X was added to each well to dissolve the crystal violet adherent to the wells. Each sample of solubilized crystal violet was then transferred to a new, sterile microtiter plate and the absorbance of the solutions was read at 570 nm using a Molecular Devices Thermo microplate reader. Sterile TSBG treated with crystal violet was measured as a control. For this assay, biofilm formation was defined as those wells measured at an $OD_{570} > 0.05$.

***C. elegans* survival assay.** All of the following procedures involving *C. elegans* were performed by members of Dr. Costi Sifri's laboratory, University of Virginia. Fourteen ml culture tubes containing 3 ml of TSB were inoculated with one colony of either K56-2 or *pgaC::tp* from a 'fresh' plate (TSA grown overnight at 37°C with added 150 µg/ml of tp for *pgaC::tp*), and incubated overnight at 37°C. Tp was not added to liquid culture in case the presence of the antibiotic slowed bacterial growth (i.e. to eliminate possibility that the change in worm killing was due to altered growth and not active pathogenesis). Saturated cultures were diluted in fresh TSB to an OD_{600} of 1.7, and 10 ul were plated on 3.5 cm-diameter plates and incubated for 24 h at 37°C. Plates were then removed from the incubator and allowed to cool at room temperature for 30 min. Nematodes were maintained on modified NGM (0.3% NaCl, 1.7% Bacto-agar, 3.5g Bacto-peptone, 1ml of 5mg/ml cholesterol in ethanol, 1 ml of 1M MgSO4, 1 ml of 1M $CaCl_2$, and 25ml of 1M KPO_4) until reaching the proper larval stage. Then 25 L4 worms were transferred to each plate. This was performed in triplicate for each bacterial strain. The worms were periodically transferred to newly made assay plates to ease the continued monitoring the original animals. Otherwise the plates would be overrun by progeny growth. After approximately 5 days, the worms no longer produced progeny and were followed until death. These killing plates were maintained at 25°C and monitored for death at least every 24 h. A worm was considered dead when it failed to respond to plate tapping or gentle touch with a platinum wire. Worms that died as a result of getting stuck to the walls of the plate were censored from analysis.

Microscopy of *C. elegans*. Bacterial colonization of the nematode digestive tract was observed by differential interference contrast imaging with Nomarski optics using an Axioplan2 microscope (Zeiss, http://www.zeiss.com).

Quantification of *C. elegans* colonization. For quantification of nematode colonization, worms were allowed to feed on either mutant or wild type *B. cenocepacia* plates under standard killing conditions for 72 h. Approximately 30 nematodes were manually removed from killing plates and added to 250 µl of M9 buffer (6 g Na_2HPO_4, 3 g KH_2PO_4, 5 g NaCl, 0.25 g of $MgSO_4 7H_2O$ per liter), divided into three pools, and washed 5 times with 200 µl M9 buffer supplemented with 1mM sodium azide by serial transfer in a 96 well microtiter plate. Nematodes were then transferred to 2 ml conical tubes and the solution volume was increased to 250 µl with fresh M9 buffer (without sodium azide). The worms were homogenized by adding 200 µl of sodium carbide beads (BioSpec Products, http://www.biospec.com) and vortexing for 1 min. Serial dilutions of the supernatant were made to determine the number of viable bacteria.

Table 6. Primers used for this study

Label	Primer Name	Sequence 5' to 3'	Purpose
A	pgaC external F	ATGACCACCCACAGCCTGATCC	Confirmation of *pgaC::tp* insertion
B	pgaC external R	GCGCGTTCTTCATGTTGAATGCCT	Confirmation of *pgaC::tp* insertion
C	Tpcass F	ATGAAGGCACGAACCCAGTTGACA	Generation of SOE clone insertion
D	Tpcass R	AGCGAATTGTTAGGCCACACGTTCAA	Generation of SOE cloning insertion
E	pgaC internal F	ACTATCCGTTCTTCATGTCG	Southern confirmation of insert
F	pgaC internal R	ATGCAGTATCCGAACTACGA	Southern confirmation of insert
G	pgaC 5' int + tail F	*TTGAACGTGTGGCCTAACAATTCGCT*GCCGCTGTTCGCCGAATACCTG*	Generation of SOE clone insertion
H	pgaC 3'int + tail R	*TGTCAACTGGGTTCGTGCCTTCAT*CAGCCGATCGCATCGTGCGCG*	Generation of SOE clone insertion
I	Internal pgaC F	ACTATCCGTTCTTCATGTCG	Southern confirmation of insert
J	Internal pgaC R	ATGCAGTATCCGAACTACGA	Southern confirmation of insert

*****Nucleotides in italics denote the tails used to generate SOE insertions and correspond to the trimethoprim cassette.

Results and Discussion

Confirmation of *pgaC::tp* in K56-2. **Figure 10** is a schematic of the mutation of the *pgaC* gene generated via SOE cloning. The loss of some original sequence of *pgaC* and the addition of the trimethoprim resistance (tpR) cassette (*pgaC::tp*) yielded a predicted product of approximately 1.4 kb, whereas the *pgaC* gene product from K56-2 was approximately 1.2 kb (see **fig. 11**) using primers **A** and **B**. DNA sequencing using these same primers confirmed the chromosomal insertion of the tpR cassette, which was absent from the parent strain (data not shown). Southern analysis was performed using two different primer sets; both confirming the generation of a chromosomal mutation in the *pgaC* gene. The insertion of the tpR cassette into the *pgaC* gene resulted in the loss of a Pst1 restriction site. **Figure 12** depicts a Southern blot using ^{32}P-end labeling and probe generated using PCR amplification of genomic DNA (gDNA) using primers **A** and **B**. **Figure 13** shows ECL labeling of a probe generated from primers internal to *pgaC* (primers **I** and **J**). These results refute the possibility of unresolved merodiploidy within the *pgaC::tp* strain, and confirm that the insertion of the trimethoprim cassette into *pgaC* was made in the predicted location.

