DISSERTATION

FOLATE PATHWAY INHIBITOR RESISTANCE MECHANISMS IN *BURKHOLDERIA PSEUDOMALLEI*

Submitted by

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ABSTRACT

FOLATE PATHWAY INHIBITOR RESISTANCE MECHANISMS

IN *BURKHOLDERIA PSEUDOMALLEI*

Antimicrobials are invaluable tools used to facilitate the treatment of infectious diseases. Their use has saved millions of lives since their introduction in the early 1900's. Unfortunately, due to the increased incidence and dispersal of antimicrobial resistance determinants, many of these drugs are no longer efficacious. This greatly limits the options available for treatment of serious bacterial infections, including melioidosis, which is caused by *Burkholderia pseudomallei*, a Gram-negative saprophyte. This organism is intrinsically resistant to many antimicrobials. Additionally, there have been reports of *B. pseudomallei* isolates resistant to several of the antimicrobials currently used for treatment, including the trimethoprim and sulfamethoxazole combination, co-trimoxazole. The overarching goal of this project was to identify and characterize mechanisms of trimethoprim and sulfamethoxazole resistance in clinical and environmental isolates, as well as in laboratory induced mutants. Prior to these studies, very little work has been done to identify and characterize the mechanisms by which *B. pseudomallei* strains are or could become resistant to folate-pathway inhibitors, specifically trimethoprim and sulfamethoxazole.

During the initial phases of these studies, we determined the antimicrobial susceptibilities of a large collection of clinical and environmental isolates from Thailand and Australia (n = 65). A high frequency of naturally-occurring resistance to trimethoprim alone (40%) was observed. However these strains were susceptible to sulfamethoxazole and to the co-trimoxazole combination. Trimethoprim resistance in a subset of these strains was due to increased

expression of an efflux pump belonging to the resistance nodulation and cellular division (RND) superfamily, BpeEF-OprC, in the presence of trimethoprim. This efflux pump had been previously shown to efflux trimethoprim, chloramphenicol and tetracyclines when expressed in surrogate bacterial strains. The molecular mechanism of increased BpeEF-OprC expression in these isolates remains unknown. Similarly, decreased susceptibility in laboratory mutants selected on trimethoprim were due to mutations leading to amino acid substitutions in BpeT, which caused overexpression of BpeEF-OprC, or FolA, the trimethoprim drug target. This is the first description of mutations to FolA conferring trimethoprim resistance in *B. pseudomallei*, though similar mutations have been observed in *B. cenocepacia* and *Escherichia coli*. A similar study to select for sulfamethoxazole resistance, instead suggested that *B. pseudomallei* may be able to tolerate high concentrations of the drug.

Studies to characterize laboratory induced mutants selected on co-trimoxazole led to the identification of two novel resistance determinants. Mutations to BpeS, a newly named LysR-type regulator with high similarity to the cognate BpeEF-OprC efflux pump regulator, BpeT, cause increased BpeEF-OprC expression in these strains. Additionally mutations to Ptr1, an annotated pteridine reductase, partially contributed to the decreased co-trimoxazole susceptibility. However, it is unclear what function Ptr1 has in the folate synthesis pathway, as deletion of this gene also caused slight decreases in antimicrobial susceptibility. Finally, in a collection of co-trimoxazole resistant clinical isolates from Thailand, high-level expression of the BpeEF-OprC was found in the resistant isolates. A mutation to BpeS was also observed in two of the clinical isolates that had BpeT-independent BpeEF-OprC overexpression. Co-trimoxazole resistant isolates were each resistant to both trimethoprim and sulfamethoxazole individually. However, deletion of the *bpeEF-oprC* efflux pump structural genes in all isolates resistant to co-

trimoxazole or isolates resistant to trimethoprim alone (except those with a mutant FolA) resulted in antimicrobial susceptibility to trimethoprim, co-trimoxazole and sulfamethoxazole. These data suggest that sulfamethoxazole is also a substrate of the BpeEF-OprC efflux pump and this RND pump is the major resistance determinant contributing to clinically relevant folate pathway inhibitor resistance in *B. pseudomallei.*

To summarize, we have identified and described several resistance determinants in *B. pseudomallei* causing decreased susceptibilities to trimethoprim, sulfamethoxazole and/or co-trimoxazole; these include drug target and metabolic pathway modifications and overexpression of the BpeEF-OprC efflux pump. Further characterization of these mechanisms and the development of specific detection assays could allow for rapid determination of antimicrobial resistance and provide useful information for the development of novel antimicrobials against *B. pseudomallei.*

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DEDICATION

To my parents Josef & Janet Podnecky who taught me how to solve problems, to not be intimidated by challenging tasks, to never accept anything but my best, and to strive to constantly improve the world around me.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PUBLICATIONS

Nicole L. Podnecky, Vanaporn Wuthiekanun, Sharon J. Peacock, & Herbert P. Schweizer. 2013. The BpeEF-OprC efflux pump is responsible for widespread trimethoprim resistance in clinical and environmental *Burkholderia pseudomallei* isolates. *Antimicrobial Agents & Chemotherapy.* 57(9):4381-4386.

Ediane B. Silva, Andrew Goodyear, Marjorie D. Sutherland, Nicole L. Podnecky, Mercedes Gonzalez-Juarrero, Herbert P. Schweizer, and Steven W. Dow. Correlates of immune protection following cutaneous immunization with an attenuated *Burkholderia pseudomallei* vaccine. *Infection & Immunity.* (in press).

CHAPTER 1: Introduction to *Burkholderia pseudomallei*

1.1 The Organism of Interest

1.1.1 Discovery and characterization.

Melioidosis, also called Whitmore's disease, was first described in 1911 in Rangoon (presently the city of Yangon in Burma) (1, 2). Whitmore and Krishnaswami described their findings from observations of patients and autopsies performed on several diseased victims. The authors isolated a bacterium on potato slants and peptone agar. They characterized a Gram-negative, motile, rod-shaped organism, and confirmed it as the pathogen of interest by fulfilling Koch's postulates. They named this bacterium *Bacillus pseudomallei* (1), based on the Greek adjective pseudês meaning false, as it was similar to a previously described *Bacillus mallei* bacterium. This was the first discovery of the organism that was re-named several times throughout its history (3), but was finally named *Burkholderia pseudomallei* in 1992 (4).

The bacteria are relatively small in size, measuring approximately 0.8 μm by 1.5 μm (5) with rounded ends (6). *B. pseudomallei* is a Gram-negative and non-spore forming bacterium, but can display bipolar staining resulting in a safety-pin appearance microscopically (1, 5, 7). In contrast to other *Burkholderia spp.*, including *B. thailandensis*, *B. pseudomallei* cannot assimilate the monosaccharide arabinose (8). Macroscopically, it grows well on general media in ambient air. *B. pseudomallei* typically forms opaque, white creamy colonies that display a very textured, rugose, appearance after extended incubation on standard nutritional media, but they also can display diverse morphologies that can switch based on environmental conditions, such as nutrition depletion, reduced iron availability, increased temperature, and sub-inhibitory concentrations of antimicrobials (9). Interestingly, this organism is also associated with a distinct

odor that has been described as sweet and earthy or milky (5). *B. pseudomallei* is a catalase and oxidase positive organism that is capable of utilizing citrate as a sole carbon-source and growth on MacConkey agar (4). It is a hardy organism that is very resistant to degradation, can survive in the environment for extended periods of time, and is able to tolerate variation in both pH and temperature despite being a non-spore former (10). This may be due to the ability of the bacteria to enter a viable but non-culturable form (11, 12). It is, however, quite sensitive to ultra-violet light and dehydration (10). *B. pseudomallei* requires the water content in soil to be above 10% and is more sensitive to ultraviolet radiation than other soil-borne bacteria (10).

1.1.2 The *Burkholderia* genus.

The *Burkholderia* genus was proposed by Yabuuchi *et al*. in 1992 based on similarities of a number of species in 16S rRNA, DNA homology, lipid and fatty acid composition, and phenotype (4). This genus originally included seven species: *B. cepacia* (the reference organism), *B. caryophylli*, *B. gladioli*, *B. mallei*, *B. pickettii*, *B. pseudomallei*, and *B. solanacearum* (4). As of October 2013 in the List of Prokaryotic Names with Standing Nomenclature (LPSN) the *Burkholderia* genus consists of 75 distinct species of bacteria and an additional 8 bacteria listed as *Candidatus Burkholderia spp*. (13, 14). The *Candidatus* taxon is used to describe prokaryotes that have not been well characterized (15, 16) or those that are characterized but unculturable with current methods (17). The *Burkholderia* genus is continuing to grow with the discovery of novel organisms.

Burkholderia spp. originate from diverse ecological niches including: soils, surface waters, and a variety of hosts, including humans, animals and plants (18). Several *Burkholderia spp.* have been identified as significant plant pathogens, including *B. caryophylli*, *B. plantarii*, *B. glumae* and *B. andropogonis* (18). *B. pseudomallei* possesses several gene clusters and type III

secretion systems similar to those identified in plant pathogens, which suggests an interaction with plants and the rhizosphere in endemic regions (19, 20).

The vast majority of *Burkholderia spp*. are of little public health concern in humans with several major exceptions in addition to *B. pseudomallei* (18). *B. mallei* is an obligate mammalian pathogen (21), as it has undergone genome reduction and can no longer adapt to an autonomous life style (22-24). The reservoir for this organism is primarily horses (21), but also includes closely related donkeys and mules (25). *B. mallei* is the highly pathogenic etiologic agent of the disease glanders (21), which results in high mortality rates despite rapid and appropriate treatment (26). Several other *Burkholderia spp.* have been found to cause serious infections in immune compromised individuals, particularly those struggling with cystic fibrosis; these include: the *B. cepacia* complex (*B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. stabilis*, *B. vietnamiensis*, *B. dolosa*, *B. ambifaria*, *B. anthia*, *B. pyrrocinia* and *B. ubonensis*), *B. fungorum*, *B. gladioli*, B. *oklahomensis* and *B. thailandensis* (18, 27-29).

The *Burkholderia* genus includes two frequently used surrogate organisms with reduced virulence for studies of *B. pseudomallei*, *Burkholderia thailandensis* (30, 31) and *Burkholderia cepacia* (32). However, studies in surrogates without confirmatory testing in *B. pseudomallei* and/or *B. mallei* are becoming uncommon due to inherent differences between these species both genetically and phenotypically. Two attenuated strains of *B. pseudomallei* have recently become available, and are preferred for testing over surrogate species (33, 34).

1.1.3 Geographic distribution.

Historically the distribution of *B. pseudomallei* was thought to be confined between 20°N and 20°S in the moist tropical regions of Southeast Asia and regions of tropical Australia (6, 35-37). We now know that while *B. pseudomallei* is widely considered endemic to Southeast Asia

and northern Australia, it is also considered emerging in other tropical and sub-tropical regions of the world (**Figure 1.1**) (38, 39). Cases of melioidosis have occurred in the Indian subcontinent, regions of the Middle East, Africa, South America and Central America (6). However, the true distribution is likely larger than documented, as melioidosis is an often under-diagnosed condition especially in areas of the world with poorly developed health infrastructure and where *B. pseudomallei* is not a well known pathogen (38).

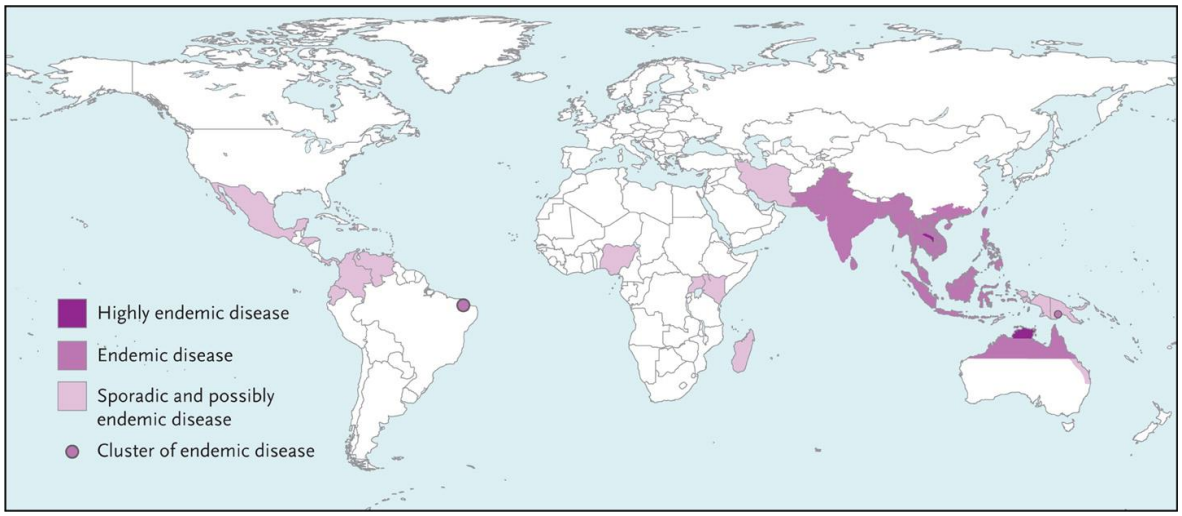

Figure 1.1 Global geographic distribution of reported melioidosis cases. A recent study illustrates the distribution of reported *Burkholderia pseudomallei* infections. **Source** (39).

Also of note are outbreaks caused by the importation of *B. pseudomallei* to non-endemic areas. Some notable outbreaks include Paris, France in 1975 (40), London, England in 1992 (41, 42), and South America (38, 43, 44). While it is suggested that *B. pseudomallei* will remain confined to the environments of warmer regions, such as Columbia (44), in Europe it is assumed that *B. pseudomallei* did not, and would not, survive in the environment in more temperate regions. This is because temperatures drop below 0°C and these temperatures cannot be tolerated for extended periods of time by *B. pseudomallei* (10).

1.1.4 Genomics.

Burkholderia spp. are unique compared to other prokaryotes in that they have multiple circular chromosomes, whereas most bacteria typically have one (45). For instance the *B. pseudomallei* strain K96243 genome consists of two chromosomes that are 4.07 and 3.17 megabase pairs in size and encode for 3,460 and 2,395 predicted genes, respectively (45, 46). Of these, a predicted 2,590 genes are shared with other members of the *Burkholderia* genus, and as a result, are suggested to represent the essential core genome (47-49). Interestingly, many of the genes on the larger chromosome, Chromosome 1, are associated with cellular growth and metabolism, while those on the smaller chromosome, Chromosome 2, are linked with survival and adaptation to changing environments (45).

High genetic variability has even been observed in *B. pseudomallei* isolated from the same patient throughout the course of infection and treatment, suggesting rapid adaptation of this organism to its environment (50). This has also been observed following longer periods leading to relapsing infections (51), and may be the result of frequent recombination events. *B. pseudomallei* appears to be a genetically promiscuous organism. It is known to integrate large regions of DNA, genomic islands, into its chromosomes (48). These genetic features are likely obtained from other bacterial species possibly during interactions in the environment. In contrast to this, certain strains of *B. pseudomallei* have been found lacking large regions of their chromosomes (52). Both the integration of foreign DNA and loss of genetic regions is likely due to the bacteria's natural homologous recombination mechanisms (53). Based on advanced statistical analysis, *B. pseudomallei* is expected to have a high rate of recombination and a low rate of mutation (54). This is additionally supported by the large number of *B. pseudomallei* strains and *Burkholderia spp*., suggested by Hanage *et al*. to be an indication of increased

recombination rates (54). *B. pseudomallei* has a very high guanine and cytosine (gc) composition compared with other bacterial species. The 69% gc content (35) greatly increases the difficulty of genetic manipulation, primer design, and DNA sequencing.

1.1.5 Virulence factors.

Burkholderia pseudomallei is well adapted for its life as a facultative intracellular pathogen. *B. pseudomallei* has been shown to modulate its own uptake or engulfment by host cells including macrophages, at which point the bacterium escapes the endosome and replicates within the host cytoplasm (55). From this point it uses actin-based motility to spread between adjacent cells and create multi-nucleated giant cells (56). This route of molecular pathogenesis is similar to that of *Salmonella* and *Listeria* (57). At least 28 specific genes have been identified that play a role in *B. pseudomallei* adhesion, invasion and escape from endosomes, intracellular survival, cell to cell spread, actin-based motility and the formation of multinucleated giant cells (57, 58). Among these are the Bsa type III secretion system (59), which is necessary for escape from endosomes, and BimA, which is essential for actin-based motility and thus required for cell-to-cell spread (60).

In addition to its ability to invade host cells, *B. pseudomallei* has demonstrated its ability to effectively evade host defenses, as it is resistant to complement and lysosomal defensins (61). It also has the ability to synthesize proteases, lipase, catalase, peroxidase, superoxide dismutase, lecithinase, haemolysins, siderophores, a capsule, numerous type III and type VI secretion systems, and flagella as virulence factors (62-64).

Many Gram-negative bacteria including *Burkholderia spp.* are capable of producing *N*-acyl-homoserine lactone (AHL), a signaling molecule that allows bacteria to monitor their population density, known as quorum sensing (65). Once the cell recognizes a quorum,

transcriptional activation and up-regulation of virulence factors occurs. These virulence factors are commonly associated with pathogenesis, and may include: siderophores, proteases, chitinases, lipases, swimming motility, biofilm production, etc. (66). *Burkholderia spp.* have been shown to produce biofilms (67), which may contribute to decreased efficacy of antimicrobial therapy and play a role in latency.

1.1.6 Select Agent status.

B. pseudomallei is categorized as Tier 1 (formerly Category B) Select Agent by the Centers for Disease Control and Prevention (CDC) (68) and the Animal and Plant Health Inspection Service (APHIS) based on criteria set forth by the Public Health Security and Bioterrorism Preparedness and Response Act (69) and the Agricultural Bioterrorism Protection Act of 2002 (68). Primarily these criteria identified agents with increased potential for public health impact based on morbidity and mortality, ease of dispersion and transmission, public perception and requirements for surveillance, diagnostics and treatment (68). As a result, research with *B. pseudomallei* is required to be performed in a Biosafety Level 3 (BSL-3) registered space with strict biosecurity and biosafety requirements. Additionally, the Select Agent status places restrictions on some types of experiments that can be performed; primarily restricting those that would increase virulence or impair the effectiveness of currently recommended treatments. Current *B. pseudomallei* research is focused on developing a better understanding the epidemiology, prevalence and transmission and to improve prevention, diagnosis and treatment of melioidosis (70). While this has greatly increased interest and availability of funding for some types of research on this organism, it has also limited the available tools and techniques that are approved for research on *B. pseudomallei* (71).

Recently several attenuated strains of *B. pseudomallei* have been constructed that are now considered excluded from the Select Agent program (33, 34). The Bp82 attenuated strain (1026bΔ*purM*) has been shown to be completely avirulent in immune deficient mice including those with severe combined immune deficiency (SCID) (33). These strains can now be used for studies that would not be possible with virulent *B. pseudomallei*.

1.2 Melioidosis

Melioidosis is a serious, often fatal, infectious disease that occurs in the tropics and subtropics, primarily in the hyper-endemic regions of Southeast Asia and Northern Australia. It is largely a seasonal disease, and typically infects immune compromised individuals especially those with frequent contact with soils and water. The average rate of incidence in highly endemic regions is between 3.6 and 5.5 cases per 100,000 people (72). Melioidosis is not commonly known in the Western world, however there are cases of travelers returning with infections, concerns of the broadening distribution of *B. pseudomallei* environmentally in a warming climate and the risk of intentional release of this agent as a possible bioweapon.

1.2.1 Routes of infection.

Burkholderia pseudomallei has a relatively low infectious dose; when administered intraperitoneally the 50% lethal dose (LD_{50}) for *B. pseudomallei* in Syrian hamsters is less than ten bacteria (73), however the infectious dose varies dependent on the route of infection and bacterial strain (74). It is also important to note that there is no data available about the LD_{50} for humans and animal models may not be an accurate estimation of this dose (19).

There are several known routes of infection for melioidosis; these include 1) cutaneous, typically exposure to the organism through broken skin and open wounds, 2) inhalation, such as

the aspiration of contaminated water and soils during extreme weather events and heavy rainfall, 3) ingestion of contaminated water or food, and 4) iatrogenic inoculation (74-78).