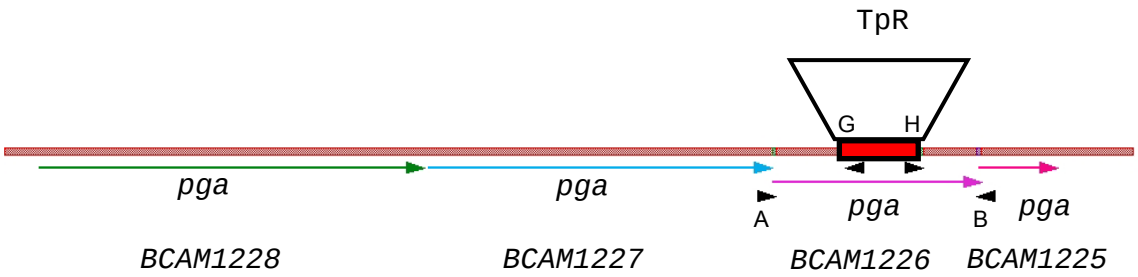

Figure 10. A schematic map of the *pgaABCD* locus with a tpR cassette insertion in the gene *pgaC*. A, B, G and H denote the primers used to generate the insertion (see **table 6**).

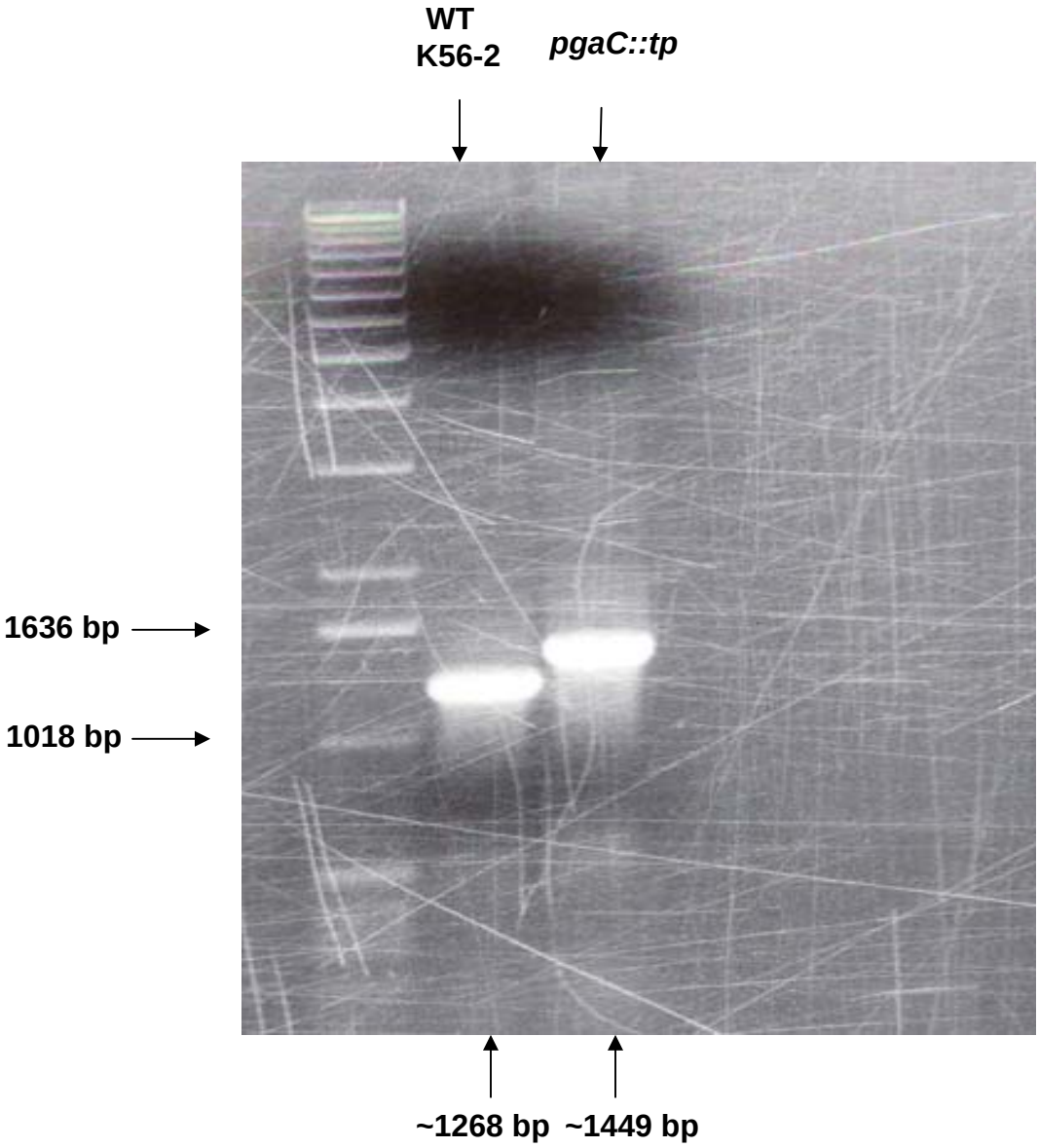

Figure 11. PCR confirmation using primers **A** and **B** (as shown in **fig. 10**) of tpR cassette insertion into *pgaC*, generating the strain, *pgaC::tp*. Ladder band sizes are marked on the left, and estimated PCR sizes are denoted on the bottom.

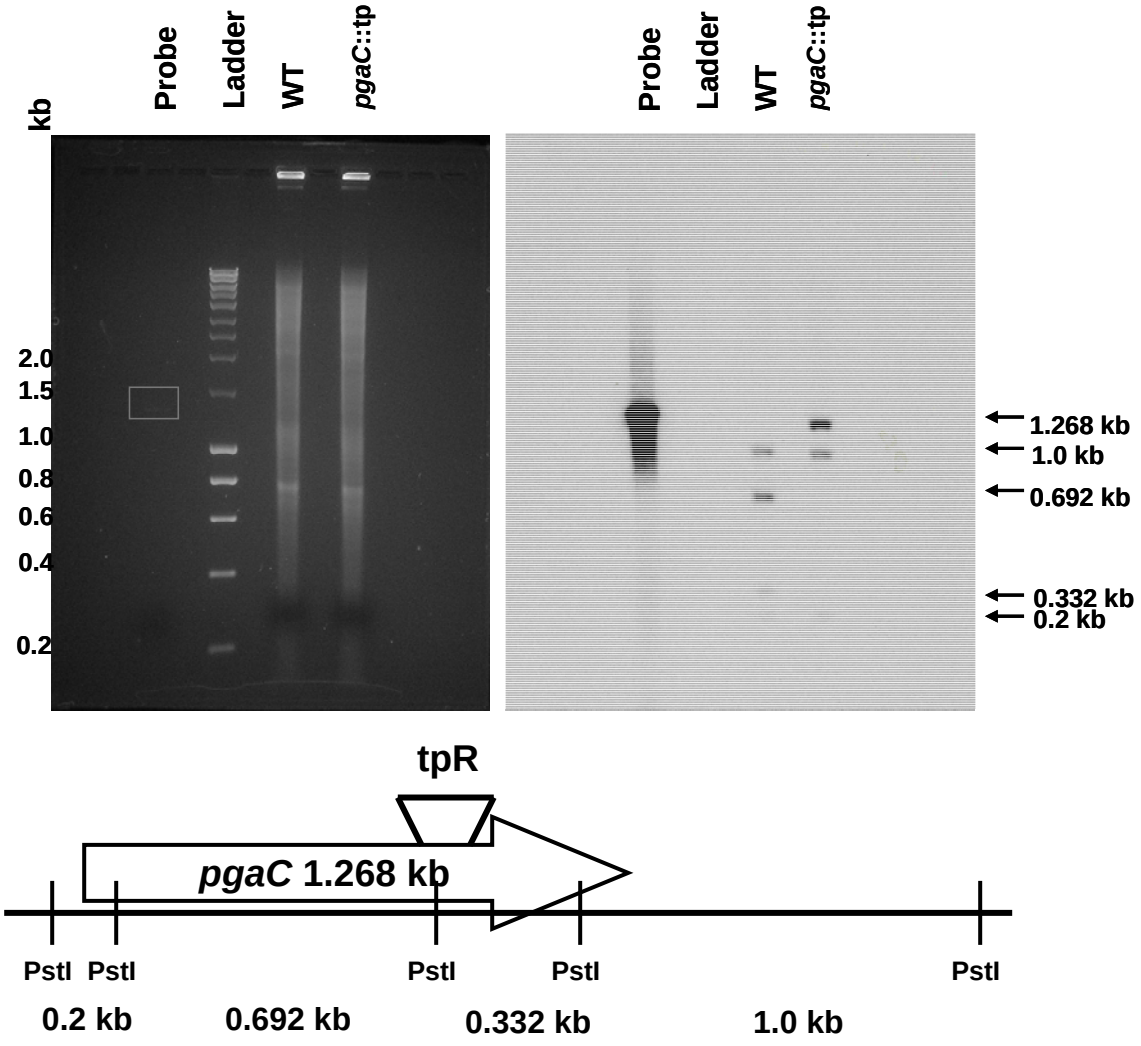

Figure 12. Southern blot confirmation of *pgaC::tp* using ^{32}P-end labeling. The picture on the left depicts the total digests of genomic DNA using the restriction enzyme, Pst1. Insertion of the tpR cassette results in the loss of a Pst1 restriction site. The picture on the right represents the result of the Southern blot using the PCR product derived from primers **A** and **B** as the probe. At the bottom is a schematic of the fragments derived from the Southern blot.