Since *B. pseudomallei* is found primarily in moist soils and surface water in endemic areas (79, 80), it is not surprising that an epidemiological link is established between cases of melioidosis and exposure to mud and/or surface waters (3). Cutaneous exposure is suggested to be the most common route of infection (6); typically resulting from the exposure of cuts and abrasions with contaminated soils and surface water often resulting from occupational hazards (37, 81).

Experimental research has shown that laboratory animals can be infected by aerosol challenges (74). Infection by inhalation leads to rapid onset of disease, and this route of infection is thought to be responsible for the infection of helicopter crews during the Vietnam War, as contaminated soils and water were aerosolized by air currents from the helicopter blades (36). The incidence of melioidosis in returning soldiers and helicopter crew members from the Vietnam War resulted in the nicknaming of melioidosis as the "Vietnamese time bomb" (104, 122, 123). The incubation time for cases associated with extreme weather is relatively short, which again suggests inhalation as the route of infection (82). Increased cases of inhalation-related melioidosis cases have been reported following heavy rain and wind from monsoons (83). There is quite a bit of evidence that *B. pseudomallei* causes infections following near drownings (37, 84, 85) and the ingestion of contaminated drinking water or from other bodies of water in both humans and animals (76, 86). The lack of chlorination in water sources has been implemented in case clusters of melioidosis (87). Several studies have been able to culture *B. pseudomallei* from the gastrointestinal tract and in feces (88-90). One can thus speculate that food grown or rinsed with contaminated water may be a common source of infection. However,

contrary to this, Cheng *et al*. found no correlation between chlorination of drinking water and incidence in the endemic Northern Territory of Australia (91).

While there is increasing focus on *B. pseudomallei* research and diagnostics there have only been a few cases of iatrogenic inoculations. A study following 60 laboratorians over 15 years showed only a few individuals had changes in serology, which suggested a low rate of subclinical infection. However, this incidence rate was similar to that of the local population, and thus suggests the seroconversion was likely due to environmental factors other than working in a laboratory, as described in the editorial note (92). Infection with *B. pseudomallei* is unlikely in a laboratory setting under normal work conditions with safe practices (92, 93).

While the most likely routes of infection with *B. pseudomallei* are known, the mode of infection in individual patients is often unknown. Melioidosis in tourists to endemic regions often occurs without any apparent contact with soils (94). In fact, only in 25% of cases is there sufficient evidence to suggest a likely route of infection in melioidosis patients (81).

1.2.2 Transmission.

Melioidosis is typically acquired from interactions with the environment; there is very little evidence of person-to-person, animal-to-person, and vector transmission of this disease (77). Person-to-person transmission of melioidosis occurs very infrequently (95), a case of venereal transmission was the first report in 1975 (96). Additionally, there have been two reported cases of mother to child transmission in Australia (97). In one of these cases, *B. pseudomallei* cultures obtained from breast milk were identical to those found in the infant's blood and cerebrospinal fluid by pulsed-field gel electrophoresis (97). Zoonotic transmission of melioidosis to humans has been very rarely documented; several cases were discussed by Choy *et al*. in 2000 (98). These infections primarily resulted from occupational exposure to infected

animals and presented as wound infections (98). Vector-transmission of melioidosis, by *Xenopsylla cheopis,* the oriental rat flea, and *Aedes aegypti* mosquitoes from infected to naïve guinea pigs, was demonstrated by Blanc and Baltazard in 1949 (99). There is limited evidence to support these findings, as very few studies investigating vector transmission have been done since (100). However, even if vector transmission does occur, one can argue that its contribution to new infections of melioidosis would be limited, as septic melioidosis is of short duration, therefore there would only be a very narrow window for a vector to feed on an infected individual, become infected and transmit disease.

1.2.3 Risk factors.

Individuals with chronic health conditions including diabetes mellitus, renal disease, thalassemia, and alcoholism have been shown to have significantly higher risk of melioidosis infection (37, 101-103). Additionally increased risk of melioidosis is also linked to gender, extreme weather events, and occupational and recreational contact with soils and waters in endemic regions (6).

In 2000, a prospective 10 year epidemiologic study of melioidosis in Darwin, Northern Australia included of 252 melioidosis patients ranging in age from 16 months to 91 years (104). In this study male cases outnumbered female, accounting for 75% of the cases. This gender bias was further supported by other studies including a 14-year prospective study in the Northern Territory of Australia (105) and in a 10-year study at the University of Malaya Medical Centre in Malaysia (106). Additionally, Currie *et al.* observed that the vast majority of these patients had similar medical complications, predisposing risk factors for melioidosis (80%). These included diabetes mellitus, history of hazardous alcohol use, and chronic disease (lung or renal) (104). Cystic fibrosis has also been shown to be a significant risk for the development of pulmonary

melioidosis (3, 107-109). However, HIV infection has not been shown to increase risk of melioidosis (110, 111). Interestingly many of these health-related risk factors can been attributed to impaired antimicrobial function of neutrophils (6) and altered function of macrophages (112). Additionally there is an observed decrease in T-cell mediated immune function in patients with diabetes mellitus (113, 114).

As a result of *B. pseudomallei* being a soil and water-borne pathogen, extreme weather events such as tsunamis, typhoons, and unusually heavy monsoon rainfalls during the rainy season have been associated with outbreaks of melioidosis (104, 105, 115-117). On July 18-19, 2005 the Typhoon Haitang hit Taiwan, resulting in very heavy rainfall. From July 21 through August 24, 2005 there were 54 reported cases of melioidosis in Taiwan, which far exceeded the 9.4 average annual cases that were seen from 2001 to 2004 (82). These cases had molecular diversity, which suggested that the outbreak was not due to a common contaminated source. In the Northern Territory of Australia, 13 cyclones occurring in the summer of 1998-1999 were linked with several case clusters of molecularly diverse melioidosis (6). Extreme weather related melioidosis clusters only contribute to a small portion of the public health burden, however Currie *et al.* found that 85% of individual melioidosis cases in northern Australia also occur during the tropical monsoon season (104). Global climate change has the potential to increase the frequency and severity of extreme weather events. These extreme weather events may also play a role in melioidosis distribution, moving *B. pseudomallei* by water and wind currents within a region. A warming and wetter climate could increase the geographic distribution of *B. pseudomallei* worldwide (118).

Finally, melioidosis appears to be an occupational hazard to those who work outdoors including farmers in endemic regions. There is an observed 6-fold higher incidence rate of

melioidosis in farmers compared to other populations (101). Rice farmers are often the model population for these studies in Southeast Asia (117). However, those with other occupations where frequent contact with the soil, such as construction workers and those who are frequently outdoors for recreational reasons, are at increased risk as well (119). These populations, especially those with other underlying health conditions, would greatly benefit from vaccination (101).

1.2.4 Clinical manifestations.

The incubation time of *B. pseudomallei* infection is highly variable and largely dependent on the route of infection, bacterial strain, and size of inoculum (6). Incubation times can be very short when high concentrations of bacteria are encountered, such as in the case of near drownings (84, 120). In about 25% of cases where an exposure was able to be determined, there was a 1 to 21 day incubation time to the onset of clinical symptoms (81). However, historically latent periods have been documented as long as 62 years in a former prisoner of war from World War II (121).

Melioidosis is a disease with incredible variability of clinical presentation; in fact, it has been referred to as the "the remarkable imitator" (124) and "the great mimicker" (125, 126). Melioidosis most commonly presents as a respiratory infection or pneumonia (3), however acute presentation of disease also includes: cutaneous or wound infection, urinary tract infection, septicemia, empyema, septic arthritis, visceral abscess or suppurative parotitis in children (3, 7, 127). Typical disease presentation is categorized into five states: localized, transient bacteremia, non-disseminated septicemia, disseminated septicemia and unconfirmed infection (128).

Serological assays suggest that most *B. pseudomallei* infections are in fact asymptomatic (129-131). However, melioidosis is one of the most commonly fatal infectious diseases in

northeast Thailand (132) and the most fatal pneumonia in the Top End region of northern Australia (133). *B. pseudomallei* has the capacity to disseminate hematogenously causing overwhelming infection with foci in the liver, spleen and brain (3, 134). In the severe form of disease, melioidosis can manifest as a septicemia and can lead to septic shock. Without prompt diagnosis and treatment, this condition is fatal in about 90% of cases (3, 117). Between 35% and 50% of severe septicemic melioidosis cases are fatal within the first 48 hours of patient admission (6, 135). Typically such severe cases of melioidosis occur in individuals with one or more risk factors (37, 102, 136). As mentioned previously, *B. pseudomallei* is resistant to many broad spectrum antimicrobials commonly used for sepsis (3) this greatly necessitates prompt diagnosis to allow treatment with effective antimicrobials in severe cases.

1.2.5 Chronic infection.

Sadly, despite what appears to be effective treatment, recurrent infections with *B. pseudomallei* are very common among survivors (137). Approximately 6% of patients relapse within the first year, and 13% report relapses within 10 years post-initial infection (116, 138). Not surprisingly, higher rates of relapse were observed in individuals who were immune-suppressed (139, 140), those who did not follow treatment recommendations (139, 141), were prescribed shorter eradication phase treatments (142), or had more severe disease (137). More recently, lower rates of relapses have been reported, this is likely due to improved eradication phase treatments (140, 142), to be further discussed in **Chapter 2**.

It is not well understood how *B. pseudomallei* establishes latent infections in the host, but it may involve the production of biofilms, and is most likely characterized by reduction or arrest of cellular growth and replication. *B. pseudomallei* avoids the immune response and persists in a host by residing within professional phagocytic cells (143). Latent organisms can reside in the

host until a time of stress and immune-suppression, at which point the infection becomes reactivated and can result in severe disease with mortality rates comparable to acute infection (137, 144). While a study by Chaowagul *et al*. in 1993 did not observe increased antimicrobial resistance in organisms recovered from relapses (137), decreased antimicrobial susceptibilities were observed in several other studies (145, 146), suggesting *B. pseudomallei* can adapt based on selective pressure *in vivo*. A better understanding of how *B. pseudomallei* is capable of persisting in the host and evading both host immune defenses and treatment would likely lead to the development of efficacious vaccines, improved treatments for chronic and latent infections and better diagnostic methods.

1.2.6 Diagnostics.

Traditional microbiological methods of selective culture and biochemical analysis remain the gold standard for the diagnosis of melioidosis (135, 147). While this method is highly specific, it has low sensitivity and is time intensive, as it requires several days to determine the result (135, 147). There has been much research into the development of rapid detection assays with high specificity and sensitivity. Serologic assays including enzyme-linked immunosorbent assays (ELISA) and indirect hemagglutination assays (IHA) are valuable diagnostic assays, but unfortunately yield frequent false positives when used in endemic regions, as there are elevated antibody levels even in healthy populations. Nearly 100% of children over the age of 4 years in Thailand are seropositive (3, 117, 131, 148, 149).

There are many molecular detection techniques, including polymerase chain reaction (PCR) assays, developed and validated to detect *B. pseudomallei*, which have greatly improved specificity compared to immunological techniques, and improved sensitivity compared to culture (11, 150-154). However, the concentration of *B. pseudomallei* in clinical samples, especially

blood, is highly variable and typically too low for reliable detection (155, 156). Molecular detection would likely be improved with the use of other clinical specimens such as sputum, urine or pus (156). The development and, most importantly, the validation and implementation of modern molecular techniques for diagnosis will hopefully allow for earlier detection of melioidosis, resulting in prompt treatment with appropriate antibiotics, which is essential to reduce mortality (3).

1.2.7 Treatment.

B. pseudomallei is naturally resistant to many antimicrobials often used for the treatment of sepsis, including β-lactams, penicillins, aminoglycosides, macrolides, and polymyxin B (73). Timely diagnosis of melioidosis is imperative to ensure treatment with efficacious antimicrobials (3, 6), and has shown to greatly reduce mortality (117, 157).

At the 2010 U.S. Department of Health and Human Services *Burkholderia* workshop, subject matter experts from around the world gathered to develop recommendations for the treatment of *B. pseudomallei* and *B. mallei* infections (158). These guidelines were developed based on past experimentally determined drug regimen efficacies and current knowledge of antimicrobial susceptibilities of these agents, this will discussed further in **Chapter 2**. The current recommendations for melioidosis include two phases of treatment, the intensive and eradication phase (93, 94, 158, 159). The initial intensive phase consists of intravenous ceftazidime or a carbapenem (meropenem or imipenem) for a minimum of 10-14 days (158). During this phase of treatment it is expected that the clinical state of the patient will markedly improve, though this process may be very slow (158). The intensive phase is then followed by an extended eradication phase of oral co-trimoxazole or co-amoxiclav (a combination of amoxicillin and the β-lactamase inhibitor clavulanic acid) for 12-20 weeks (158, 159). Co-amoxiclav should

be used for the treatment in the case of pregnancy (158, 160). This stage of treatment was intended to reduce the risk of relapsing infection (141, 161).

1.2.8 Prevention and control.

The development of a vaccine has been long awaited and is greatly needed. However, despite intensive research and the investigation of multiple candidates, there is still no vaccine available to prevent melioidosis in humans (162). A number of vaccine candidates have been tested, but so far no vaccine has been able to fully protect against melioidosis in both its acute and chronic form. This work is underway and must include research to understand strain variation in endemic areas and identify conserved antigens for vaccine development, or the use of a live-attenuated or whole cell vaccine (162).

Since *B. pseudomallei* is a saprophytic organism that is primary transmitted from contact with contaminated soil and surface water in endemic areas, one of the primary initiatives should be education of the public. There could be reduced morbidity and mortality caused by melioidosis in endemic regions if the public was educated to understand the importance of immediately cleaning wounds, covering wounds, and wearing proper foot and hand protection that would reduce injuries and potential exposure when working outside (118, 126). Rice paddy workers, rubber plantation farmers, and other outdoor workers, especially those with pre-existing chronic health conditions, are the greatest population at risk. Education of these workers and their employers would likely reduce the incidence rate of melioidosis among this population. Those individuals with pre-existing health-related risk factors for melioidosis should also be educated, especially considering the more severe outcome of melioidosis that these patients can suffer (106). It also would be important to warn travelers to endemic regions, especially those with risk factors, of this disease. At a community level, heightened awareness of the potential for

melioidosis to be transmitted through contaminated water sources and through body fluids, though rarely reported, could reduce the number of cases (118). Large animal die-offs should be investigated; since *B. pseudomallei* can infect both human and animal populations they may be sentinel events indicating a risk of human exposure (118).

A recent study indicated that environmental management of the pathogen maybe possible. Inglis and Buller in 2001 found that in an area where *B. pseudomallei* was highly prevalent in soil and surface water, the reintroduction of native vegetation and remediation of chemical fertilizers helped to eradicate *B. pseudomallei* in the soil (12). There are currently studies ongoing to further investigate this potential in rice paddies and rubber plantations (118). Additionally, the reduced use of fertilizers and restoration of endogenous vegetation should be further investigated as a potential method for environmental remediation of *B. pseudomallei* (163).

Melioidosis is a notifiable disease in some regions of Australia, which has led to the development of a standard case definition (164). It is likely that melioidosis is emerging in other tropical and sub-tropical regions of the world where this infection is unrecognized (118). Improved diagnostic capacity in rural areas and other regions of the world would improve surveillance of the distribution and prevalence of *B. pseudomallei*. Better surveillance of melioidosis is necessary for control and prevention of this serious tropical disease.

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CHAPTER 2: Antimicrobials and Antibiotics

Antibiotics are naturally occurring antimicrobials that are produced by living organisms (bacteria, protozoa, fungi and plants) to eliminate their competition for space and resources (1). These compounds are toxic to living organisms at high concentrations (3). Typically organisms that produce antibiotics also possess mechanisms to protect themselves, this will be discussed further in **Section 2.2.4**. However, recent studies have suggested that the concentrations of antibiotics in the environment would likely be too low to negatively affect nearby competitors (3). Instead it is suggested that these compounds may serve as signaling molecules for communication between organisms, as these compounds modulate the expression of a wide array of genes (3).

Antimicrobial is a broad term used to describe synthetically made or modified antibiotics, but also includes naturally-occurring antibiotics as well. Often both terms are used interchangeably or antibiotic will be used to describe clinically relevant antimicrobials. Careful design of antimicrobial compounds by medicinal chemists for clinical use allows for optimization of the drugs. Prokaryotic cells are unique from eukaryotic cells in many ways, which would require too much time and space to discuss here. But many of these differences can be exploited for the design of antimicrobials that will specifically inhibit bacteria with limited toxicity to the person or animal being treated. Proteomic, genomic and advanced chemical modeling techniques are used to ensure that new compounds target highly conserved regions, often the active site, of essential compounds that are unique to prokaryotes. The high affinity to the drug target with minimal cross-reactivity is important, as any negative effect on eukaryotic cells could cause toxicity in a patient.

As marvels of modern medicine, antimicrobials have drastically altered our ability to combat bacterial infections that challenge our immune systems. Since their introduction and widespread use, there have been dramatic reductions in the morbidity and mortality caused by many infectious diseases.

2.1 Antimicrobial Classes

Typically antimicrobials can be grouped into classes based on several factors: route of administration, antimicrobial effect, chemical structure and the drug target. The route of administration is important in the clinical setting, as most antimicrobials are optimized for oral administration, which facilitates outpatient care; however, for more serious infections intravenous antimicrobials are preferred as there is generally greater bioavailability of the drug. Some antimicrobials are bactericidal, meaning that they cause lysis or cellular death, while others that are bacteriostatic only cause arrest of cellular growth and replication when testing *in vitro* (4). This is a traditional method of antimicrobial classification that is determined by performing a kill-curve and monitoring the number of viable bacteria over time in the presence of the antimicrobial. In the presence of a bacteriostatic compound there will be minimal change in the number of viable cells over time, while a bactericidal drug will leave only a very small percentage or no viable cells. However, this does not allow for discrimination between cell death and persistence of the organism in a viable but non-culturable state, which could indicate antimicrobial tolerance.

A common way to group antimicrobials is based on the essential cellular process that they inhibit, or their mode of action. These separate classes target cell wall synthesis, protein synthesis, genetic material (DNA and/or RNA) synthesis, and folate synthesis (5). These will be discussed and structural groups will be used as examples for each antimicrobial mechanism.

2.1.1 Cell wall biosynthesis inhibitors.

The integrity of the bacterial cell wall is essential for survival and proliferation. Additionally, the bacterial cell wall is unique from those of other organisms, making it an ideal drug target. Cell wall biosynthesis is targeted by the β-lactam family of antimicrobials, including penicillins (structurally similar or modified compounds derived from the first described antibiotic, a product of *Penicillium notatum*; *e.g*. amoxicillin and carbenicillin) (6) cephalosporins (*e.g* ceftazidime) (5), carbapenems (*e.g*. meropenem and imipenem) and the monobactams (5). The β-lactam drugs target transpeptidases and carboxypeptidases known as penicillin-binding proteins (7). These proteins were so named as they bind penicillin, however the name does not aid in describing the function of these compounds. There are a number of penicillin binding proteins that are essential for polymerization, crosslinking, and elongation of the cell, which are all necessary processes for peptidoglycan crosslinking and thus cellular division and survival.

In addition to the β-lactams, glycopeptides and lipoglycopeptides, such as vancomycin, also prevent cell wall synthesis by inhibiting the synthesis of peptidoglycan, transglycosylation and transpeptidation (5) These antimicrobials are very effective against Gram-positive bacteria, but have limited efficacy against Gram-negatives, as not all of these compounds can cross the outer membrane of Gram-negatives (5).