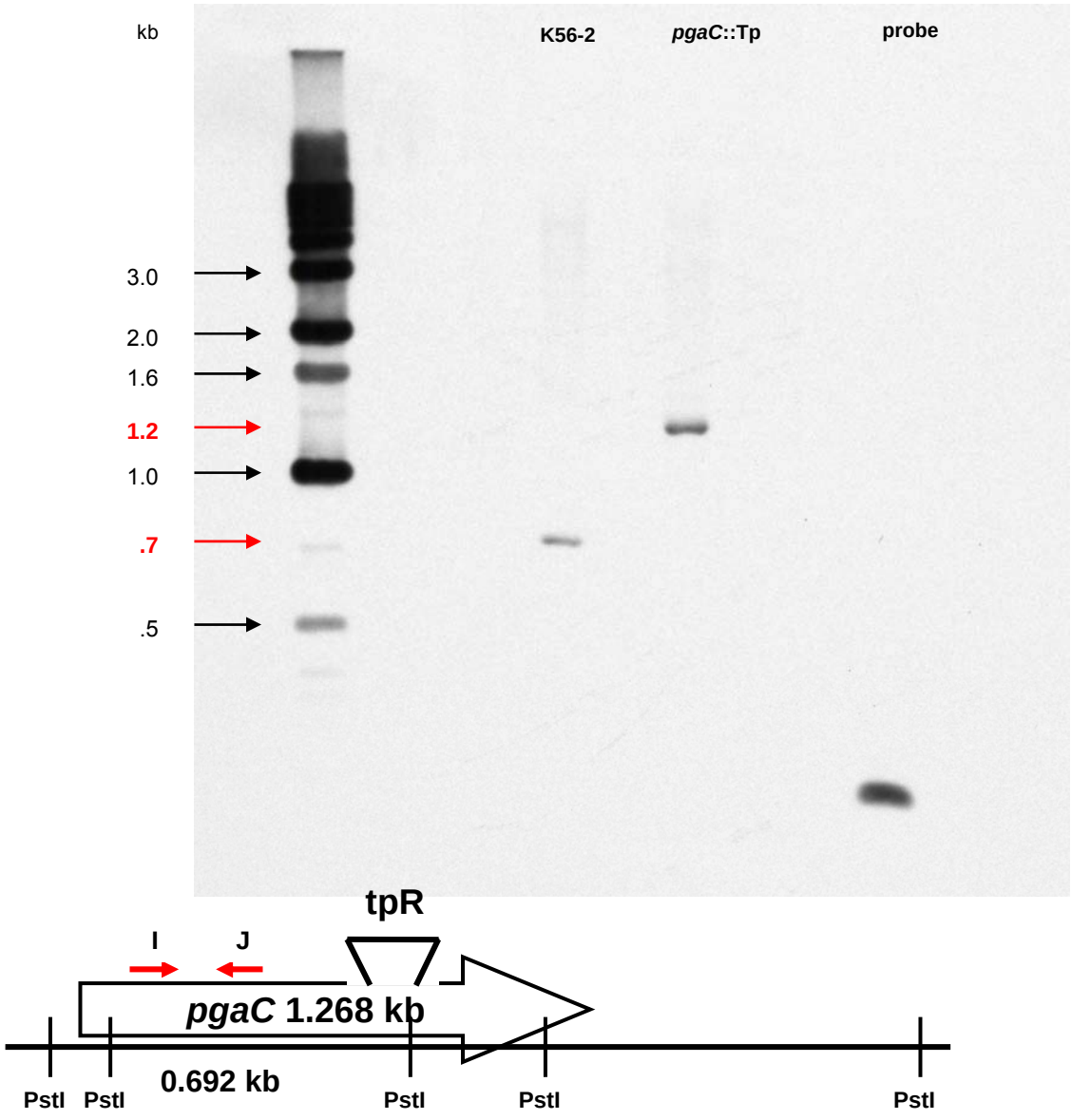

Figure 13. Southern confirmation of the tpR insert into *pgaC* using electrochemical luminescence (ECL) and the PCR product from primers **I** and **J** as the probe. Insertion of tpR results in the loss of a Pst1 restriction site.

Exopolysaccharide quantification. To assess the importance of the *pgaC* gene in polysaccharide secretion in *B. cenocepacia* K56-2, a standard EPS quantification procedure was used to measure biofilm generation in the strain, *pgaC*::*tp*. **Figure 14** outlines results typical for this experiment. Although *B. cenocepacia* K56-2 is known to generate relatively small amounts of biofilm *in vitro* (12; 142), there was a statistically significant decrease in crystal violet staining of 48 h cultures at 37°C, indicating a decrease in polysaccharide production in this strain compared to the parental strain, K56-2.

PNAG as a virulence factor of *C. elegans*. To determine whether *pgaC* plays a role in the virulence of *B. cenocepacia* in *C. elegans* infection, nematodes were plated onto both wild type K56-2 and *pgaC::tp* and monitored for their ability to kill worms. **Figure 15** outlines the representative results for this assay. The common nematode food-source bacterium, *E. coli* OP50, is completely avirulent in this assay, as highlighted by 100% survival of nematodes for more than 10 days. In contrast, plating of nematodes on lawns of *B. cenocepacia* K56-2 resulted in no survival after 110 hours, supporting the notion that *B cenocepacia* strain K56-2 is indeed pathogenic to *C. elegans*. However, when *C. elegans* were fed on the *pgaC::tp* mutant, they had an increased rate of survival, nearly twice as long, compared to the parental K56-2 strain.