2.1.2 DNA and RNA biosynthesis inhibitors.

In addition to the cell wall synthesis, DNA synthesis is also essential for replication of the bacterium and maintenance of the genetic information. RNA synthesis is necessary for transcription of mRNA and other RNAs that are necessary for protein production and transcriptional regulation. This machinery is targeted by several structural classes. Quinolones

(*e.g.* nalidixic acid) and the derived fluoroquinolones (*e.g.* ciprofloxacin and norfloxacin) target DNA gyrase (8) and topoisomerase IV (9). These compounds prevent the supercoiling of DNA and inhibit the ligase activity of gyrase and topoisomerase IV but not the endonuclease activity. This ultimately results in double-stranded DNA breaks (10). Double-stranded DNA breaks initiate the SOS response in bacteria, which causes large genetic recombination events and mutagenic polymerase activity. Rifampicins inhibit the activity of DNA-directed RNA polymerase, thereby inhibiting transcription (5). Integrity of DNA and RNA synthesis is important, as is the translation of that genetic material into functional proteins and enzymes.

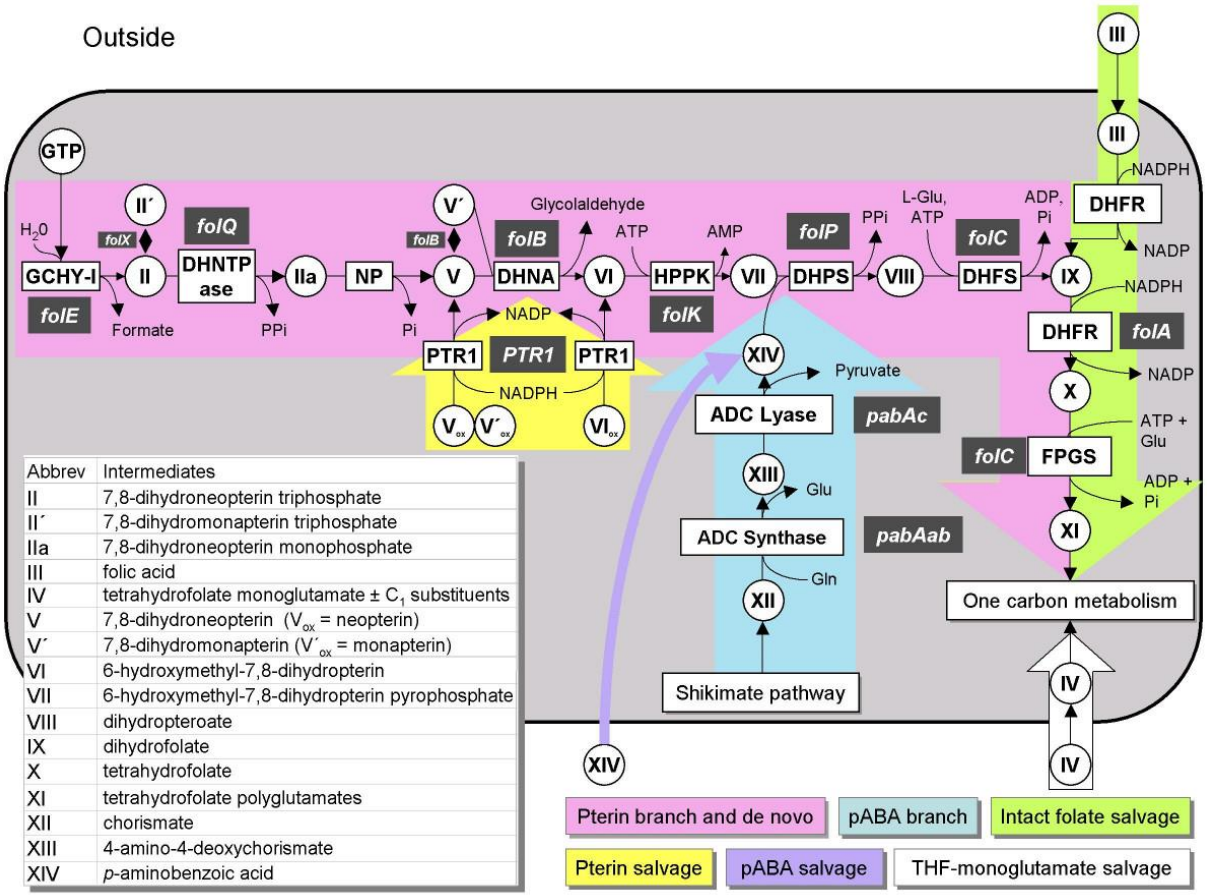
2.1.3 Protein biosynthesis inhibitors.

Protein synthesis is essential in bacteria for many cellular processes including maintenance of homeostasis, genetic material and the cell wall, as well as growth and replication. The bacterial ribosome is unique consisting of 30S and 50S subunits, whereas eukaryotic cells have ribosomes composed of 40S and 60S ribosomal subunits. This distinction provides unique targets for antimicrobials. The protein synthesis machinery is targeted by several classes of drugs. Aminoglycosides (*e.g.* gentamicin and kanamycin) specifically act on the 16S rRNA of the 30S subunit preventing ribosomal translocation and thus translation. Additionally aminoglycosides have been shown to impair the proofreading capacity of the ribosome causing errors and early termination (11, 12). Tetracyclines (*e.g.* tetracycline and doxycycline) also target the 30S ribosomal subunit, but prevent aminoacyl-tRNA from associating with the ribosome (5). Macrolides (*e.g.* erythromycin and cethromycin) on the other hand, bind the 50S subunit and in doing so prevent peptidyl transferase activity and ribosomal translocation (13, 14). Similarly, chloramphenicol prevents peptidyl transferase activity by binding the 23S rRNA of the 50S subunit (13).

2.1.4 Folate biosynthesis inhibitors.

Folate is essential in all living organisms (15), as it plays a critical role in the synthesis of purine nucleotides for DNA and RNA synthesis. Tetrahydrofolate (H_4folate) is a one-carbon donor in a number of biosynthetic pathways, including the synthesis of methionine, purines and thymine (15). Animals have the ability to scavenge folate from the environment and thus in animals folic acid (a B vitamin) is an essential nutrient, though there are also pterin and *para*-aminobenzoic acid (pABA) salvage pathways (2). In contrast, most pathogenic bacteria cannot utilize exogenous folates *in vitro* (16) or *in vivo* (17); instead prokaryotes must synthesize H_4folate starting with GTP processing by GTP cyclohydrolase I (GCHY-I) (2) **(Figure 2.1**). This pathway is complex and highly conserved in prokaryotes. Both the salvage and biosynthetic folate pathway have the same final steps, which includes processing of dihydrofolate (H_2folate) into H_4folate, though the dihydrofolate reductase (DHFR) enzyme responsible is structurally dissimilar in bacteria compared with that found in animals. Within the folate synthesis pathway there are a several steps that have been targeted by synthetic antimicrobials including the DHFR and the enzyme responsible for a previous step, dihydropteroate synthetase (DHPS) (2).

Sulfonamides were actually the first antimicrobial drugs used. Prontosil was developed by Bayer and was used clinically in the early 1930's. Since that time there have been many sulfonamides (*e.g.* sulfamethoxoazole) developed and used for the inhibition of the DHPS (**Figure 2.1**). The DHPS enzyme catalyzes the synthesis of 7,8-dihdropteroate from 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (DHPPP) using pABA to condense DHPPP (15). Sulfonamides are structurally similar to pABA and competitively bind DHPS preventing its



Abbrev	Intermediates
II	7,8-dihydroneopterin triphosphate
II´	7,8-dihydromonapterin triphosphate
IIa	7,8-dihydroneopterin monophosphate
III	folic acid
IV	tetrahydrofolate monoglutamate ± C_1 substituents
V	7,8-dihydroneopterin (V_{ox} = neopterin)
V´	7,8-dihydromonapterin ($V´_{ox}$ = monapterin)
VI	6-hydroxymethyl-7,8-dihydropterin
VII	6-hydroxymethyl-7,8-dihydropterin pyrophosphate
VIII	dihydropteroate
IX	dihydrofolate
X	tetrahydrofolate
XI	tetrahydrofolate polyglutamates
XII	chorismate
XIII	4-amino-4-deoxychorismate
XIV	*p*-aminobenzoic acid

Figure 2.1 Folate biosynthesis and salvage pathways. This illustration shows the bacterial folate synthetic pathway (pink) and the *para*-aminobenzoic acid (pABA) synthesis pathway (blue). Some organisms possess folate salvage pathways (green) while other salvage necessary precursors including pterin (yellow) and pABA (purple). **Source** (2).

activity. Benzylpyrimides (*e.g.* trimethoprim) inhibit the activity of dihydrofolate reductase (DHFR)further down the synthesis pathway (**Figure 2.1**). They were discovered by Hitchings *et al.* in the late 1940's (18, 19). The DHFR enzyme is necessary for the synthesis of H_4folate from H_2folate. Benzylpyrimidies are similar in structure to a portion of the H_2folate and will competitively bind the DHFR enzyme inhibiting its activity. Trimethoprim, and other diaminopyrimidines are specific inhibitors of bacterial DHFRs, with nearly 10^4 times higher binding affinity due to structural differences between bacterial and mammalian DHFRs (20-22). In 2000, Quinlavin *et al.* reinvestigated the mechanisms of action of trimethoprim (20). They

observed that in trimethoprim treated cells H_4folate levels diminished initially, then H_2folate levels declined as the H_2folate was catabolized or oxidized into more stable precursors, including *para*-aminobenzoyl-glutamate (pABGlu) and folic acid (20). This suggests that trimethoprim inhibits both DHFR and the conversion of folic acid to H_2folate (20). Trimethoprim, 2,4-diamino-5-(3,4,5-trimethoxybenzyl) pyrimidine is an synthetic antimicrobial and is the preferred inhibitor of its class because it is broad spectrum, highly specific for the bacterial enzyme, and exhibits good pharmacokinetic and pharmacodynamic properties for use as a therapeutic (23). Trimethoprim was first used in the early 1960's in the United Kingdom and since then has become a commonly used antimicrobial agent (24), though it is now often prescribed in combination with sulfonamides, such as sulfamethoxazole.

Sulfonamides and benzylpyrimides are commonly used in combination (*e.g.* trimethoprim and sulfamethoxazole), as studies have shown significant increases in the efficacy of treatment. This combination approach is recommended for clinical use when there is concern about the emergence of antimicrobial resistance, such as when lengthy treatment regimens are required, including that of tuberculosis (25) or melioidosis (26). Combination therapy is also encouraged when there is an observed synergist effect, meaning the antimicrobial effect of the combination is greater than the sum of the effect of each individual drug (25). Additionally combination therapy is often prescribed when the etiological agent of a critically ill patient is still unknown and to improve the antimicrobial spectrum (25).

2.2 Antimicrobial Resistance

Recently the threat of "super bacteria" has become a concern in the media primarily due to the emergence of several high profile, community-acquired, drug resistant organisms, including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant

Staphylococcus aureus (VRSA), vancomycin-resistant *Enterococcus spp.* (VRE), multidrug-resistant *Mycobacterium tuberculosis* (MDR TB), extensively drug resistant *Mycobacterium tuberculosis* (XDR TB), and most recently carbapenem-resistant *Enterobacteriacea* (CRE). Overall there has been a very sharp increase in the frequency of multi-drug resistance in Gram-negative organisms (27). In 2001 the World Health Organization (WHO) published a set of recommendations on the efficacious use of antimicrobials to slow the emergence of resistance (28). Their recommendations include improved **1)** surveillance of antimicrobial resistant strains (to monitor the distribution of resistance and allow for treatment with appropriate antimicrobials), **2)** infection control and sanitation in the clinical setting (to reduce nosocomial infections), **3)** antibiotic use in animals (to reduce the environmental prevalence of antibiotics and prevent the emergence of antibiotic resistance in this setting) and **4)** public awareness (to improve patient compliance with drug regimens and education to enhance understanding on when antimicrobial prescription is appropriate). While steps have been made forward in all of these areas, there is much work remaining to be done.

The widespread distribution of antimicrobial resistant bacteria has made many of the first discovered antibiotics obsolete (29). This has resulted in increased use and reliance on synthetic antimicrobials, for which there are likely no naturally-occurring enzymes that would inactivate these drugs. However, antimicrobial resistance in quick to emerge even with synthetically constructed or modified drugs (**Figure 2.2**).

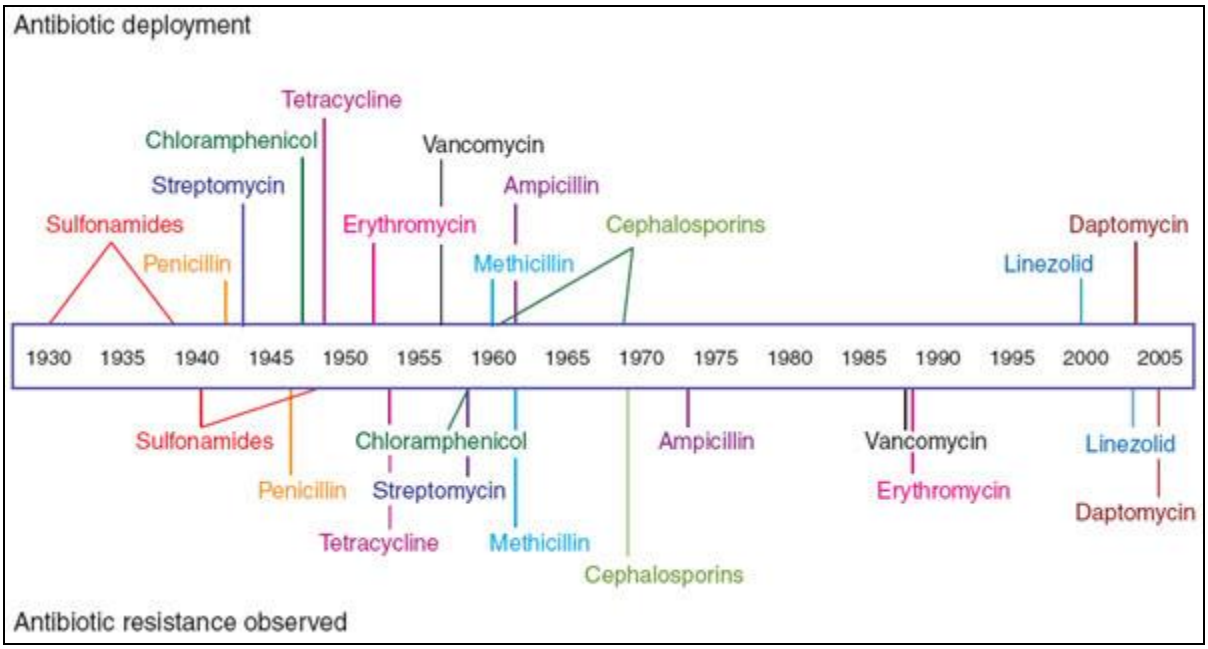


Figure 2.2 Timeline of antimicrobial drug emergence. This illustration shows the time between the introduction of novel antimicrobials into clinical use and the emergence of resistant strains. **Source** (30).

The underlying causes of antimicrobial resistance to novel therapeutics are debated in the literature. Many have argued that increased exposure of a microorganism to an antimicrobial due to recurrent infections, very long treatments, patient non-compliance, inappropriate drug choices or environmental contamination will cause frequent mutations leading to the emergence of resistance (31, 32). Other studies have shown that spontaneous genetic mutations lead to resistance just as readily as with exposure to the antimicrobial (33). Cirz *et al.* recently demonstrated that in strains of *E. coli* with a mutant LexA protein incapable of proteolysis, there was a 250-fold decrease in the emergence of ciprofloxacin resistance (29). Their study suggests that activation of the SOS response in *E. coli*, which among other things, leads to the expression of a highly mutagenic DNA polymerase, increased the rate at which ciprofloxacin resistance arose, though this finding may largely be a result of the fluoroquinolone propensity to cause double-stranded DNA breaks, which trigger an SOS response.

2.2.1 Antimicrobial susceptibility testing.

Antimicrobials are commonly tested for their anti-bacterial activity *in vitro*, this is known as antimicrobial susceptibility testing or minimal inhibitory concentration (MIC) testing. The MIC of a drug can be determined by dilution, microdilution, disk diffusion or most recently by Etest® technique. In the United States, the Clinical and Laboratory Standards Institute (CLSI) publishes guidelines for how MIC testing should be performed for many pathogens (34). The MIC is used to predict the likelihood of successful treatment with the recommended dosage of the antimicrobial tested against the organism of interest. Based on the MIC value for a given genus and species, the tested isolate is categorized as "susceptible", "intermediate" or "resistant". *In vivo,* these groupings are related to the range of drug concentration that can be achieved without significant toxicity, called the therapeutic window. This is largely dependent on the pharmacokinetic and pharmacodynamic properties of the drug. If the MIC is shown to fall within the therapeutic window, the organism is "susceptible" and it is expected that treatment with the tested antimicrobial will be effective (25).

However, it is complicated to relate the MIC *in vitro* to the true activity of the drug *in vivo*. In the laboratory setting, bacteria are given all the nutritional components they need and are tested during active growth and replication (35). Many antimicrobial drugs, such as gentamicin and rifampin are only inhibitory during active growth of the organism. During latency it can then be assumed that organisms such as *B. pseudomallei* would be tolerant to all of the above-mentioned antibiotics as well as others, though this would not be evident by *in vitro* MIC testing. Other mechanisms such as biofilm production and stress-induced responses may impair the efficacy of antimicrobial therapy, but these factors may be considered drug tolerance rather than resistance (36).

2.2.2 Mechanisms of drug resistance.

Bacteria have versatile mechanisms that protect them from the toxic effects of antimicrobials. Antimicrobial resistance can be intrinsic, meaning that reduced drug susceptibility is a function of the inherent physiological properties of the bacterium (37), or acquired in populations that were previously susceptible, usually by horizontal gene transfer or by random mutation(s). Acquired resistance mechanisms are especially concerning as they can rapidly disseminate across bacterial populations, potentially making the specific drug ineffective against many pathogens.

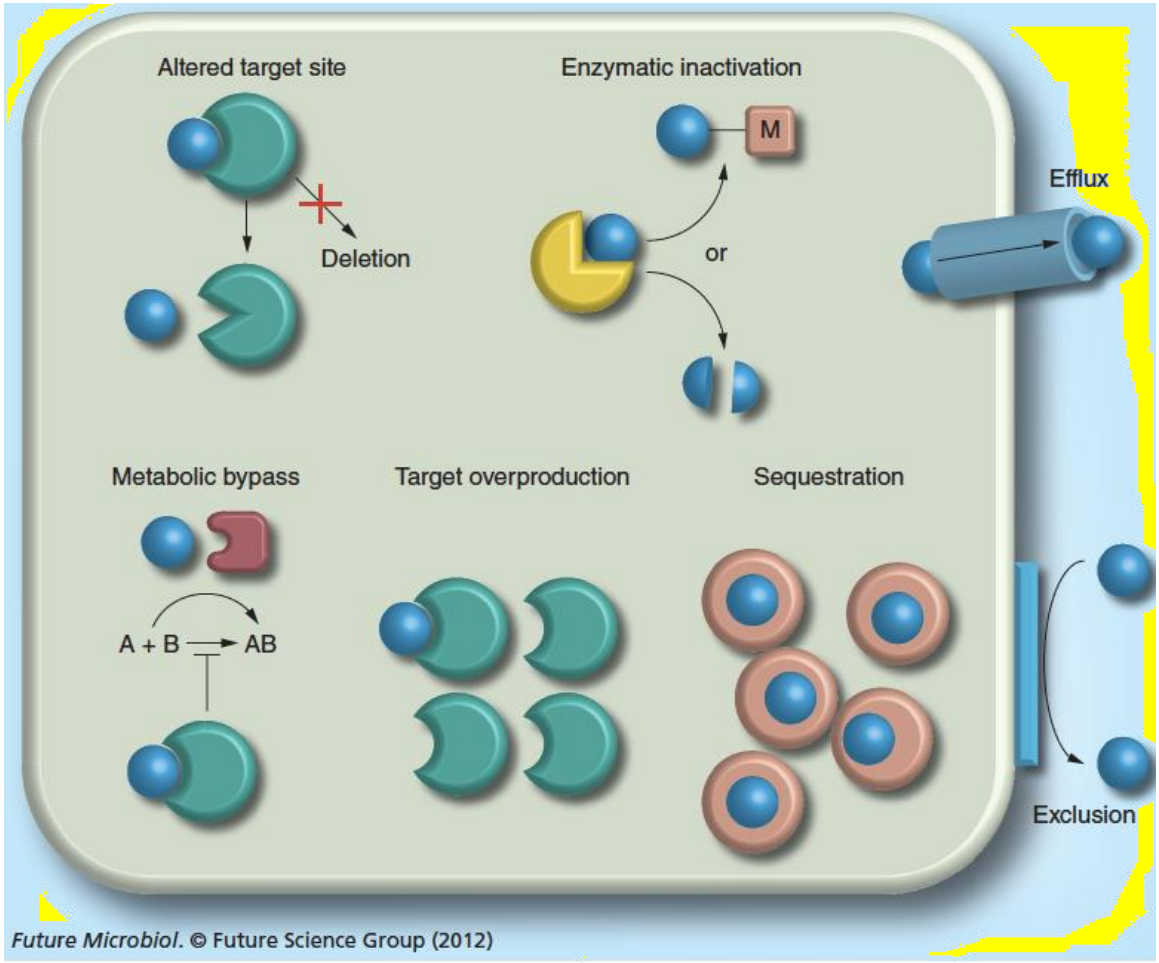


Figure 2.3 Illustration depicting known mechanisms of antimicrobial resistance. This illustration shows the major mechanisms of antimicrobial resistance in bacteria, including reduced drug accumulation (efflux and exclusion), alteration of the drug target or targeted pathway, overexpression of the target, and inactivation or sequestration of the drug. **Source** (36).

Antimicrobial resistance is generally a function of one or several of the following, which will be discussed in more detail below: modification of the drug target, inactivation of the drug, active efflux, changes to the targeted pathway(s), overproduction of the target, sequestration of the drug, reduced drug permeability or a combination of mechanisms (**Figure 2.3)** (36, 38).

2.2.3 Drug target modification.

Modification of the antimicrobial drug target can prevent interactions of the antimicrobial with the target. However, it is important that these modifications maintain function of the drug target, as many antimicrobial targets are enzymes involved in essential cell processes. Mutations that cause major changes in an enzyme structure might allow the bacteria to be resistant to the drug; however the cell will likely be negatively affected, *i.e*. the mutation will result in a fitness cost. Typically drug target modifications are acquired as a result of accumulated chromosomal mutations (39, 40) and cause slight distortions in structure. These mutations may occur spontaneously or by selective pressure. An example is the modification of the trimethoprim drug target, DHFR. This enzyme is required for folate synthesis, an essential process in bacteria (**Section 2.1.4**).

There are currently over 20 different trimethoprim resistant DHFRs known to be transferable between organisms (42, 43). Some of these DHFRs have originated from organisms other than bacteria, such as the *Plasmodium falciparum* parasite (44), while others originate from various bacterial species, including *Bacteriodes spp*., *Clostridium spp*. *Neisseria spp.* and *Mycobacterium catarrhalis* (24). These trimethoprim resistant DHFRs are commonly used in molecular cloning as selectable markers for the maintenance of plasmids and other genetic constructs.

Other than mutations to the structure of the drug target itself, small molecules can competitively bind to the target and modify it to prevent or disrupt the association of the target with the antimicrobial or absence of the drug target. This mechanism confers resistance to tetracycline, where Tet(O) binds the post-translocational ribosome and causes conformational changes to the ribosome allowing for the dissociation of tetracycline, ultimately resuming protein synthesis (45). Deletion of the target is a rare but very effective resistance mechanism. Chromosomal deletions of large regions containing the penicillin-binding protein 3 (PBP 3) have been observed in several clinical *B. pseudomallei* isolates resistant to ceftazidime (41). The absence of this gene, while conferring resistance, affected cellular division and resulted in long filamentous growth and reduced growth rates (41). This mechanism of ceftazidime resistance will be discussed further in **Section 2.3.2**.

2.2.4 Enzymatic drug inactivation or modification.

Naturally occurring antibiotics are produced by many genera of bacteria. Interestingly, the organisms that produce antimicrobials would also be negatively affected by the compounds. However, the production of such antimicrobials is often coupled with expression of mechanisms for protection. These defenses tend to cause inactivation or destruction the molecular structure of the drug. A well-known example is the hydrolysis of the β-lactam ring by β-lactamase enzymes. There are a number of different β-lactamases that target various drugs including penicillins and cephalosporins, and are generally classified by the drugs they inhibit (*e.g.* penicillinases, cephalosporinases and oxacillinases) (46). The first identified penicillinase was described in 1940 in *Escherichia coli* prior to the use of penicillin clinically (47). Most β-lactamases have broad activity but do not cause inactivation of the carbapenems (*e.g.* meropenem and imipenem). However, a number of carbapenemases have recently been identified in Gram-negative bacteria

(48) and these resistance determinants are spreading rapidly in the clinical setting (49-51). Efforts are ongoing to prevent further dissemination of these resistance genes and preserve the efficacy of carbapenems, which are often used for the treatment of severe or multi-drug resistant infections (49). In *B. pseudomallei* the chromosomally-encoded PenA β-lactamase is linked with resistance to co-amoxiclav (106). The PenA β-lactamase will be discussed further in **Section 2.3.**

In addition to enzymes that degrade the antimicrobial, in some cases enzymes alter the drug by the addition of chemical groups. For example acetyltransferase (52) and phosphotransferase (53) will add acetyl or phosphate groups, respectively, to chloramphenicol causing a loss of its antimicrobial properties. Similarly, plasmid-encoded adenylyltransferases can modify aminoglycosides, such as streptomycin and spectinomycin (54).

2.2.5 Drug target pathway modification.

Modification of the essential pathway targeted by an antimicrobial is complicated and typically involves horizontal gene transfer of a replacement enzyme for the susceptible drug target. There is much un-cited mention of metabolic bypass resulting in trimethoprim and sulfamethoxazole resistance in the literature. However, recently the presence of a *folM* gene in *E. coli* was shown to complement strains lacking dihydrofolate reductase (55). It is feasible that such a gene with limited structural homology, but similar function, could replace an antimicrobial inhibited enzyme (FolM will be further discussed in **Chapters 5** & **6)**. Additionally one could speculate that replacement of the entire pathway would result in a resistant phenotype. For example, an isolate that acquires the eukaryotic genes necessary for scavenging folic acid from the environment and subsequent processing of the compound into the essential H_4folate would be resistant to sulfamethoxazole and potentially trimethoprim as well.

2.2.6 Drug target overproduction.

Antimicrobials are designed to have high affinity for their respective targets, which provides effective inhibition. However, mutations that cause increased expression of the drug target can result in resistance to the antimicrobial. This resistance is a function of an imbalance caused by a high concentration of drug target overwhelming the inhibitory capacity of the available antimicrobial. Theoretically this problem could be alleviated by further increasing the concentration of the antimicrobial; however this is not typically feasible as it could cause toxicity to the patient. Similarly, the overproduction of drug target substrates would improve competitive binding of the substrate over the structurally similar antimicrobial. An example of this was described in the 1940's, where the overproduction of pABA was found to result in sulfonamide resistance in clinical *Staphylococcus aureus* isolates (56, 57). Sulfonamides, as described in **Section 2.1.4**, are structural analogs of pABA and competitively bind to DHPS causing inhibition of the enzyme function. However, the reported 100-fold overproduction of pABA improved the frequency at which DHPS bound pABA instead of the sulfonamide. The overproduction of drug target and substrates may not be possible for resistance to all antimicrobials, as often overproduction can slow growth and replication in addition to having toxic effects on the cells.

2.2.7 Drug sequestration.

Among rare antimicrobial resistance mechanisms, proteins that selectively bind antimicrobials preventing their association with the intended drug target have been noted. An example of this was described in *E. coli* strains that were resistant to coumermycin A_1 (58). The authors found that overexpression of the wild-type *gyrB* gene resulted in lower available drug concentrations. This was not observed in strains expressing a truncated version of the *gyrB* gene.

Interestingly, GyrB is the drug target for coumermycin A_1, and its overexpression is thought to allow binding between the 2 compounds, resulting in sequestration of the drug and thus allowing other functioning DNA gyrase components to remain unaffected (58). Though this particular example could potentially be considered target overproduction as well, target overproduction would not necessarily allow for sequestration of the drug. Sequestration would require high affinity binding and sufficient levels to cause a significant decrease of the unbound available antimicrobial.

2.2.8 Reduced drug permeability.

Reduced cell permeability is primarily a function of the outer membrane found in Gram-negative organisms (59, 60). Some bacteria have significantly reduced outer membrane permeability compared to others (*e.g. B. cepacia* membrane permeability is 89% lower than that of *E. coli* (61)). The cell permeability is largely dependent on the structure and chemical modifications to the lipid A component of the lipopolysaccharide, found in the outer membrane of Gram-negative bacteria (62) and protein channels, porins, in the outer membrane (63). Porins, such as OmpD, are necessary for the transport of solutes across the outer membrane (59, 64). Porins allow for size exclusion of many large compounds, including some antimicrobials (65). Reduced expression of the outer membrane porins can further inhibit the influx of antimicrobials (66). The reduced rate of influx decreases the accumulation of the drug in the bacterial cell, and thus its antimicrobial properties. Siritapetawee *et al.* demonstrated this in 2004 as they determined the permeability rates of a number of antimicrobials in *Burkholderia spp.* (65). Small molecule diffusion can be reduced by 5 to 100 fold (67, 68), thereby reducing the uptake of hydrophobic agents including β-lactams and rifamycin (69).

Biofilms are also known to reduce the inhibitory effect of antimicrobials against bacteria, either directly by interactions with the drug or indirectly by alterations to the cell membrane permeability or general increased fitness of the bacteria. The genes stimulated by quorum sensing include those necessary for the production of biofilms. Biofilms are multicellular communities that are attached to surfaces (such as bone, tissue, catheters, etc.) and consist of bacteria embedded in a biopolymeric extracellular matrix (70). Bacteria within biofilms are overall more resistant to antimicrobials, likely due to the inability of the drug to readily diffuse through the extracellular matrix. In some cases MIC changes of 1,000 fold were observed (71). Biofilm production within the host organism impairs the efficacy of clinical treatment and may provide an explanation for chronic infections.

2.2.9 Drug efflux.

Efflux pumps are membrane associated protein complexes that actively mediate the extrusion of potentially harmful substrates, such as heavy metals, toxins and antimicrobials, from the bacterial cytoplasm or periplasmic space to the exterior of the cell (37). This efflux is an energy-dependent process that is driven by either the proton motive force (72) or ATP hydrolysis (73). There are five major classes of bacterial efflux pumps: ATP-binding cassette (ABC) family (74), the major facilitator superfamily (MFS) (75), multidrug and toxic compound extrusion (MATE) family (76), the resistance-nodulation-division (RND) family (77) and the small multidrug resistance (SMR) family (**Figure 2.4**) (78).

While other efflux pumps are functional as a single component, RND efflux pumps are unique in that they are tripartite. The RND transporter protein is located in the cytoplasmic membrane and recognizes specific substrates for extrusion (80). The outer membrane protein (OMP) consists of β-barrels that anchor the protein to the outer membrane (79). Finally the

membrane fusion protein (MFP) interacts with both the RND transporter and the OMP either to hold the complex together or bridge the gap between these two components (81-83). Typically the transporter, OMP and MFP are expressed from the same operon, which is tightly regulated by local and/or global regulators (79). Overexpression of efflux pumps can be triggered by effectors including the drug itself or by mutations to regulators or promoters (84-87). Interestingly, in some organisms there is a negative correlation between expression of RND efflux pumps and outer membrane porins, such as with increased AcrAB-TolC production and

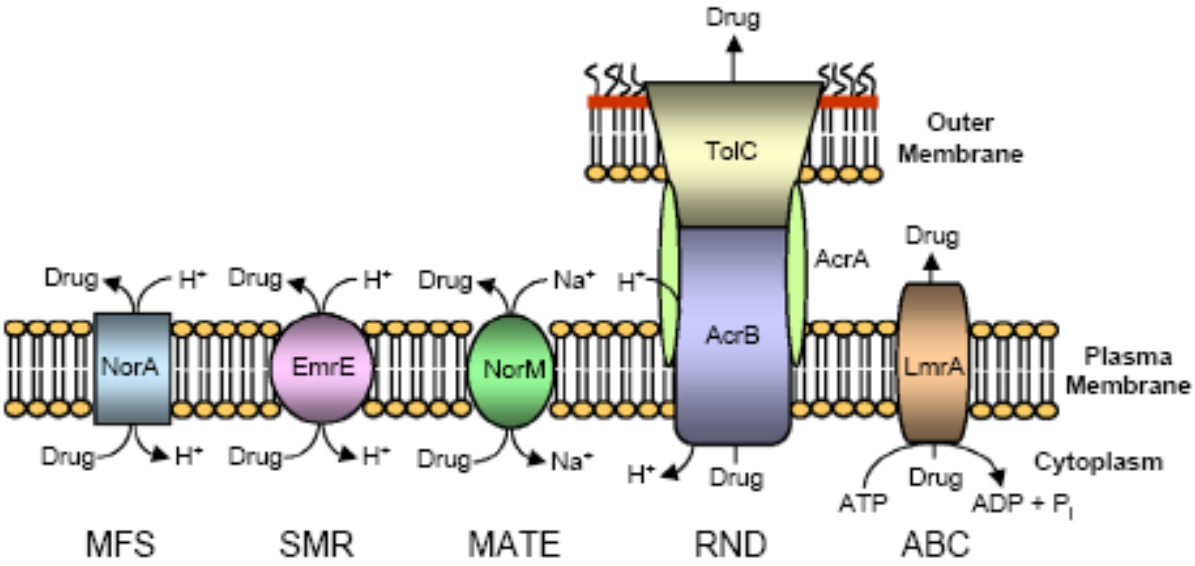


Figure 2.4 Illustration depicting the various superfamilies of efflux pumps. **Source** (79). **Abbreviations:** MFS, major facilitator superfamily; SMR, small multidrug resistance superfamily; MATE, multidrug and toxic compound extrusion superfamily; RND, resistance-nodulation-cell division superfamily; ABC, ATP-binding cassette superfamily.

decreased OmpF levels in *Escherichia coli* (79). This combination of increased efflux and decreased influx has a strong synergist effect on the antimicrobial susceptibility of the bacterium. The combination of reduced cell permeability and efflux has been implicated for the extensive intrinsic resistance of *B. pseudomallei* to many diverse classes of antimicrobials (36, 88). This synergy is demonstrated by the differential antimicrobial susceptibility results observed from

expression of the BpeEF-OprC efflux pump in various gram-negative organisms. When expressed in *B. pseudomallei* this efflux pump caused significantly higher reductions in chloramphenicol and trimethoprim susceptibilities compared to that observed in *Pseudomonas aeruginosa* (36, 89).

The natural physiological role of efflux pumps in bacterial populations is not well understood (90). Several functions other than antimicrobial efflux were discussed by Piddock and Poole in their recent reviews of the subject. These included response to cellular stress, response to environmental stress, and the dispersal of virulence factors (91, 92). Interestingly, soil dwelling bacteria on average have many more efflux pumps than those from other niches (93). It may be a function of efflux pumps to protect the cell from molecules produced by competing bacteria and plants (94).

2.3 Clinically Relevant Antimicrobial Resistance in *B. pseudomallei*

2.3.1 Intrinsic resistance.

Burkholderia pseudomallei, as described in **Chapter 1**, is a Gram-negative pathogen responsible for the severe disease melioidosis. The antimicrobial therapy for melioidosis is difficult due to the intrinsic resistance of *B. pseudomallei* to various antimicrobial classes including aminoglycosides, macrolides, cephalosporins, penicillins, and tetracyclines, which will be discussed briefly.

The primary mechanism of resistance to penicillins (including amoxicillin and carbenicillin) and 1st and 2nd generation cephalosporins is the enzymatic inactivation of the antimicrobials by a β-lactamase PenA (33, 95-97).

Aminoglycosides, including gentamicin, kanamycin, spectinomycin, streptomycin and tobramycin, are very effectively expelled from *B. pseudomallei* by the AmrAB-OprA RND

efflux pump and to a lesser extent by the BpeAB-OprB RND efflux pump (98, 99). Recent studies have identified and characterized strains naturally lacking the AmrAB-OprA efflux pump which are susceptible to these antimicrobials (98, 100). Similarly macrolides, including clarithromycin and erythromycin, are good substrates for the AmrAB-OprA efflux pump and are also effluxed at low levels by BpeAB-OprB (36). The BpeAB-OprB efflux pump expels quinolones including rifampicin (101).

Intrinsic resistance to tetracyclines occurs as a result of efflux. Doxycycline has been shown to be a substrate of the AmrAB-OprA, BpeAB-OprB and BpeEF-OprC efflux pumps (36, 89, 101). Finally, *B. pseudomallei* is naturally insensitive to polymyxin B, likely due to the lipopolysaccharide component of the outer membrane (36, 102). Because *B. pseudomallei* is naturally resistant to so many classes of antimicrobials there are currently very few choices, and the development of an efficacious treatment regimen is complicated. The current recommendations for treatment of melioidosis are largely the result of which antimicrobials *B. pseudomallei* is not intrinsically resistant to and provide the greatest success.

2.3.2 Acquired resistance.

Acquired resistance in *B. pseudomallei* is relatively rare, though resistant isolates have been identified clinically following the acute and chronic phases of treatment. The initial treatment for melioidosis consists of intravenous ceftazidime (a 3rd generation cephalosporin) for a minimum of 10-14 days (26). The use of ceftazidime as an initial therapeutic for melioidosis has improved mortality rates drastically from 80% to 43% (103). Currently, resistance to ceftazidime is rare in *B. pseudomallei*, however several clinical cases have been reported in response to treatment with ceftazidime (41, 97, 104). In a recent study characterizing several ceftazidime resistant clinical isolates, deletion of *BPSS1219*, encoding PBP 3 the ceftazidime

drug target was observed (41). Large chromosomal deletions were noted in the clinical strains, which included three penicillin-binding proteins. However, the testing of targeted deletions of each gene indicated that *BPSS1219* deletion was responsible for the observed high-level resistance. This gene also appears to be important for cellular division, as isolates lacking this gene require glycerol in the media and are very slow growing, and interestingly, isolates lacking *BPSS1219* were found to have increased susceptibility to imipenem (41), a secondary drug choice for the intensive phase treatment.

In other clinical isolates, resistance to ceftazidime was found to be a result of either mutation to the PenA β-lactamase (*i.e.* C69Y or P167S) (97, 105) or due to overexpression of PenA (105). In addition to causing ceftazidime resistance, PenA is also linked with resistance to amoxicillin and clavulanic acid (co-amoxiclav). The PenA β-lactamase is effective at hydrolyzing the β-lactam ring of amoxicillin, and mutations to the PenA β-lactamase, including S72F, were shown to cause significant decreases in co-amoxiclav susceptibilities (97, 106). Co-amoxiclav is a secondary choice for the eradication phase of melioidosis treatment and is recommended for use in pregnant women (26). This combination therapy is synergistic as the amoxicillin β-lactam is protected by the β-lactamase inhibitor clavulanic acid. However, resistance to co-amoxiclav was reported at 1.5% of clinical *B. pseudomallei* isolates in 1998 (107).

In such cases where ceftazidime cannot be used, the alternatives for the intensive phase of treatment are the carbapenems meropenem and imipenem (26). Meropenem is very effective for the treatment of melioidosis, though is much more expensive than ceftazidime and has lower stability at ambient temperature (26). Imipenem is also highly effective against *B. pseudomallei* but is currently not recommended due to higher frequency of severe side effects (26). To my

knowledge there have been no reports of resistance to these carbapenems in *B. pseudomallei*. But interestingly, our lab is currently investigating a potential carbapenemase present in *Burkholderia ubonensis* (L. Randall and H. P. Schweizer, unpublished work).