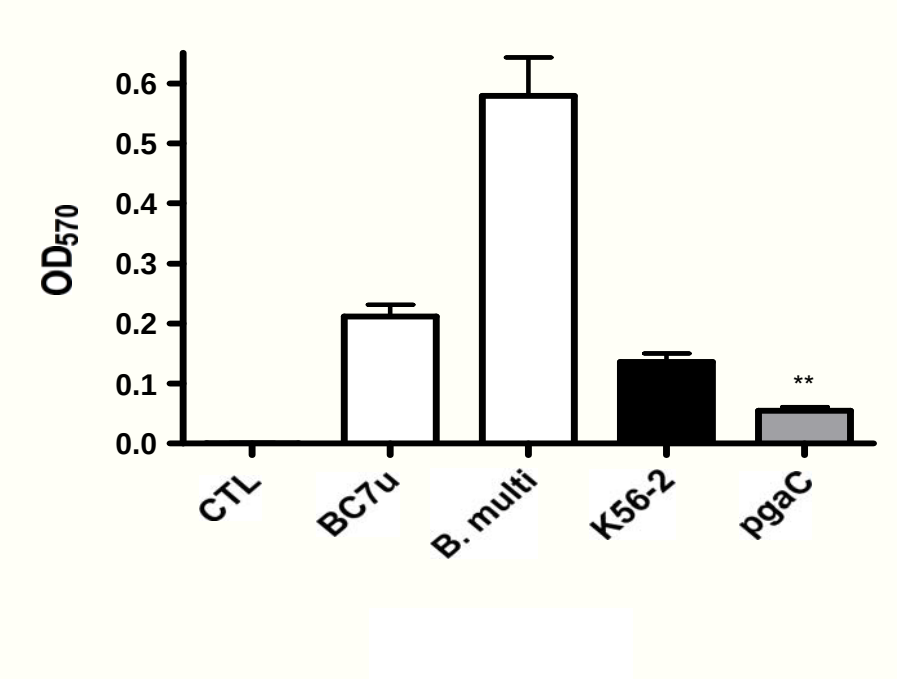

Figure 14. Crystal violet staining of EPS generated in 96-well microtiter plates after 48h at 37°C. The ctl lane is TSBG alone, BC7u is a moderate EPS producing *B. cenocepacia* strain, B. multi is a highly mucoid isolate of the Bcc bacterium, *B. multivorans*, ATCC 17616. ** = $p > 0.008$ determined by Mann Whitney comparison of four replicates of K56-2 and pgaC.

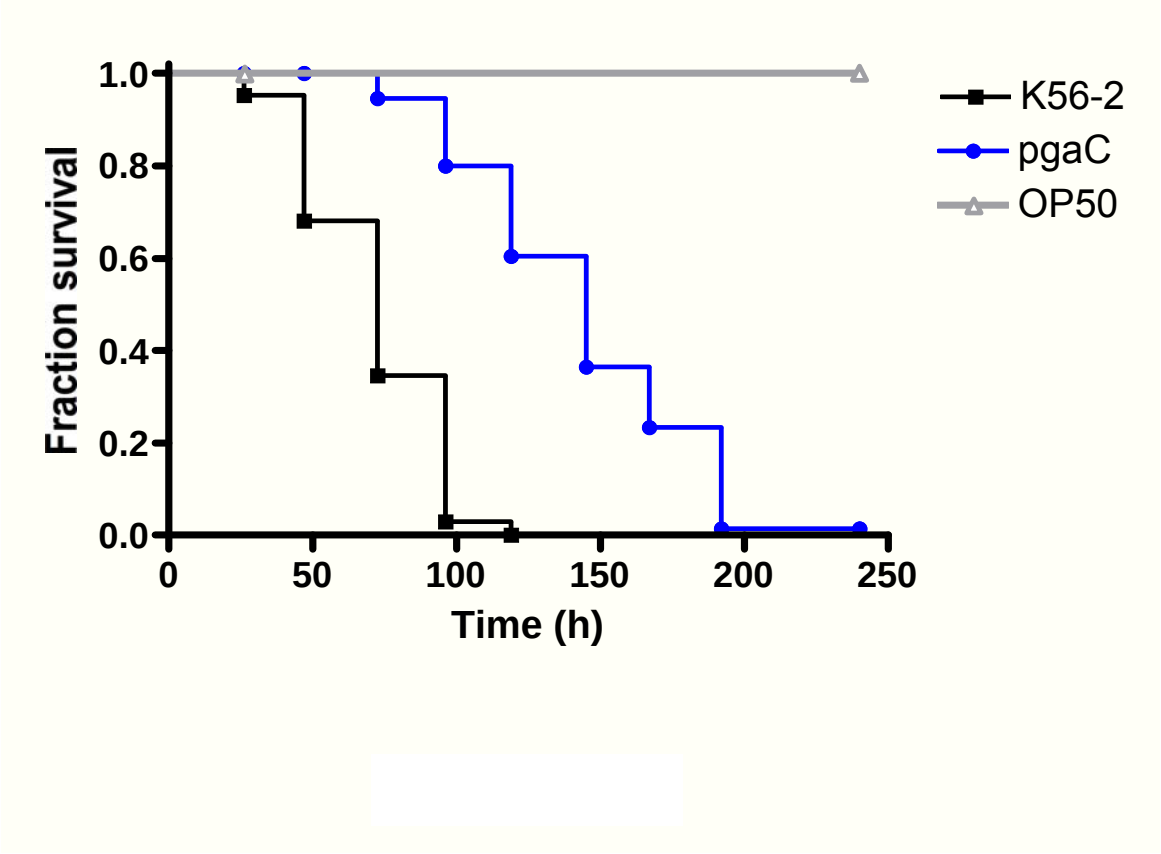

Figure 15. Survival of *C. elegans* fed on *E. coli* OP50, *B. cenocepacia* K56-2, and *B. cenocepacia* K56-2 *pgaC::tp*. $p < 0.001$ using log rank analysis of pgaC and K56-2. This experiment was performed in triplicate with similar results.