The eradication phase of melioidosis treatment typically consists of at least 12-20 weeks of co-trimoxazole (26). Co-trimoxazole, a combination of trimethoprim and sulfamethoxazole, provides a sequential blockade of the folate synthesis pathway. Combination therapies are often used because of their synergist effect and the reduced risk of developing resistance. Despite this, there have been reported co-trimoxazole resistance frequencies of 2.5% in Australia (108) and 13-16% in Thailand (109, 110). These resistance frequencies may be over-estimated due to the inherent technical difficulties associated with sulfonamide susceptibility testing. There has been very little work done to determine the causative mechanisms of this resistance. In other organisms, drug target mutations to the DHFR and DHPS are known to confer resistance to trimethoprim and sulfamethoxazole, respectively (42 1276, 111), however no such mutations have been documented in *B. pseudomallei*. A study characterizing the BpeEF-OprC efflux pump from *B. pseudomallei* showed that expression of this efflux pump in *Pseudomonas aeruginosa* resulted in efflux of trimethoprim and chloramphenicol (89), and is also known to efflux tetracyclines, including doxycycline (T. Mima and H.P. Schweizer, unpublished results). Doxycycline was previously used in combination with other drugs for the treatment of melioidosis, but is no longer recommended (26). Chloramphenicol is occasionally used in the case of neurologic melioidosis infection as it can cross the blood-brain barrier, but was shown to be ineffective for oral treatment of melioidosis (112). This BpeEF-OprC efflux pump is controlled by the BpeT lysR-type transcriptional regulator. Point mutations to BpeT have been shown to cause increased expression of the efflux pump (T. Mima and H.P. Schweizer,

unpublished results), as did the interruption of *bpeT* by a recombination event in a clinical *B. pseudomallei* isolate with decreased chloramphenicol and trimethoprim susceptibilities (104).

2.4 Dissertation Aims and Preview of Chapters

This introduction has provided an overview of our basic understanding of the *Burkholderia pseudomallei* bacterium and problems encountered clinically in the treatment of melioidosis. *B. pseudomallei* is resistant to many antimicrobials, however no work has been done to date has to elucidate the mechanisms of trimethoprim, sulfamethoxazole and co-trimoxazole resistance. Improved understanding of the underlying mechanisms leading to co-trimoxazole resistance would allow for appropriate antimicrobial usage to prevent the emergence of resistance and allow surveillance and monitoring of the resistance determinants. This information would also be of value to those working to improve the current treatment strategies and develop enhanced and novel therapeutics for the treatment of melioidosis. In this context, the dissertation describes experiments and results of 4 major research projects to investigate *B. pseudomallei* resistance mechanisms. The work that will be described focuses on the investigation and characterization of folate pathway inhibitor resistance in *B. pseudomallei* clinical and environmental isolates, as well as laboratory-induced mutants, as it particularly relates to the molecular mechanisms of trimethoprim and sulfamethoxazole resistance.

The overarching questions to be addressed by this work are:

- What resistance mechanisms are responsible for trimethoprim and sulfamethoxazole resistance in clinical isolates?
- Are environmental isolates also resistant to trimethoprim and sulfamethoxazole, and if so are the same resistance mechanisms responsible?

- Are the mechanisms of trimethoprim and sulfamethoxazole resistance the same in laboratory induced mutants compared to clinical and environmental isolates?
- Is co-trimoxazole resistance simply the result of a combination of trimethoprim and sulfamethoxazole resistance mechanisms?
- What mechanism(s) are responsible for co-trimoxazole clinical and environmental isolates, and are these different than those in laboratory induced mutants?

These questions lead to the hypothesis that the definition and characterization of trimethoprim and sulfamethoxazole resistance mechanisms will allow for the rapid identification of these resistant determinants in clinical isolates and maliciously engineered strains, which will enable timely administration of appropriate treatments and prophylaxis.

To test this hypothesis, the following specific aims were pursued:

I. Identify and characterize trimethoprim resistance mechanisms in clinical and environmental *Burkholderia pseudomallei* isolates in **Chapter 3**: "The BpeEF-OprC Efflux Pump is Responsible for Widespread Trimethoprim Resistance in Clinical and Environmental *Burkholderia pseudomallei* Isolates".

II. Generate and characterize trimethoprim and sulfamethoxazole resistant laboratory induced mutants of Bp82 in **Chapter 4**: "Mechanisms Responsible for Acquired Trimethoprim and Sulfamethoxazole Resistance in *Burkholderia pseudomallei*".

III. Generate and characterize co-trimoxazole resistant laboratory induced mutants of Bp82 in **Chapter 5**: "Investigation of Adaptive Co-Trimoxazole Resistance Mechanisms in *Burkholderia pseudomallei*".

IV. Identify and characterize co-trimoxazole resistance mechanisms in clinical and environmental *Burkholderia pseudomallei* isolates in **Chapter 6**: "The BpeEF-OprC Efflux Pump is a Major Contributor to Co-Trimoxazole Resistance in *Burkholderia pseudomallei* Clinical Isolates".

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CHAPTER 3: The BpeEF-OprC Efflux Pump is Responsible for Widespread Trimethoprim Resistance in Clinical and Environmental *Burkholderia pseudomallei* Isolates[*]

The work presented in this chapter and published paper describes the frequency of trimethoprim resistance in a collection of clinical and environmental *B. pseudomallei* isolates from endemic regions. We identified BpeEF-OprC efflux pump expression as the essential determinant for trimethoprim resistance in the clinical and environmental isolates tested.

3.1 Summary

Trimethoprim-sulfamethoxazole (co-trimoxazole) is the primary drug used for oral eradication therapy of *Burkholderia pseudomallei* infections (melioidosis). Here, we demonstrate that trimethoprim resistance is widespread in clinical and environmental isolates from northeast Thailand and northern Australia. This resistance was shown to be due to BpeEF-OprC efflux pump expression. No dihydrofolate reductase target mutations were involved although frequent insertion of IS*Bma2* was noted within the putative *folA* transcriptional terminator. All isolates tested remained susceptible to trimethoprim-sulfamethoxazole, suggesting that resistance to trimethoprim alone in these strains probably does not affect the efficacy of co-trimoxazole therapy.

[*] The majority of this work is presented in: **Nicole L. Podnecky**, Vanaporn Wuthiekanun, Sharon J. Peacock, & Herbert P. Schweizer. 2013. *Antimicrobial Agents & Chemotherapy.* 57(9):4381-4386.

3.2 Introduction

Burkholderia pseudomallei is a saprophytic Gram-negative bacterium mostly found in soil and water in many subtropical and tropical regions of the world including northern Australia and southeast Asia (1, 2). *B. pseudomallei* is the etiologic agent of the multifacted disease melioidosis (2-6). Treatment of melioidosis is complicated by the intrinsic resistance of *B. pseudomallei* to many classes of antimicrobials (7, 8). The current recommended therapy includes an initial intensive phase followed by a lengthy eradication phase to prevent relapse (6, 9, 10). Most patients require at least 10-14 days of parenteral ceftazidime or a carbapenem followed by 12-20 weeks of oral trimethoprim-sulfamethoxazole with or without doxycycline. Trimethoprim and sulfamethoxazole inhibit the folic acid biosynthetic pathway by targeting dihydrofolate reductase (FolA) and dihydropteorate synthase (FolP), respectively (11). The synergistic trimethoprim-sulfamethoxazole combination, co-trimoxazole, has a potent antimicrobial effect. *B. pseudomallei* co-trimoxazole resistance has been previously documented in endemic regions (12-16) and rates range from 2.5% in Australia (13) to 13-16% in Thailand (12, 14). Previous studies have identified and characterized trimethoprim resistance mechanisms including resistant diyhdrofolate reductases in other organisms, such as *Escherichia coli* (11, 17), but in *B. pseudomallei* trimethoprim resistance has only been studied indirectly in surrogate (18) or closely related bacteria (19) showing that efflux could play an important role in resistance. The objective of this study was to identify and characterize the mechanism responsible for trimethoprim resistance in clinical and environmental *B. pseudomallei* isolates from northeast Thailand and northern Australia.

3.3 Materials and Methods

3.3.1 Bacterial strains.

B. pseudomallei 1026b was used as a prototype strain for all experiments in this study (20-22). Additionally, a collection of 30 clinical and 30 environmental isolates from Thailand (isolated in 2001 and 1990-2001 respectively) and 4 clinical and 1 environmental isolate from Australia (isolated between 1994-1997) were examined, see **Table 3.1**. All procedures involving *B. pseudomallei* were performed in Select Agent approved Biosafety Level 3 (BSL3) facilities in the Rocky Mountain Regional Biosafety Laboratory at Colorado State University using approved Select Agent compliant procedures and protocols. *Escherichia coli* strain DH5α (23) was primarily used for cloning experiments, in addition to HPS1, while RHO3 (24) was used as a conjugation donor strain to mobilize plasmids into *B. pseudomallei* (**Table 3.2**). The *E. coli* and *B. pseudomallei* strains were grown in Lennox Luria-Bertani (LB) broth or agar media (MO BIO Laboratories, Carlsbad, CA). Media was supplemented with 400 μg/mL of diaminopimelic acid (DAP) (SIGMA, St. Louis, MO) for growth of RHO3. For selection of desired plasmids in *E. coli* strains 100 μg/mL of ampicillin (SIGMA, St. Louis, MO) and 35 μg/mL of kanamycin (Km; SIGMA) were added to the media as necessary, while selection for kanamycin resistant markers in *B. pseudomallei* was performed at on media containing Km at 1 mg/ml. All strains were incubated at 37°C with aeration.

Table 3.1 Clinical and environmental *B. pseudomallei* isolates.

Thai Clinical *B. pseudomallei* Isolates			**Thai Environmental *B. pseudomallei* Isolates**			**Australian *B. pseudomallei* Isolates**		
Strain	**Specimen**	**Isolation**	**Strain**	**Specimen**	**Isolation**	**Strain**	**Specimen**	**Isolation**
1026b[1]	blood	1993	E0008	environmental	1990	MSHR 305[2]	brain	1994
2613a	blood	2001	E0016	environmental	1990	MSHR 491	environmental	1997
2614a	pus	2001	E0021	environmental	1990	MSHR 435	skin	1996
2617a	pus	2001	E0024	environmental	1990	MSHR 465a	blood	1997
2618a	pus	2001	E0031	environmental	1990	MSHR 668[2]	blood	1995
2625a	blood	2001	E0034	environmental	1990			
2637a	pus	2001	E0037	environmental	1990			
2640a	pus	2001	E0181	environmental	1990			
2650a	blood	2001	E0183	environmental	1990			
2660a	blood	2001	E0235	environmental	1990			
2661a	blood	2001	E0237	environmental	1990			
2665a	blood	2001	E0241	environmental	1990			
2667a	blood	2001	E0279	environmental	1990			
2668a	blood	2001	E0342	environmental	1990			
2670a	tracheal suction	2001	E0345	environmental	1990			
2671a	blood	2001	E0350	environmental	1990			
2673a	blood	2001	E0356	environmental	1990			
2674a	blood	2001	E0366	environmental	1990			
2677a	sputum	2001	E0371	environmental	1990			
2682a	blood	2001	E0372	environmental	1990			
2685a	pus	2001	E0377	environmental	1990			
2689b	blood	2001	E0378	environmental	1990			
2692a	blood	2001	E0380	environmental	1990			
2694a	sputum	2001	E0383	environmental	1990			
2698a	blood	2001	E0384	environmental	1990			
2704a	blood	2001	E0386	environmental	1990			
2708a	pus	2001	E0393	environmental	1990			
2717a	pus	2001	E0394	environmental	1990			
2719a	tracheal suction	2001	E0396	environmental	1990			
2764b	blood	2001	E0411	environmental	1990			
2769a	pus	2001						

[1] 1026b **source** (20).
[2] **Source** (25).

Table 3.2 *Escherichia coli* strains and plasmids used in this study.

Strain	Description		Reference
DH5α	*E. coli* general cloning strain F⁻ Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA*1 *endA*1 *hsd*R17 (r_K^- m_K^+) *phoA supE*44 *thi*-1 *gyrA*96 *relA*1 λ⁻		(23)
HPS1	*E. coli* general cloning strain F⁻Δ(*lac-proAB*) *endA*1 *gyrA*96 *hsdR*17 *supE*44 *relA*1 *recA*1 *thi* zzx::mini-Tn5Lac4		(26)
RHO3	*E. coli* conjugation donor strain F⁻ *thi*-1 *thr*-1 *leuB*6 *lacY*1 *tonA*21 *supE*44 *recA* Δ*asd* Δ*aphA* (chr::RP4-2-Tc::Mu) λ*pir*⁺		(24)
Plasmid	**Descriptive Name**	**Relevant Properties**[1]	**Source**
pGEM-T Easy		Amp^r; TA cloning vector	Promega (Madison, WI)
pCR2.1		Amp^r; Km^r; TA cloning vector	Invitrogen (Carlsbad, CA)
pEXKm5		Km^r; Allelic exchange vector	(24)
pTNS3		Amp^r; Tn*7* transposase expression vector	(24)
pRK2013		Km^r; triparental mating helper plasmid, Mob⁺ Tra⁺	(27)
pPS2497	pEXKan4Δ*bpeEF-oprC*	Km^r; Allelic exchange vector for knockout of *bpeEF-oprC*	C. López, unpublished
pPS2591	pEXKm5Δ*bpeEF-oprC*	Km^r; 1696 bp *Eco*RI band from pPS2497 (Δ[*bpeEF-oprC*]) ligated into pEXKm5	This study
pPS1679	pUC20-*bpeEF-oprC*	Amp^r; 1026b *bpeEF-oprC* source	(18)
pPS2481	pUC18T-mini-Tn*7*T-P_{tac}	Amp^r; Km^r; transposable element for complementation with P_{tac} promoter	(28)
pPS2670	pPS2481-*bpeEF-oprC*	Km^r; 6,054 bp *Eco*RI+*Hin*dIII fragment from pPS1679 (*bpeEF-oprC*) ligated into pPS2481	This study
pPS2647	pEXKm5Δ*bpeT*	Km^r; Allelic exchange vector for knockout of *bpeT*	T. Mima, unpublished
pPS2280	pUC18T-mini-Tn*7*T	Amp^r; Km^r; transposable element for complementation	(29)
pPS2778	pCR2.1-P_{bpeT}-*bpeT*	Amp^r; 1,135 bp PCR product (P_{bpeT}-*bpeT*) from 1026b ligated into pCR2.1	This study
pPS2787	pPS2280-P_{bpeT}-*bpeT*	Km^r; 1,234 bp *Kpn*I+*Nsi*I blunt-ended fragment from pPS2778 (P_{bpeT}-*bpeT*), ligated into *Sma*I site of pPS2280	This study

[1] **Abbreviations:** Amp^r, ampicillin resistant; Km^r, kanamycin resistant.

3.3.2 Antimicrobial susceptibility testing.

Trimethoprim, sulfamethoxazole and co-trimoxazole susceptibilities were assessed by determining minimal inhibitory concentrations (MIC) using the Etest® method following manufacturer's instructions (AB Biomérieux, Marcy l'Etoile, France). Briefly, strains were grown to mid-log phase (OD_{600nm} = 0.6-0.8) and diluted to a 0.5 McFarland standard in 0.85% sterile saline. The resulting bacterial cell suspension was then used to swab Mueller Hinton II agar plates (Becton Dickinson and Company, Sparks, MD) to which the Etest strips were applied. MIC results were determined following 16-20 h incubation at 37°C. Results were read at 80% inhibition, again following the manufacturer's guidelines. Since there are no established breakpoints for non-*Enterobacteriaceae,* the following *Enterobacteriaceae* MIC cut-offs were used to define susceptibility and resistance for trimethoprim alone (≤ 8 μg/mL susceptible and > 8 μg/mL resistance) from Table 2A of the CLSI guidelines (30). The non-*Enterobacteriaceae* sulfonamide cutoffs were used for sulfamethoxazole alone (≤ 256 μg/mL susceptible and > 256 μg/mL resistant) from CLSI Table 2B-5, while the standard MIC cut-offs for *B. pseudomallei* were used for co-trimoxazole (≤ 2/38 μg/mL susceptible and > 2/38 μg/mL resistant) from CLSI Table 2K (30).

MICs for other antimicrobials were determined by standard microdilution in Mueller Hinton II broth (Becton Dickinson), following CLSI guidelines (30). The antimicrobials used and their respective suppliers are listed: acriflavine (Acr, SIGMA), carbenicillin (Car, Gemini Bio-Products, West Sacramento, CA), chloramphenicol (Chl, SIGMA), erythromycin (Ery, SIGMA), gentamicin (Gen, SIGMA), norfloxacin (Nor, SIGMA), and tetracycline (Tet, SIGMA). Assays were incubated at 37°C in ambient air for 16-20 h before MIC determination.

Microdilution MICs was read at 100% inhibition of growth, as judged by eye. All MICs were tested in a minimum of biological triplicate. Results are reported as the mode of the replicates.

3.3.3 DNA sequencing and analysis.

The *folA* coding sequence was PCR amplified in four independent PCR reactions from genomic DNA isolated with the PureGene Core kit A (QIAGEN, Valencia, CA) using primers P1966 and P1967 (Integrated DNA Technologies, Coralville, IA), see **Table 3.3**, and Platinum Taq DNA Polymerase High Fidelity (Life Technologies Corporation, Grand Island, NY). The PCR products were pooled for each strain and purified from agarose gels using the GenElute gel extraction kit (Sigma-Aldrich, St. Louis, MO). The PCR products were sequenced using P1966 and P1967 at the Colorado State University Proteomics and Metabolomics facility. Alignments of *folA* sequences from experimental samples and comparison with the 1026b *folA* sequence were performed using ClustalW2 (31). In strains that had an ISB*ma*2 insertion downstream of *folA*, primer set P2182 and P2183 (**Table 3.3**) were used to amplify and sequence the upstream region of *folA*.

Similarly, *llpE*), *bpeE* (P2169-2172), *bpeF* (P2094-2101, P2109-P2110) and *oprC* (P2173-P2167) (**Table 3.3**) were PCR amplified and sequenced as described above. A specific primer set (P2222-P2223) was designed for strain E0235, as this strain had several single nucleotide polymorphisms in the region where the primers annealed.

The *bpeT* gene in the clinical and environmental isolates was sequenced either as described above using primers P1790 & P1791 (**Table 3.3**) or PCR products from this primer set were TA cloned into pGEM T-easy or PCR2.1 (**Table 3.2**). The plasmids were then sequenced using the M13F-20 and M13R primers.

Table 3.3 Oligonucleotide primers used in this study.