Growth curves of both K56-2 and *pgaC::tp* indicate that insertion of the trimethoprim cassette into the *pgaC* gene does not have any effect on growth compared to the wild type strain (data not shown). These results suggest that the *pgaC::tp* is deficient in worm killing, likely due to a deficiency in PNAG excretion.

Colonization of *C. elegans*. To compare the ability of *B. cenocepacia* K56-2 and *pgaC::tp* to colonize *C. elegans*, approximately 30 nematodes were manually disrupted at varying time points after feeding on either strain. The resulting liquor was plated onto TSA plates for CFU analysis. **Figure 16** depicts the average CFU/worm estimated from this study at 4, 24 and 48 hrs. At each time point analyzed, there was at least a 0.5 log difference in viable bacteria within the nematodes fed either K56-2 or *pgaC::tp*. These results suggest that the mutant strain, *pgaC::tp*, is significantly deficient in the ability to colonize *C. elegans* compared to the parental strain.

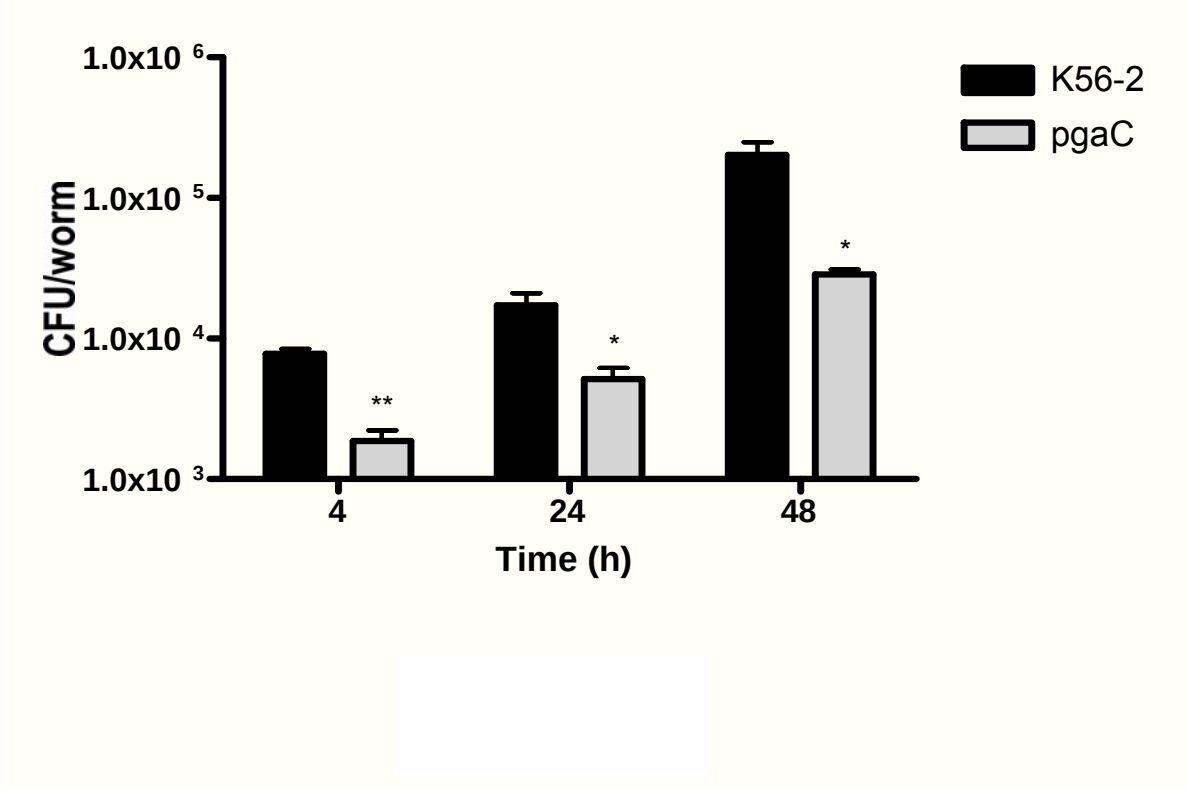

Figure 16. Colonization of *C. elegans* fed either K56-2 or *pgaC::tp*. This experiment was performed in triplicate and the values represent the average CFU per worm per experiment. * = p > 0.034 and ** = p > 0.002 using Students unpaired t test.

Localization of K56-2 and *pgaC::tp* within *C. elegans*. Some bacteria, like *Y. pestis* and *Y. pseudotuberculosis*, that are pathogenic to *C. elegans* colonize the cuticle of the worm, forming an obstructive biofilm that inhibits the ability of the worm to feed. Others, such as *S. aureus*, are intestinal pathogens that accumulate in the gut, causing severe intestinal distension that hinders both feeding and excretion. Additionally, a species of actinomycetes have been shown to be directly invasive to the nematode cuticle causing disseminated infection (97). To determine the pathogenic mechanism of K56-2 and the mutant strain *pgaC::tp* on *C. elegans*, differential interference imaging of nematodes fed either pathogen was used to visualize the infection directly. **Figures 17a-c** are representative micrographs of worms fed on either bacterium for 72 hrs. The micrographs depict severe distention of the upper and lower intestinal tract when the nematodes are fed K56-2 and that distension is absent in worms fed *pgaC::tp*. However, the mid-intestinal tract of both cohorts of *C. elegans* appear equally distended after 72 hours (**fig. 17c**). Neither K56-2 nor *pgaC::tp* were able to invade or colonize the cuticle of the worms (data not shown). These results suggest that *pgaC::tp* may be attenuated in worm killing compared to the parental strain due to its reduced ability to colonize *C. elegans*, but that colonization deficiency is location-specific within the nematode intestinal tract.

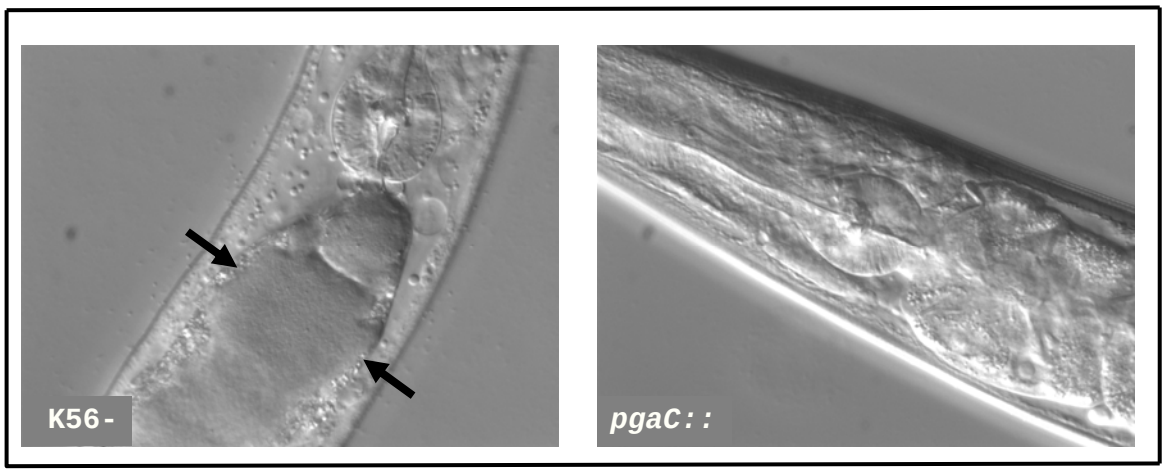

Figure 17a. Colonization of the pharynx and upper intestine of *C. elegans* fed either *B. cenocepacia* K56-2 or K56-2 *pgaC::tp* for 72 hrs. The arrows in the left panel point to severe distention of the upper intestine track when the nematodes are fed K56-2. This distention is absent when the worms are fed K56-2 *pgaC::tp*.

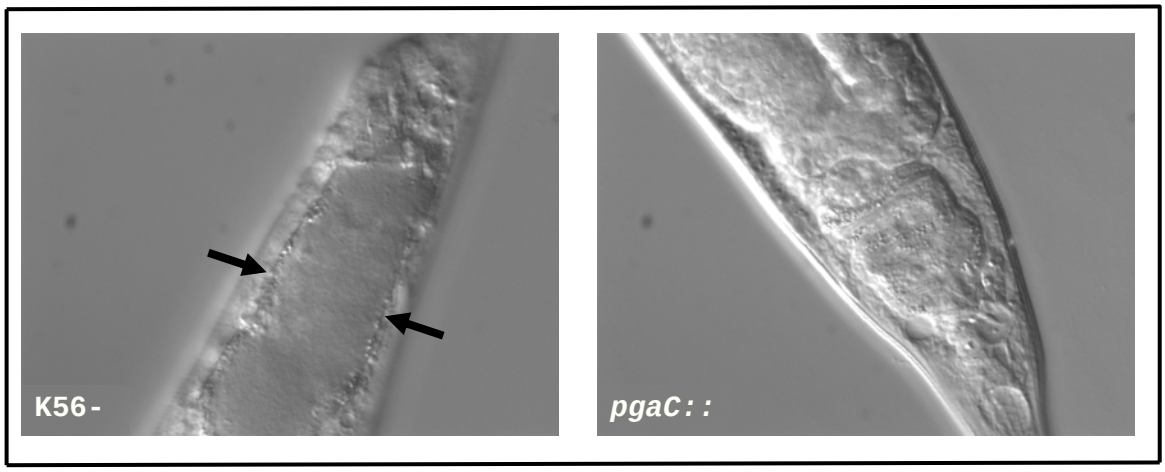

Figure 17b. Colonization of the lower intestine of *C. elegans* fed either *B. cenocepacia* K56-2 or K56-2 *pgaC::tp* for 72 hrs. The arrows in the left panel point to severe distention of the upper intestine track when the nematodes are fed K56-2. This distention is absent when the worms are fed K56-2 *pgaC::tp*.