Primer	Descriptive Name	Primer Sequence	Source
Dihydrofolate reductase (*folA*) experiments			
P1966	*folA*-F	5'-CTTCCGGCCTCTTTTCTTTC-3'	This study
P1967	*folA*-R	5'-GTGCTGATCGAGCAGATGAC-3'	This study
P2182	Upstream-*folA*-F	5'-CTGTATCGGCTGATGGTGTC-3'	This study
P2183	Upstream-*folA*-R	5'-AGGCCTTCCTCGTACAGTTG-3'	This study
P2578	IS*Bma*2-orientation-F	5'- CCAACGATTTCACGTACGC-3'	This study
P2569	IS*Bma*2-orientation-R	5'-CCGTACAGCACGACCAATC-3'	This study
P2579	IS*Bma*2-orientation-R2	5'-GACGTTGACCTGGACCTCAC-3'	This study
Construction of *bpeEF-oprC* and *bpeT* deletion strains and genetic complements			
P1989	Δ*bpeEF-oprC*-F	5'-GGAAGTACGCGGACTTCGC-3'	(24)
P1990	Δ*bpeEF-oprC*-R	5'-GCATCAACCTCGGCTACACG-3'	(24)
P1791	*bpeT*-R	5'-CGACGCATCGCGATGGAAAC-3'	T. Mima, unpublished
P1790	*bpeT*-F	5'-ATGGACCGGCTGCAAGCCAT-3'	T. Mima, unpublished
P2224	P_{bpeT}-*bpeT*-F	5'-TTACGCCACCCACTCGTTC-3'	This study
P2225	P_{bpeT}-*bpeT*-R	5'-CAGACATCGGGATAAATGCC-3'	This study
P 479	Tn*7*L	5'-ATTAGCTTACGACGCTACACCC-3'	(29)
P1509	*BpglmS*-1	5'-GAGGAGTGGGCGTCGATCAAC-3'	(29)
P1510	*BpglmS*-2	5'-ACACGACGCAAGAGCGGAATC-3'	(29)
P1511	*BpglmS*-3	5'-CGGACAGGTTCGCGCCATGC-3'	(29)
DNA sequencing of *llpE-bpeEF-oprC, bpeT* and the *bpeT* to *llpE* intergenic region			
	M13F-20	5'- GTAAAACGACGGCCAGT-3'	(32)
	M13R	5'- AACAGCTATGACCATG-3'	(32)
P2142	*bpeT-bpeE*-F	5'- TCTGAATGATCGTCGTCACC-3'	This study
P2143	*bpeT-bpeE*-R	5'- AATCGGTGATCGTCTTCGAC-3'	This study
P2169	*bpeE*-F1	5'-TTCTTCCAGTTCCGCTTCAG-3'	This study
P2170	*bpeE*-R1	5'-TGCAGGTAAGTCTGCTCGTC-3'	This study
P2222	*bpeE*-F1 (E0235)	5'-CTATCGGGACGTGTCGCATG-3'	This study
P2223	*bpeE*-R1 (E0235)	5'-CGACACGACGTTGCCGAG-3'	This study
P2171	*bpeE*-F2	5'-CATCAACCTCGGCTACACG-3'	This study
P2172	*bpeE*-R2	5'-TCGATCGATGAAGAATTTCG-3'	This study
P2109	*bpeF*-F1	5'-GCATCTCGTGCCGATGAC-3'	This study
P2110	*bpeF*-R1	5'-CGAACTCGTCCTCGTTCTG-3'	This study
P2094	*bpeF*-F2	5'-ACATGACGTATCTGCGCAAC-3'	This study
P2095	*bpeF*-R2	5'-CATCGCGAACTGCTTGTAGA-3'	This study
P2096	*bpeF*-F3	5'-AACGTCGAGCGCAACATC-3'	This study
P2097	*bpeF*-R3	5'-CGTTGATCTGGTAGCTCGTG-3'	This study
P2098	*bpeF*-F4	5'-GCGGCTTCAAGATGCAG-3'	This study

Primer	Descriptive Name	Description	Source
P2099	*bpeF*-R4	5’-ACCACACCCATGATGAACG-3’	This study
P2100	*bpeF*-F5	5’-AGGGCGACAACAACATCTTC-3’	This study
P2101	*bpeF*-R5	5’-GGCCTTCAGGTTCTGGTTC-3’	This study
P2173	*oprC*-F1	5’-GGTGTTCTTCGGGATGCTC-3’	This study
P2174	*oprC*-R1	5’-GCCGGTACAGATCCTGGTC-3’	This study
P2175	*oprC*-F2	5’-GTCGTACGAAGCGGACCTG-3’	This study
P2176	*oprC*-R2	5’-CACCTGCTGCCGGTAGTTC-3’	This study
P2177	*oprC*-F3	5’-AACCTGTTCCTGTGGTCGAG-3’	This study
P2178	*oprC*-R3	5’-CCGCCTCTCTCAGGTTCTC-3’	This study
Reverse transcription quantitative PCR experiments			
P1516	Bp23S-F	5’-GTAGACCCGAAACCAGGTGA-3’	(33)
P1517	Bp23S-R	5’- CACCCCTATCCACAGCTCAT-3’	(33)
P1524	*bpeF*-F1-RT	5’-TCCGAGTATCCGGAAGTCGT-3’	(33)
P1525	*bpeF*-R1-RT	5’-GTCCTCGACACCGTTGATCT-3’	(33)
P1814	*bpeT*-RT-for	5'-GAGCTTTCAGGTCAACAACC-3’	T. Mima, unpublished
P1815	*bpeT*-RT-rev	5'-GTGAGTGGAATTCGCAGAG-3’	T. Mima, unpublished

3.3.4 Multiplex IS*Bma*2 PCR.

Primers P2578, P2569 and P2579 (Integrated DNA Technologies) (**Table 3.3**) were designed and used in multiplex PCR to determine the orientation of IS*Bma*2 in the clinical and environmental strains. P2578, P2569 and P2579 were all added at final concentrations of 0.6 pmol/µl and standard Taq polymerase (New England Biolabs, Waltham, MA) was used. PCR conditions were an initial denaturation at 95°C for 2 minutes, 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1.5 min, followed by a final extension at 72°C for 10 min.

3.3.5 Markerless deletion of *bpeEF-opC* and *bpeT*.

A 4,314 bp region of the *bpeEF-oprC* operon was deleted in several of the clinical and environmental isolates by allelic exchange using a knockout construct integrated into the pEXKm5 vector system, pPS2497 (**Table 3.2**) (24). A 573 bp region of the *bpeT* gene was

deleted in the strains of interest, again using the pEXKm5 vector system, pPS2647. The pEXKm5 knockout constructs were introduced into *B. pseudomallei* by electroportation as previously described (34) or by conjugation using either a tri-parental mating helper plasmid (pRK2013) or the RHO3 strain (**Table 3.2**) as previously described (24). Transformants and exconjugates were selected on LB medium supplemented with 1 mg/ml Km. These merodiploids were then resolved by sucrose counter-selection as previously described (24) and screened by PCR using primers P1989 & P1990 for *bpeEF-oprC* and P1790 & P1791 for *bpeT* deletions.

3.3.6 Complementation of *bpeEF-oprC* and *bpeT* deletions.

Genetic complementation was accomplished utilizing the mini-Tn*7* system, which allows for stable and site-specific single-copy insertions into the *B. pseudomallei* genome at three possible *glmS*-associated Tn*7* insertion sites (34). Tri-parental conjugation with RHO3 was necessary to introduce the pTNS3 transposition helper plasmid and one of the following plasmids into the *B. pseudomallei* knockout strains: pPS2481, pPS2670, pPS2280 or pPS2787 (**Table 3.2**). Single *glmS*2 insertions were confirmed by PCR with the *glmS* primer sets (P479 & P1509, P479 & P1510 and P479 & P1511) as previously described (**Table 3.3**) (34).

The inducible *E. coli lac* operon P_{tac} promoter was used to express the *bpeEF-oprC* operon, which originated from strain 1026b (18). BpeEF-OprC expression in the complemented strains was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG; Gold Biotechnology, St. Louis, MO) at a final concentration of 1 mM. Complementation of *bpeT* gene deletions were performed using the endogenous *bpeT* promoter amplified from the 1026b strain using primers P2224 & P2225 (**Table 3.3**).

3.3.7 Reverse transcription quantitative PCR (RT-qPCR).

Expression levels of *bpeF* and *bpeT* mRNA were analyzed in bacteria grown to mid-log phase (OD_{600nm} = 0.6-0.8) in Lennox Luria Broth (MO BIO Laboratories, Carlsbad, CA) at which point *bpeEF-oprC* expression remained either uninduced or was induced for 1 hour by addition of trimethoprim to a final concentration of 32 μg/mL. Trimethoprim stock solution was made in dimethylacetamide at a concentration of 100 mg/mL. RNA extraction, cDNA synthesis and RT-qPCR were done as previously described (33, 35) except that the RNeasy Protect Bacteria Mini Kit (QIAGEN, Valencia, CA) was used for RNA extraction. 23S rRNA was used as the housekeeping control. The primer sets used were P1516 & P1517 for 23S rRNA, P1524 & P1525 for *bpeF* (33), and P1814 & P1815 for *bpeT* (**Table 3.3**). RT-qPCR samples were tested in a minimum of technical and biological triplicate. Technical replicates were averaged for each biological replicate.

3.3.8 Statistical analysis.

The relative fold expression of each gene in the clinical and environmental isolates, compared to 1026b, was determined by the Bio-Rad iCycler iQ™ Optical System Software version 2.0 software using specific reference and target gene primer set amplification efficiencies. Subsequent comparisons of the relative expression data were analyzed by two-way analysis of variance (ANOVA) with Tukey's multiple comparisons test (comparisons across strains) or Sidak's multiple comparisons test (comparisons across conditions) using GraphPad Prism version 6.0c for Mac OSX (GraphPad Software, La Jolla, California). P-values < 0.05 were considered significant.

3.4 Results

3.4.1 Initial characterization of isolates.

To evaluate the innate frequency of trimethoprim, sulfamethoxazole, and co-trimoxazole resistance in *B. pseudomallei* clinical and environmental isolates, MICs were determined for a collection of 66 isolates from Northeast Thailand and Northern Australia.

Using the susceptibility criteria explained in **Section 3.3.2**, MIC testing of strains from Thailand showed that 47% of clinical (14/30) and 30% of environmental strains (9/30) were trimethoprim resistant with MICs ranging from 12 μg/mL to the detection limit of ≥ 32 μg/mL (**Figure 3.1**). None of these isolates were resistant to sulfamethoxazole or co-trimoxazole. The frequency of trimethoprim resistance in Australian isolates was 60% (3/5) and these 5 isolates were all susceptible to sulfamethoxazole and co-trimoxazole. These data show that although trimethoprim resistance was highly prevalent in our collection of *B. pseudomallei* isolates, none of the strains tested showed clinically significant co-trimoxazole resistance.

3.4.2 Further characterization of the isolates of interest.

A sub-population of isolates, shown as black bars in **Figure 3.1**, was selected for further studies. These strains include the 1026b reference strain, a trimethoprim sensitive environmental isolate (E0237), a clinical isolate with a trimethoprim MIC of 16 μg/mL (2769a) and 10 other trimethoprim resistant isolates with MICs above the limit of detection that were chosen at random (**Table 3.4**). In addition to the trimethoprim, sulfamethoxazole and co-trimoxazole MICs reported for these isolates, the MICs of seven more antimicrobials, representing various drug classes, were determined (**Table 3.4**). In some of the strains, there was up to a 3-fold increase in

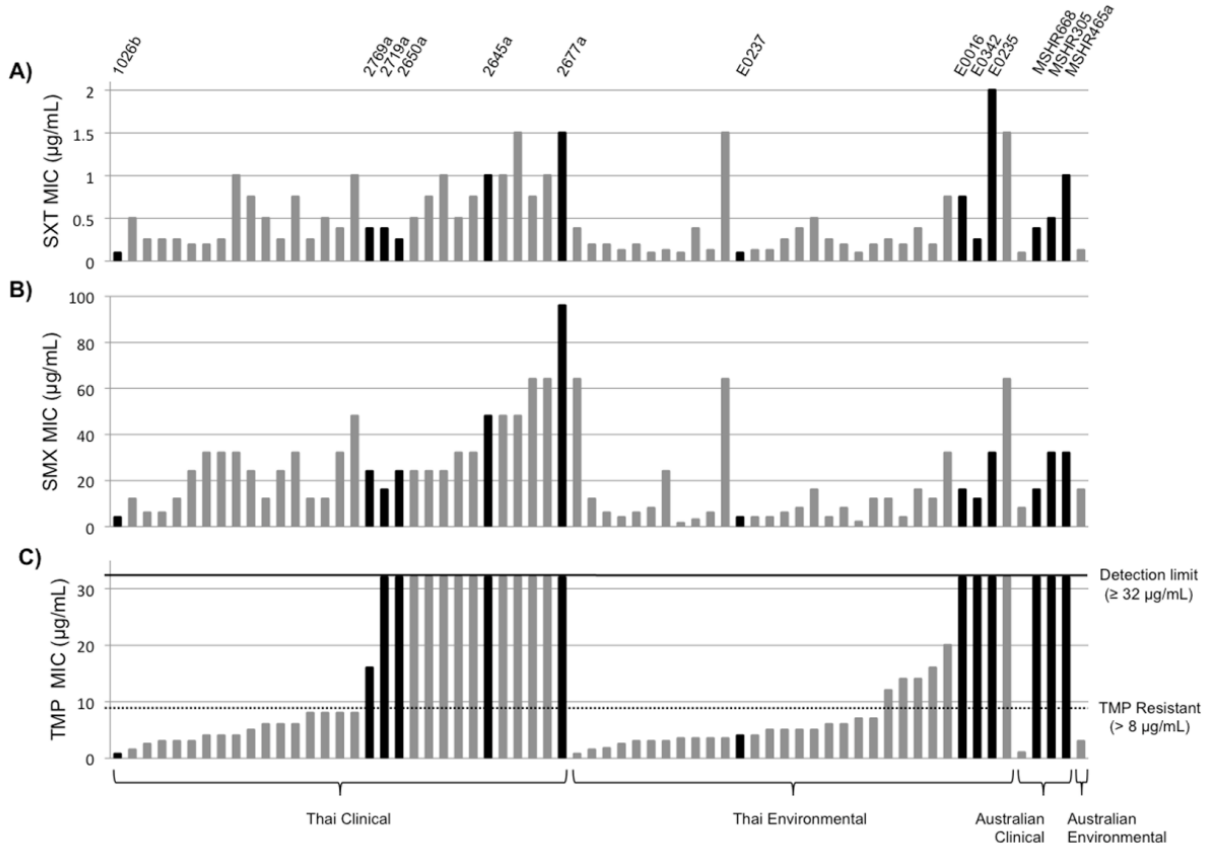

Figure 3.1 Distribution of folate pathway inhibitor MICs in *B. pseudomallei* clinical and environmental isolates. Minimal inhibitory concentrations (MICs) for the co-trimoxazole (SXT) drug combination (**panel A**), sulfamethoxazole (Smx) (**panel B**) and trimethoprim (Tmp) (**panel C**) were determined in a collection of 35 clinical and 31 environmental isolates from Thailand and Australia. All 66 isolates tested were susceptible to co-trimoxaozle (**panel A**) and sulfamethoxazole (**panel B**). However, isolates in **panel C** with values above the dotted line are classified as trimethoprim resistant, while others below the cutoff are trimethoprim susceptible. Several isolates from this collection were selected for further testing (black bars, ■), the names of these 13 strains of interest are presented above **panel A**.

MIC for norfloxacin and tetracycline compared to 1026b, but for others there was no change (**Table 3.4**). Unfortunately, these data did not have a lot of deviation from 1026b, and due to the complex genetic variability of each isolate compared to another; we could not draw any meaningful conclusions from this testing.

Table 3.4 Antimicrobial susceptibilities of selected *B. pseudomallei* isolates.

	Minimal Inhibitory Concentration (μg/mL)									
Strain	Tmp[1]	Smx	SXT	Acr	Chl	Nor	Tet	Car	Ery	Gen
1026b	0.75	4	0.094	32	8	4	0.5	256	128	256
Thai Clinical Isolates										
2650a	≥ 32	8	1	32	32	32	4	512	256	64
2665a	≥ 32	16	1	32	8	16	2	512	256	128
2677a	≥ 32	24	1.5	32	8	32	2	512	128	128
2719a	≥ 32	12	1	32	8	16	2	512	256	128
2769a	16	12	0.75	32	8	8	2	512	256	64
Thai Environmental Isolates										
E0016	≥ 32	12	0.75	32	16	32	2	512	256	256
E0235	≥ 32	24	1.5	32	16	32	4	512	256	256
E0237	4	8	0.5	32	16	8	2	512	128	64
E0342	≥ 32	24	0.75	32	8	32	2	512	256	512
Clinical and Environmental Isolates from Australia										
MSHR305	≥ 32	32	0.5	ND	ND	ND	ND	ND	ND	ND
MSHR465a	≥ 32	16	0.38	ND	ND	ND	ND	ND	ND	ND
MSHR668	≥ 32	32	1	ND	ND	ND	ND	ND	ND	ND

[1] The detection limit for the Etest® method for trimethoprim is ≥ 32 μg/mL.

Minimal inhibitory concentrations (MIC) for trimethoprim, sulfamethoxazole and co-trimoxazole of 12 clinical and environmental isolates of interest are shown in comparison to the 1026b reference strain (**left panel**). The majority of these strains had trimethoprim MICs above the detection limit. While all had increase sulfamethoxazole and co-trimoxazole MICs compared to 1026b, none of these strains were resistant to those antimicrobials.Additionally we tested the MICs of known BpeEF-OprC efflux pump substrates (**middle panel**) and non-substrates (**right panel**). Abbreviations: Car, carbenicillin; Ery, erythromycin; Gen, gentamycin; Acr, acraflavine; Chl, chloramphenicol; ND, no data; Nor, norfloxacin; Tet, tetracycline; Tmp, trimethoprim; SXT, trimethoprim:sulfamethoxazole (1:19).

3.4.3 DNA sequencing of the trimethoprim drug target, *folA*.

Mutations affecting the trimethoprim target dihydrofolate reductase (FolA) can cause resistance to this antimicrobial. We compared the *folA* sequences of 12 *B. pseudomallei* strains to the trimethoprim susceptible 1026b reference strain (**Table 3.4**). Two mutations, V77A and A144T, were found in 5 of the trimethoprim resistant isolates compared to reference strain 1026b. However, these conservative mutations were not linked to trimethoprim resistance in these isolates.

3.4.4 Identification and investigation of an IS*Bma*2 insertion element.

While attempting to PCR amplify the *folA* region we noticed that some of the strains (2665a, 2769a, E0016 and E0342) yielded the same 694 bp DNA fragment as 1026b (**Figure 3.2A**). The remaining isolates (2650a, 2677a, 2719a and E0235), however, showed a substantially larger (≈ 2.3 kb) fragment when using the same primers. DNA sequencing of this fragment with P1967 revealed the presence of an IS*Bma*2 insertion sequence (36). Further analyses were conducted using representative strains E0016 (694 bp fragment) and 2650a (≈ 2.3-kb fragment). Employing genomic DNAs as templates and PCR amplification with the subset of primers shown in **Figure 3.2A** for E0016 and **Figure 3.2B** for 2650a followed by sequencing confirmed for E0016 the identical sequence and gene order found in 1026b. In strain 2650a the 1,596 bp IS*Bma*2 was inserted in the 130 bp *folA-pmbA* intergenic region in a palindromic sequence located between nucleotides 47 and 75 downstream of *folA* that most likely serves as this gene's transcriptional terminator. The insertion site and orientation of IS*Bma*2 was identical to that found in the genome of strain K96243 (37). In both 1026b and K96243 the *folA* and neighboring genes are located on chromosome 1, but in different locations and in reverse order (**Figure 3.2**).

The two PCR amplification patterns observed in the isolates analyzed in this study indicate two distinct populations of strains, one similar to 1026b and another similar to K96243. The frequency of the IS*Bma*2 insertion at this site was determined in the 65 clinical and environmental isolates using PCR amplification of the *folA* region with P1966 and P1967. This

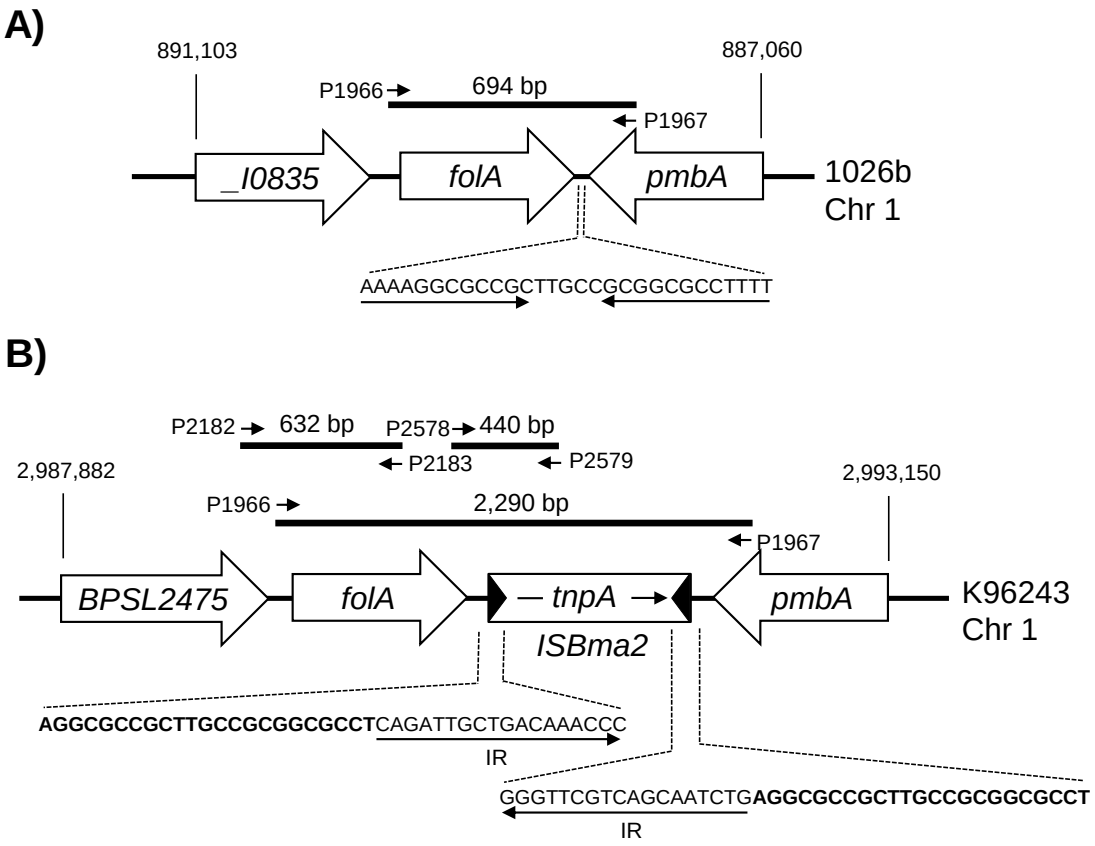

Figure 3.2 Genomic location and organization of the *folA* region of *B. pseudomallei* in the two sequenced prototypes 1026b (A) and K96243 (B). Chromosome 1 sequence coordinates and gene nomenclatures are taken from the GenBank entries for strains 1026b (accession NC_017831.1) and K96243 (accession NC_006350.1). Gene annotations indicate: *_I0835* (short for *BP1026B_I0835*) and *BPSL2475*, σ54-dependent transcriptional regulator; *folA*, dihydrofolate reductase; *pmbA*, protein belonging to the peptidase U62 family; *tnpA*, transposase. The same sequences and gene organizations were confirmed for Thai isolates E0016 (1026b-like) and 2650a (K96243-like). Horizontal black lines indicate PCR fragments obtained with the indicated primers. Primers pairs used were P1966 & P1967 that amplify the *folA* gene with and without IS*Bma*2, and P2578 & P2579 that amplify a fragment containing the *folA*-IS*Bma*2 junction sequences. In isolates where IS*Bma2* is in the opposite orientation primer set P2578 & P2579 is replaced by P2578 & P2569 and this combination yields a 647 bp fragment containing the *folA*-IS*Bma*2 junction sequences (not shown). All primer sequences are given in the text. The *folA* upstream region and 5' coding sequences can be amplified using primer pair P2182 & P2183. In **panel A** the sequence with underlined inverted repeats indicates the putative *folA* transcriptional terminator. In **panel B**, sequences that compose the 5' and 3' IS*Bma*2 inverted repeats (IR, underlined) and the 23 bp duplicated genomic DNA segments (bold letters) are indicated. The sequences of IS*Bma*2 elements and their organization as well as that of the duplicated target DNA segments is consistent with previously published data on IS*Bma*2 (36).

analysis showed that 38% of clinical and 45% of environmental isolates contain the IS*Bma*2 insertion at this locus. Additionally, DNA sequencing of this region in strain MSHR305 and comparison to the whole genome shotgun sequence (38) revealed that the IS*Bma*2 element was in the opposite orientation, relative to K96243, and thus can insert at this site in either orientation. In the strains that contained IS*Bma*2, a multiplex PCR was used to determine the orientation of the insertion element. There was a near equal distribution of IS*Bma*2 orientation, with 52% having the same orientation as K96243, resulting in a 440 bp product, and 48% having the opposite orientation, a 647 bp product. There was a slight bias in the orientation when comparing environmental isolates verses clinical isolates, where 64% of environmental and 38% of clinical isolates were in the same orientation as in strain K96243. However, a significantly larger sample size would be needed to determine the true orientation bias. The location of IS*Bma*2 most likely does not affect *folA* expression but has implications regarding the design of PCR primers for *folA* amplification for diagnostic purposes.

3.4.5 BpeEF-OprC and BpeT are necessary for trimethoprim resistance.

The BpeEF-OprC efflux pump is encoded by the *bpeEF-oprC* genes that are the distal genes of the *llpE-bpeEF-oprC* operon located on *B. pseudomallei* chromosome 2 (33, 37). Expression of this operon is governed by the LysR-type regulator BpeT that is encoded by a gene located immediately upstream of the *llpE-bpeEF-oprC* operon (T. Mima, H.P. Schweizer, unpublished observations). BpeEF-OprC has been shown to efflux trimethoprim when expressed in *Pseudomonas aeruginosa* (18), in *B. thailandensis* isolates that are resistant to chloramphenicol (19) and in *B. pseudomallei* isolates that express BpeEF-OprC as a result of a BpeT truncation (20, 21).

To investigate a potential contribution of this efflux pump to trimethoprim resistance in clinical and environmental *B. pseudomallei* isolates, a 4,314 bp segment of the *llpE-bpeEF-oprC* operon was deleted from 11 trimethoprim resistant isolates and trimethoprim susceptible reference strains 1026b and E0237. Deletion of *bpeEF-oprC* from the trimethoprim resistant strains resulted in susceptible mutant derivatives with at least 10 fold decreases in MIC. A lower trimethoprim MIC was observed in E0237 but the susceptibility of 1026b remained unchanged (**Table 3.5**). No significant trimethoprim susceptibility changes were observed in uninduced complemented strains, but induction of BpeEF-OprC expression resulted in significant MIC

Table 3.5 Reduced trimethoprim MICs are observed in the absence of BpeEF-OprC.

	Trimethoprim[1] Minimal Inhibitory Concentration (μg/mL)			
Strain	***bpeE*$^+$*F*$^+$*-oprC*$^+$**	**Δ(*bpeEF-oprC*)**	**Δ(*bpeEF-oprC*) Complemented[2] Uninduced**	**Δ(*bpeEF-oprC*) Complemented[2] +1 mM IPTG**
1026b	0.75	0.5	0.5	6
Clinical Isolates from Thailand				
2650a	≥ 32	1.5	1.5	≥ 32
2665a	≥ 32	1.5	1.5	16
2677a	≥ 32	2	2	≥ 32
2719a	≥ 32	1	1	16
2769a	16	1.5	2	≥ 32
Environmental Isolates from Thailand				
E0016	≥ 32	1	1.5	16
E0235	≥ 32	1.5	3	≥ 32
E0237	4	1	2	16
E0342	≥ 32	1	1.5	16
Clinical and Environmental Isolates from Australia				
MSHR305	≥ 32	2	2	16
MSHR668	≥ 32	2	2	32
MSHR465a	≥ 32	3	2	≥ 32

[1] The detection limit for the Etest® method for trimethoprim is ≥ 32 μg/mL.

[2] The respective strains containing the Δ(*bpeEF-oprC*) mutation have a mini-Tn*7*T-*Ptac-bpeE*$^+$*F*$^+$*-oprC*$^+$ element integrated at the chromosomal *glmS*2 locus, except for the MSHR668 derivative, which has the same element integrated at *glmS*3. The *bpeE*$^+$*F*$^+$*-oprC*$^+$ operon contained on this mini-Tn*7* element is derived from the prototype strain, 1026b.

increases such that all of the isolates, except for 1026b, became trimethoprim resistant (**Table 3.5**). Similar results were observed with strains containing deleted and complemented *bpeT*, respectively (**Table 3.6**). We should note that while complementation with the endogenous P_{bpeT} promoter did increase the trimethoprim MIC in most of the strains, this increase was lower than that observed in the *bpeEF-oprC* complementation experiment. Overall, these findings suggest that expression of the BpeEF-OprC pump is required for trimethoprim resistance in these isolates.

Table 3.6 Reduced trimethoprim MICs are observed in the absence of the *bpeEF-oprC* transcriptional regulator, BpeT.

	Trimethoprim[1] Minimal Inhibitory Concentration (μg/mL)			
Strain	***bpeT*$^+$**	**Δ(*bpeT*)**	**Δ(*bpeT*) Empty-Tn*7*[2]**	**Δ(*bpeT*) Complemented[3]**
1026b	0.75	0.25	0.38	0.5
Clinical Isolates from Thailand				
2650a	≥ 32	0.38	0.38	0.5
2665a	≥ 32	1.5	2	6
2677a	≥ 32	2	1	6
2719a	≥ 32	1.5	2	2
2769a	16	1.5	2	4
Environmental Isolates from Thailand				
E0016	≥ 32	1.5	1.5	6
E0235	≥ 32	1.5	1.5	8
E0237	4	1.5	3	2
E0342	≥ 32	1.5	2	4
Clinical and Environmental Isolates from Australia				
MSHR305	32	1.5	2	4
MSHR668	16	1.5	2	24
MSHR465a	32	1.5	2	12

[1] The detection limit for the Etest® method for trimethoprim is ≥ 32 μg/mL.
[2] The respective strains containing the Δ(*bpeT*) mutation have an empty mini-Tn*7* element integrated at the chromosomal *glmS*2 locus.
[3] The respective strains containing the Δ(*bpeT*) mutation have a mini-Tn*7*T-P_{bpeT}-*bpeT* element integrated at the chromosomal *glmS*2 locus. P_{bpeT}-*bpeT* contained on this mini-Tn*7* element is derived from the prototype strain, 1026b.

3.4.6 DNA sequencing of the BpeEF-OprC regulatory and structural components.

To assess whether mutations in structural pump components were contributing to trimethoprim resistance we sequenced the *bpeT-llpE-bpeE* and *oprC* regions in 9 Thai isolates and the *bpeF* gene in 3 of those isolates using a primer walking strategy. These analyses showed that the DNA sequence of the entire region was highly conserved with few synonymous and conservative mutations. For example, when compared to the 1026b sequence the following mutations were found in ≤ 22% of the sequenced trimethoprim resistant isolates: BpeE (A401T) and OprC (V78A, A207Q, and T508A). There were only a few conservative mutations in the *bpeT* gene and no mutations in the *bpeT-llpE* intergenic region containing the putative promoter regions for both *bpeT* and the *llpE-bpeEF-oprC* operon. Given the conservative nature of the observed mutations and the results of the complementation study, which showed that the 1026b operon sequence was sufficient for the trimethoprim resistant phenotype, it is unlikely that changes in structural BpeEF-OprC components are root causes for resistance in any of the tested isolates.

3.4.7 Relative expression of *bpeEF-oprC* and *bpeT*.

RT-qPCR was used to determine the relative expression of *bpeT* and *bpeF* mRNAs, and thus gauge expression levels of the BpeT regulator and BpeEF-OprC. Interestingly, in uninduced cells *bpeF* mRNA levels were very similar between the 9 Thai isolates tested and 1026b (**Figure 3.2**). However, when induced with 32 μg/mL trimethoprim, isolates with trimethoprim MICs ≥ 32 μg/mL had significantly increased *bpeF* mRNA levels, with fold increases ranging from 3.2 to 6.6 (**Figure 3.2**). In contrast, two isolates, E0237 and 2769a, with respective MICs of 4 μg/mL

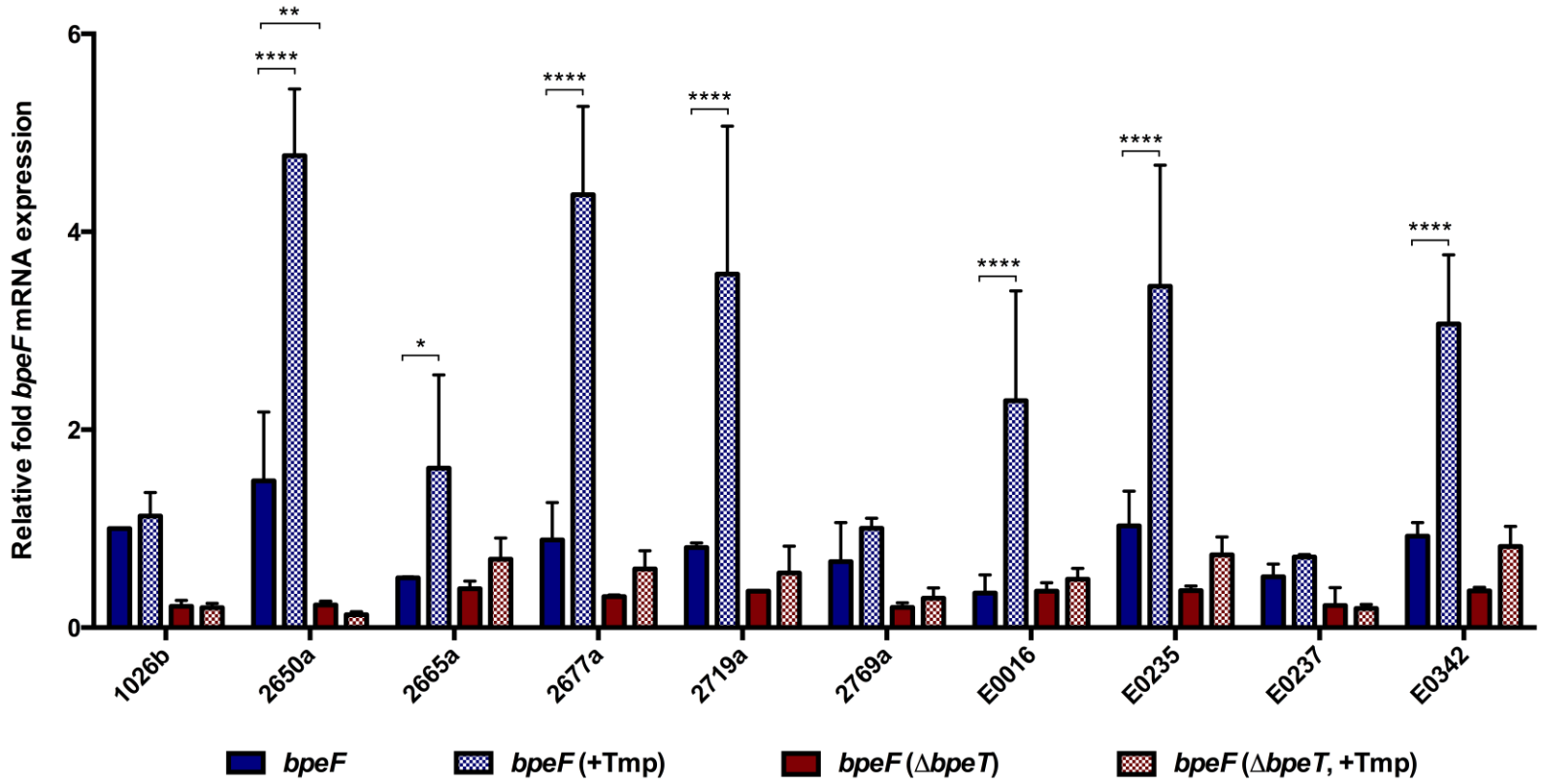

Figure 3.3 Relative expression of *bpeF* in *B. pseudomallei* clinical and environmental isolates. Relative *bpeF* mRNA levels were determined in 10 *B. pseudomallei* isolates (**blue** and **stippled blue** bars) and their respective Δ*bpeT* derivatives (**red** and **stippled red** bars). Strains were grown in LB medium (**solid** bars) or LB medium with 32 mg/ml added 1 h prior to RNA harvest (+Tmp; **stippled** bars). All fold expression values are relative to the value obtained in uninduced 1026b. Error bars indicate the standard deviation between biological replicates, which were each performed in technical triplicate. Statistical differences were analyzed by two-way ANOVA and Sidak's multiple comparisons test (****, $p < 0.0001$; **, $p < 0.01$; *, $p < 0.05$).

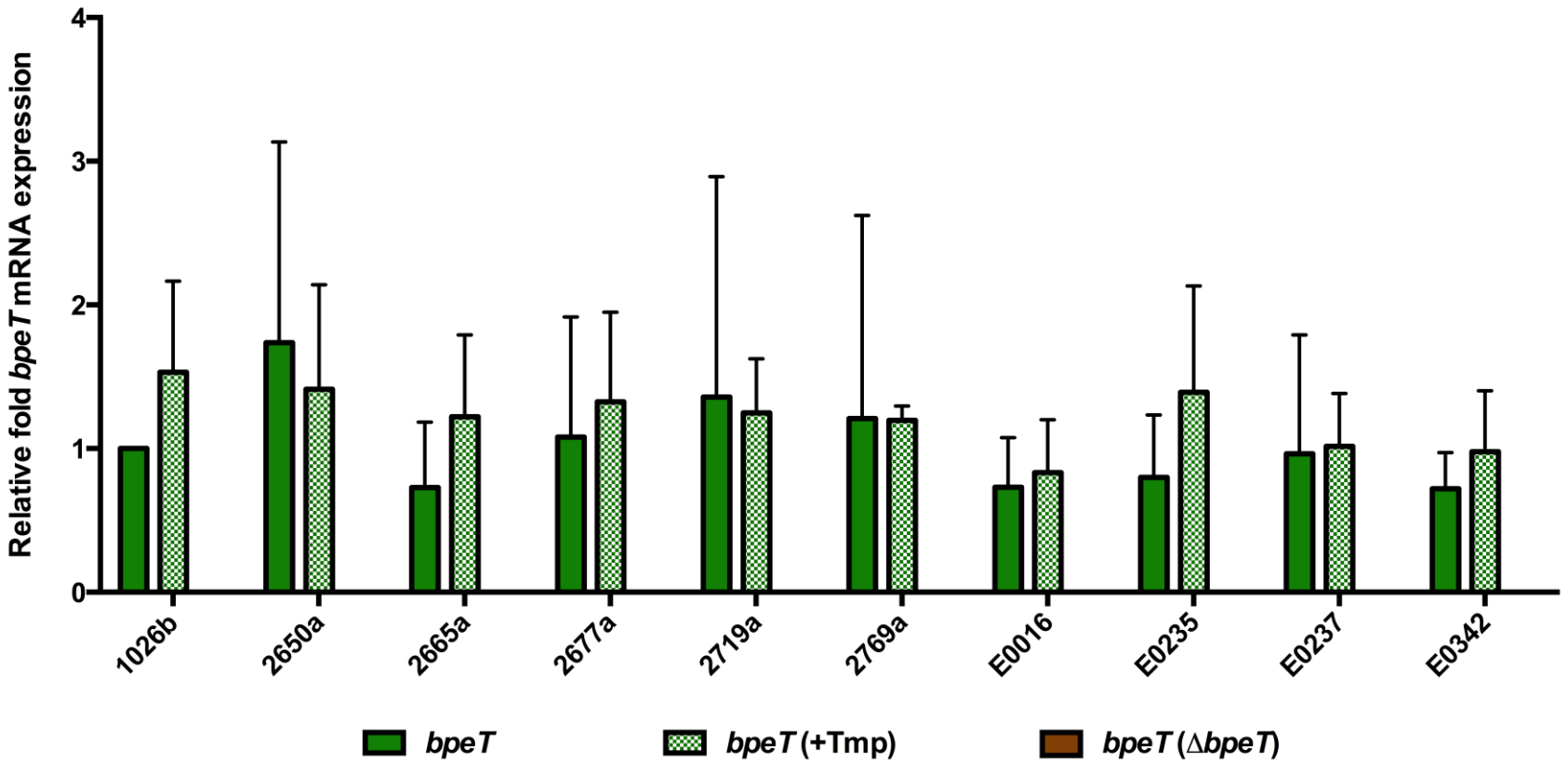

Figure 3.4 Relative expression of *bpeT* mRNA in *B. pseudomallei* clinical and environmental isolates. Relative *bpeT* mRNA levels were determined in 10 *B. pseudomallei* isolates and the respective Δ*bpeT* derivatives (**brown** bars) grown in LB medium (**solid green** bars) or LB medium with 32 µg/ml trimethoprim (Tmp) added for 1 h prior to RNA harvest (**stippled green** bars). All fold expression values are relative to the values obtained for uninduced strain 1026b. Error bars indicate the standard deviation between biological replicates, which were each performed in technical triplicate. There was no significant difference in *bpeT* mRNA expression in these strains, as compared to 1026b and to each other. As expected there was no expression of *bpeT* mRNA in the Δ*bpeT* strains.

and 16 μg/mL, and 1026b exhibited no change in *bpeF* expression levels when induced with 32 μg/mL of trimethoprim (**Figure 3.2**). As this high concentration of trimethoprim may adversely affect strains with MICs lower than 32 μg/mL, the experiment was repeated performing induction with trimethoprim at ½ the respective MICs for 1026b, 2769a and E0237 for 1 h. However, even when using ½ MIC levels of trimethoprim for induction no increases in *bpeF* expression levels were observed (data not shown). These data suggest that high-level trimethoprim resistance results from over-expression of the BpeEF-OprC efflux pump.