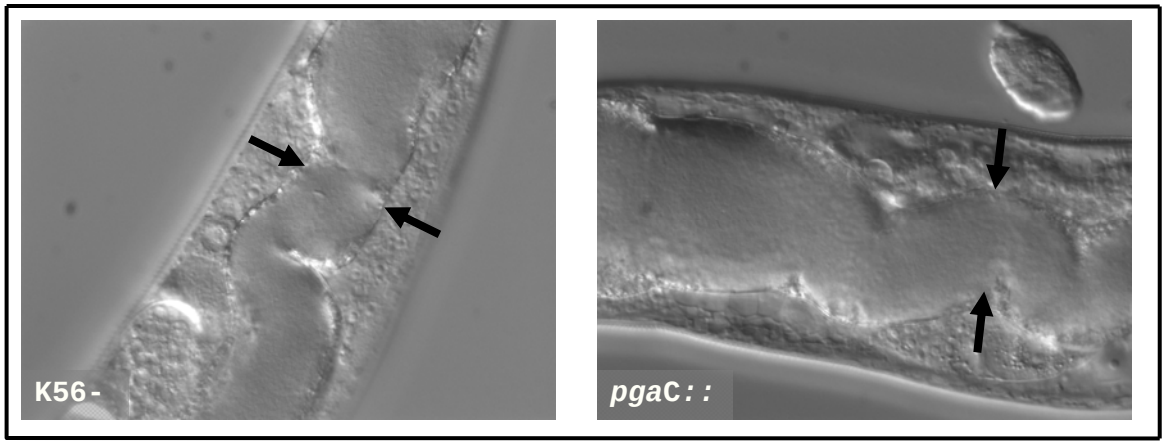

Figure 17c. Colonization of the mid-intestinal tract of *C. elegans* fed either *B. cenocepacia* K56-2 or *pgaC::tp* for 72hrs. The arrows highlight severe intestinal distention in both experimental groups.

Future Directions

The experiments outlined here describe the identification of a previously uncharacterized genetic locus of *B. cenocepacia*, potentially involved in the biosynthesis of the exopolysaccharide PNAG. Mutation of a gene in this locus, *pgaC,* resulted in decreased virulence in the *C. elegans* model of infection, which suggests that the product of this locus is an important virulence factor in the context of the nematode. However, whether the product of this locus is indeed PNAG, as is the case for homologous loci in other bacteria, remains unclear. Using antibodies specific for PNAG, it may be possible to detect differences in expression of this EPS between the wild type and mutant strains. Recently, Itoh *et al.* determined that native expression of PNAG was not abundant enough for adequate aantibody detection in *E. coli* and therefore generated a mutant strain derepressed for *pgaABCD* expression to yield more robust production of PNAG. They then used this derepressed strain to study the loss of specific *pga* genes on PNAG generation and excretion (61). Genetic analysis performed by Mike Davis revealed that the genome of *B. cenocepacia* lacks a homologue of the transcriptional regulator mutated by Itoh *et al.* However, the genome of *B. cenocepacia* does contain a homologue of the positive regulator of PNAG production in *E. coli*, called NhaR, that activates *pgaABCD* in response to high pH and increased concentration of sodium ions (49). Altering the regulation of *pgaABCD* in wild type and mutant *B. cenocepacia* may improve our ability to detect differences in PNAG production between these strains.

We successfully measured decreased nematocidal activity of *pgaC::tp* compared to the parental strain, indicating that the *pgaABCD* locus is involved in the production of an important virulence factor in this model. However, we have yet to determine if the addition of the *pgaC* gene on a plasmid restores *pgaC::tp*'s ability to kill *C. elegans* at wild type levels. The *pgaC::tp* complement strain has been generated. Preliminary results of the nematocidal assay indicate that the addition of the wild type *pgaC* gene on a plasmid to the insertional mutant is not sufficient to restore worm killing to wild type levels. As such, we hope to add back the entire *pgaABCD* locus on a plasmid to *pgaC::tp*, to determine if chromosomal insertion of the tpR cassette resulted in polar effects on downstream gene expression. We are also interested in whether overexpression of *pgaABCD* results in faster worm killing compared to wild type. This could be tested by inducible plasmid expression containing the entire locus, or by modification or activation of the positive transcriptional regulator present in the genome.

Using differential interference contrast microscopy, we were able to show that the *pgaC::tp* mutant was deficient in its ability to colonize the intestine of *C. elegans* compared to wild type *B. cenocepacia*. However, while the accumulation of bacteria in the gut of the nematode is highly correlated with worm killing, it is not sufficient for killing. Work by Garsin *et al.* demonstrated that aerobically grown *Enterococcus faecium* accumulates to high titers in the intestine of *C. elegans*, but has no effect on the longevity of the nematode (44). Therefore, the colonization defect alone is insufficient to explain the decreased virulence demonstrated by *pgaC::tp* in the worm model of infection. It is possible that

bacterial EPS production promotes more dense colonization of the intestine, which results in a higher concentration of virulence factors that are directly responsible for the worm killing phenotype. It is also possible that EPS production itself is a trigger for the upregulation of additional virulence factors, in a similar fashion to quorum sensing, and bacteria deficient in EPS production inadequately promote other virulence genes. Furthermore, PNAG in *B. cenocepacia* infection may play an immunoprotective role during the colonization of the *C. elegans* intestine, as has been demonstrated for *S. epidermidis* (7). Clearly, more attention needs to be given to the specific role of this polysaccharide in the intestine of the nematode.

While the *C. elegans* model of infection is an excellent starting point for identification of pathogenic bacteria and their mechanisms of virulence, it is critical to test our wild type and mutant strains in a mammalian model of infection to better ascertain the possible role of PNAG as a virulence factor in human infection. We have recently shipped both wild type and *pgaC::tp* strains to collaborators who have expertise with the mouse model of infection using Bcc bacteria. Their preliminary results suggest that the *pgaC::tp* mutant is deficient in its ability to colonize mice compared to the parental strain, strengthening the argument that PNAG is an important virulence factor in mammals. Future experiments include validation of the reduced virulence phenotype in the mouse model and whether the administration of PNAG specific antibodies can protect mice from infection with *B. cenocepacia* as was seen in *E. coli* infection (15). We are also interested in whether PNAG production has implications specifically in

the lung environment of healthy and CF mice. A CF mouse model is available, and testing our strains in these animals will help clarify whether PNAG production plays a role in the ability of *B. cenocepacia* to opportunistically infect the airways of CF patients.

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