Additionally we determined the relative expression of *bpeF* in strains derived from these isolates lacking the BpeT regulator. Interestingly, in all of the isolates there was a decrease in *bpeF* expression, though the only observed significant decrease was seen with strain 2650a (**Figure 3.2**). When these deletion mutants were induced with trimethoprim there was no significant increase in *bpeF* expression, as was observed in the wild-type strains (**Figure 3.2**). In the strains that overexpress *bpeF* after trimethoprim induction, the *bpeF* expression level returned to uninduced levels, with the exception of 2650a in which it was much lower than the initial expression level (**Figure 3.2**). Repetition of these experiments for *bpeT* showed no changes in expression both with and without trimethoprim induction (**Figure 3.3**).

3.5 Conclusions

This is the first study aimed at elucidation of the molecular mechanisms governing trimethoprim resistance in clinical and environmental *B. pseudomallei* isolates. The results show that resistance to trimethoprim alone is frequent in *B. pseudomallei* strains. However, resistance to sulfamethoxazole and co-trimoxazole was not detected in any of these clinical and environmental isolates tested. In all of the Australian and Thai isolates assessed, trimethoprim resistance was attributed to expression of BpeEF-OprC but not changes in the dihydrofolate

reductase target, indicating that efflux is the predominant trimethoprim resistance mechanism in *B. pseudomallei*. Increased transcription of the *bpeEF-oprC* structural genes in the presence of trimethoprim lead to high level resistance, with MICs above the detection limit of 32 μg/mL. Since DNA sequencing revealed only conservative mutations in *bpeT* and qRT-PCR analysis indicated no changes in expression levels of this regulator, we conclude that BpeEF-OprC expression in the trimethoprim resistant clinical and environmental isolates tested is governed by a to date unidentified regulatory mechanism(s).

In the course of these studies we discovered that the genomic location and organization of genes in the immediate *folA* region follow a distinct pattern that, in reference to three sequenced prototypes, allows grouping of strains into K96243-like, MSHR305-like and 1026b-like.

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CHAPTER 4: Mechanisms Responsible for Acquired Trimethoprim and Resistance in *Burkholderia pseudomallei*

This chapter describes the characterization of laboratory selected trimethoprim resistant strains derived from the Select Agent excluded Bp82 strain. Mutations to both the trimethoprim target, FolA, and the LysR-type regulator responsible for control of the BpeEF-OprC efflux pump, BpeT, were found to contribute to resistance. Additionally, antimicrobial susceptibility testing of a panel of 1026b-derived mutants expressing various combinations of efflux pumps shows that sulfamethoxazole may be a weak substrate of the BpeAB-OprB and BpeEF-OprC efflux pumps.

4.1 Summary

Burkholderia pseudomallei is a Gram-negative bacterium of interest as it is the causative agent of the disease melioidosis. Treatment of melioidosis is difficult and costly due to the organism's intrinsic resistance to many commonly used antimicrobials. Currently a combination of trimethoprim and sulfamethoxazole (co-trimoxazole) is used for the eradication phase of treatment and is an option for post-exposure prophylaxis; however resistant isolates have been reported. Mutations to the dihydrofolate reductase (DHFR), trimethoprim drug target, are well-documented mechanisms for resistance to trimethoprim. To our knowledge mutations to the DHFR have not been described previously in *B. pseudomallei*. To determine if such mutations can confer resistance to trimethoprim in *B. pseudomallei*, we used an attenuated, Select Agent excluded strain, Bp82, to isolate trimethoprim resistant mutants. These mutants were characterized by DNA sequencing of the DHFR encoding gene, *folA*. Additionally, we investigated the potential role of the BpeEF-OprC efflux pump in these induced mutants.

Trimethoprim resistance was found to be due to mutations in the *folA* gene, increased expression of the BpeEF-OprC efflux pump, or a combination of both. Similar experiments were performed to determine mechanisms of sulfamethoxazole resistance; however the ability of *B. pseudomallei* to grow at high concentration of sulfamethoxazole caused only small changes in the sulfamethoxazole susceptibilities. Antimicrobial susceptibility testing of several efflux mutant strains suggest that sulfamethoxazole may be effluxed at low levels by both the BpeAB-OprB and BpeEF-OprC pump. However, overexpression of these efflux pumps caused only slight changes to the sulfamethoxazole susceptibilites and did not confer clinically significant resistance to sulfamethoxazole.

4.2 Introduction

Burkholderia pseudomallei is a saprophyte endemic to tropical and sub-tropical regions of the world (1, 2). It is a genetically complex organism and the etiologic agent of the disease melioidosis. Melioidosis is a disease of varying severity that typically affects those with pre-existing health conditions such as diabetes mellitus and impaired renal function (3, 4), but is also a rare pathogen of healthy individuals and many diverse fauna (5). Treatment of this disease has been improved over time with the introduction of novel antimicrobials such as ceftazidime (6) and increasing comprehension of the adaptive and intrinsic resistance of *B. pseudomallei* to many clinically available antimicrobials (7). *B. pseudomallei* is widely known to be resistant to a range of drug classes, including macrolides, aminoglycosides, 1st and 2nd generation cephalosporins and some penicillins (8, 9). Currently the recommended treatment of melioidosis consists of two phases. The initial intensive phase of treatment typically involves at least 10 to 14 days of ceftazidime or alternatively a carbapenem (7, 10, 11), while the subsequent eradication phase is typically 12 to 20 weeks of co-trimoxazole (trimethoprim-sulfamethoxazole

combination) or amoxicillin-clavulanic acid (7, 10, 11). Co-trimoxazole is also the recommended therapeutic for post-exposure prophylaxis in the event of accidental exposure to *B. pseudomallei* (7, 10).

Trimethoprim is a diaminopyrimide that specifically inhibits bacterial dihydrofolate reductases (DHFR), while sulfamethoxazole, a sulfonamide, targets dihydropteroate synthetase (DHPS) (12). The combination of trimethoprim and sulfamethoxazole is synergistic (13) and efficacious for the treatment of many bacterial infections, including melioidosis. However, resistance to co-trimoxazole has been reported in endemic regions, such as Thailand (12, 14-17). Recently we described a high frequency of trimethoprim resistance approaching 40% in a collection of clinical and environmental strains from Thailand and Australia (18). This trimethoprim resistance was attributed to the expression of the BpeEF-OprC efflux pump and no DHFR target mutations were identified. The *B. pseudomallei* BpeEF-OprC efflux pump was shown to efflux both trimethoprim and chloramphenicol when expressed in *Pseudomonas aeruginosa* (19) but is also known to efflux tetracyclines and fluoroquinolones (T. Mima and H.P. Schweizer, unpublished results).

Mutations to the trimethoprim drug target, DHFR, have been described as trimethoprim resistance mechanisms in many other genera of bacteria (20-22). Primarily this has been studied in *Enterobacteriacea*, since trimethoprim is still occasionally used alone for treatment of infections with these bacteria (21). DHFR mutations in *B. pseudomallei* have not, to our knowledge, been found to confer resistance to trimethoprim. Additionally, there has been no description of *B. pseudomallei* isolates resistant to sulfamethoxazole alone or mechanisms that may contribute to such resistance. Mutations to the sulfamethoxazole drug target DHPS have been shown to confer resistance in other bacteria (23). The purpose of this study was to further

investigate potential resistance mechanisms in *B. pseudomallei* for trimethoprim and sulfamethoxazole.

4.3 Materials and Methods

4.3.1 Bacterial strains.

Escherichia coli strain DH5α was used for cloning manipulations, while the RHO3 strain was used as a conjugation donor strain to mobilize plasmids into Bp82 (**Table 4.1**). The *E. coli* strains were grown in Lennox Luria-Bertani (LB) broth or agar media (MO BIO Laboratories, Carlsbad, CA). Media were supplemented with diaminopimelic acid (DAP; SIGMA, St. Louis, MO) at 400 μg/mL for culture of RHO3. For selection of desired plasmids in *E. coli*, 100 μg/mL of ampicillin (Amp; SIGMA, St. Louis, MO) or 35 μg/mL of kanamycin (Km; SIGMA) were added to the media as necessary. *B. pseudomallei* strain 1026b and several derived efflux pump mutants were also used in the study (**Table 4.2**). All work done with *B. pseudomallei* isolates was performed in Select Agent approved Biosafety Level 3 (BSL-3) facilities at the Rocky Mountain Regional Biosafety Laboratory at Colorado State University using Select Agent compliant procedures and protocols. The *B. pseudomallei* 1026bΔ*purM* strain, Bp82, was also used in this study (**Table 4.2**).Bp82 and its derivative strains were grown in LB broth or agar media supplemented with adenine (Ade; SIGMA) at 80 μg/mL. Work with Bp82 was performed at Biosafety Level 2 (BSL-2) as approved by the Colorado State University Institutional Biosafety Committee.

Table 4.1 *Escherichia coli* strains and plasmids used in this study.

Strain	Description		Reference
DH5α	*E. coli* cloning strain (F⁻ Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA*1 *endA*1 *hsd*R17 (r_K^- m_K^+) *phoA supE*44 *thi*-1 *gyrA*96 *relA*1 λ⁻)		(24)
RHO3	*E. coli* conjugation donor strain (F⁻ *thi*-1 *thr*-1 *leuB*6 *lacY*1 *tonA*21 *supE*44 *recA* Δ*asd* Δ*aphA* (chr::RP4-2-Tc::Mu) λ*pir*⁺)		(25)
Plasmid	**Descriptive Name**	**Relevant Properties[1]**	**Source**
pGEM-T Easy		Amp^r; TA cloning vector	Promega (Madison, WI)
pEXKm5		Km^r; Allelic exchange vector	(25)
pPS2951	pGEM-T Easy *folA*(F158V)	Amp^r; 694 bp *folA* PCR product from Bp82.102 ligated into pGEM-T Easy	This study
pPS2552	pGEM-T Easy *folA*(I99L)	Amp^r; 694 bp *folA* PCR product from Bp82.104 ligated into pGEM-T Easy	This study
pPS2959	pEXKm5 *folA*(F158V)	Km^r; 712 bp *Eco*RI fragment from pPS2951, *folA* (F158V), ligated into pEXKm5	This study
pPS2960	pEXKm5 *folA*(I99L)	Km^r; 712 bp *Eco*RI fragment from pPS2952, *folA* (I99L), ligated into pEXKm5	This study
pPS3137	pEXKm5 Δ*bpeT*	Km^r; 1,010 bp Δ*bpeT* SOEing PCR product ligated into pEXKm5	This study
pPS3167	pGEM-T Easy *bpeT*(L265R)	Amp^r; 2,026 bp *bpeT* PCR product from Bp82.103 ligated into pGEM-T Easy	This study
pPS3168	pGEM-T Easy *bpeT*(C310R)	Amp^r; 2,026 bp *bpeT* PCR product from Bp82.102 ligated into pGEM-T Easy	This study
pPS3177	pEXKm5 *bpeT*(C310R)	Km^r; 2,477 bp *Pvu*II fragment from pPS3168, *bpeT* (C310R), ligated into pEXKm5	This study
pPS3178	pEXKm5 *bpeT*(L265R)	Km^r; 2,477 bp *Pvu*II fragment from pPS3167, *bpeT* (L265R), ligated into pEXKm5	This study

[1] **Abbreviations:** Amp^r, ampicillin resistant; Km^r, kanamycin resistant.

Selection for the Km resistance marker in *B. pseudomallei* was performed on media containing 1 mg/ml of Km. Colorimetric screening for the presence of the *gusA* was done on media containing 50 µg/mL of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc; Gold Biotechnology, St. Louis, MO). All strains were incubated at 37°C with aeration, unless otherwise stated.

Table 4.2 *Burkholderia pseudomallei* strains used in this study.

Strain	Description[1]	Reference
Bp82	*B. pseudomallei* prototype strain	(26)
Bp82.102	Bp82 Tmp^r isolate	This study
Bp82.103	Bp82 Tmp^r isolate	This study
Bp82.104	Bp82 Tmp^r isolate	This study
Bp82.183	Bp82 *folA* (F158V)	This study
Bp82.184	Bp82 *folA* (I99L)	This study
Bp82.253	Bp82 Δ*bpeT*	This study
Bp82.268	Bp82 *bpeT* (C310R)	This study
Bp82.269	Bp82 *bpeT* (L265R)	This study
B. pseudomallei efflux mutants		
1026b	*B. pseudomallei* prototype strain	(27)
Bp340	1026b Δ*(amrAB-oprA)*	(28)
Bp227	1026b Δ*(bpeAB-oprB)*	(28)
Bp207	1026b Δ(*amrAB-oprA*, *bpeAB-oprB*)	(28)
Bp58	1026b Δ(*amrAB-oprC*, *bpeR*)	(28)
Bp282	Bp207 *bpeT*(S280P)	T. Mima, unpublished
Bp320	Bp282 Δ(*bpeEF-oprC*)	T. Mima, unpublished

[1]**Abbreviations**: Tmp^r, trimethoprim resistant.

4.3.2 Passive selection of resistant mutants.

Bp82 (trimethoprim MIC = 0.5 µg/mL) was plated onto LB media containing 16 µg/mL of trimethoprim and incubated at 37°C for several days. Similarly, Bp82 was passively selected on solid media containing various concentrations of sulfamethoxazole, ranging from 16 µg/mL

to 2,048 µg/mL. Isolates that grew were subsequently purified on the appropriate selective media and stored at -80˚C for further studies.

4.3.3 Antimicrobial susceptibility testing.

Trimethoprim, sulfamethoxazole and co-trimoxazole minimal inhibitory concentrations (MICs) were determined using the Etest® method following manufacturer's guidelines (AB Biomérieux, Marcy l'Etoile, France). Briefly, strains were grown to mid-log phase (OD_{600nm} = 0.6-0.8) and diluted to a 0.5 McFarland standard in sterile saline. The resulting bacterial cell suspension was swabbed for confluency on Mueller Hinton II agar plates (MHA; Becton Dickinson and Company, Sparks, MD), supplemented with 40 µg/mL of Ade for Bp82 testing, to which the Etest strips were applied. MIC results were determined following 20 h incubation at 37°C. As described in the manufacturer's guidelines, the MIC results were read at 80% inhibition of growth, as judged by eye. MIC breakpoints were used as described by Podnecky *et al*. (18).

The MICs of other antibiotics were determined by microdilution method, following CLSI guidelines (29) with cation-adjusted Mueller Hinton broth (MHB, Becton Dickinson and Company) supplemented with 40 µg/mL of Ade for Bp82 derivatives. MIC results were determined following 20 h incubation at 37°C and were interpreted at 100% inhibition of growth, as judged by eye. Antibiotics for this microdilution MIC testing and the respective suppliers are listed: acriflavine (Acr; SIGMA), carbenicillin (Car; Gemini Bio-Products, West Sacramento, CA), chloramphenicol (Chl; SIGMA), erythromycin (Ery; SIGMA), gentamicin (Gen; SIGMA), norfloxacin (Nor; SIGMA), and tetracycline (Tet; SIGMA).

4.3.4 Reverse-transcriptase quantitative PCR (RT-qPCR)

Relative expression of *bpeF* and *bpeT* mRNA was determined in cultures grown to mid-log phase (O.D.600nm = 0.6-0.8) using gene specific primer sets (**Table 4.3**), as previously described (**Chapter 3**) (18). The 23S rRNA was used as the reference gene for normalization. The relative fold expression compared to the wild-type Bp82 was determined by the Bio-Rad iCycler iQ™ Optical System software version 2.0 with defined amplification efficiencies for each primer set. Comparisons of the expression data were performed by two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons tests using GraphPad Prism version 6.0c for Mac OS X (GraphPad Software, La Jolla, CA). P-values < 0.05 were considered significant.

4.3.5 DNA Sequencing and analysis.

Each of the genes of interest, *folA* and *bpeT*, were PCR amplified from genomic DNA using specific primer sets (**Table 4.3**), as previously described (18). Amplified DNA products were purified using the GenElute gel extraction kit (SIGMA), and sequenced at the Colorado State University Proteomics and Metabolomics facility. Alignments of the DNA sequences from experimental samples and subsequent comparison with the Bp82 sequences were performed using ClustalW2 (30) or Sequencher version 5.1 (31).

4.3.6 Deletion of *bpeT* in Bp82.

To build a deletion construct for the *bpeT* gene, the PCR products of primers P2636 & P2643 and P2638 & P2637 (**Table 4.3**) were assembled by splicing by overlap extension (SOEing) PCR, and the resulting 1,010 bp product was ligated into the pGEM-T easy vector (**Table 4.1**). The resulting plasmid was digested with *Not*I-HF and the 1,052 bp Δ*bpeT* construct

was ligated into the pEXKm5 allelic exchange vector to generate plasmid pPS3137. This plasmid was transformed into RHO3, which was used to mobilize the plasmid into Bp82, as previously described (25). Merodiploids were selected on media containing 1 mg/ml of Km, 50 µg/mL of X-Gluc and 80 µg/mL of Ade. Finally, sucrose counter-selection was used to resolve merodiploids as previously described (25). Putative mutants were screened by PCR to confirm deletion of the *bpeT* gene.

Table 4.3 Oligonucleotides used in this study.

Primer	Descriptive Name[1]	Primer Sequence	Source
M13F-20		5'- GTAAAACGACGGCCAGT -3'	(32)
M13R		5'- AACAGCTATGACCATG -3'	(32)
P1966	*folA*-F	5'- CTTCCGGCCTCTTTTCTTTC -3'	(18)
P1967	*folA*-R	5'- GTGCTGATCGAGCAGATGAC -3'	(18)
P1791	*bpeT*-R	5'- CGACGCATCGCGATGGAAAC -3'	Chapter 2
P1790	*bpeT*-F	5'- ATGGACCGGCTGCAAGCCAT -3'	Chapter 2
Construction of *bpeT* deletion strain			
P2636	Δ*bpeT*-F	5'- AGCGAATAATCGACCGACAC -3'	This study
P2637	Δ*bpeT*-R	5'- GATGACGGACGAGGAAAGC -3'	This study
P2638	Δ*bpeT*-SOE-F	5'- CCAATATCGCGGAGGTAGAGCGGTTAGTCG CGCAGACG -3'	This study
P2643	Δ*bpeT*-SOE-R	5'- CGTCTGCGCGACTAACCGCTCTACCTCCGCGA TATTGG -3'	This study
Reverse transcription quantitative PCR experiments			
P1516	Bp23S-F	5'- GTAGACCCGAAACCAGGTGA -3'	(33)
P1517	Bp23-R	5'- CACCCCTATCCACAGCTCAT -3'	(33)
P1524	*bpeF*-F1-RT	5'- TCCGAGTATCCGGAAGTCGT -3'	(33)
P1525	*bpeF*-R1-RT	5'- GTCCTCGACACCGTTGATCT -3'	(33)
P1814	*bpeT*-RT-F	5'- GAGCTTTCAGGTCAACAACC -3'	T. Mima, unpublished
P1815	*bpeT*-RT-R	5'- GTGAGTGGAATTCGCAGAG -3'	T. Mima, unpublished

[1] **Abbreviations:** SOE, splicing by overlap extension.

4.3.7 Allelic replacement of *folA* and *bpeT* mutations in Bp82.

The pEXKm5 allele replacement system was used to introduce mutations of interest into the Bp82 strain background. Primers P1966 & P1967 (**Table 4.3**) were used to amplify the *folA* gene and flanking regions from both Bp82.102 (F158V) and Bp82.104 (I99L) using the Platinum

Taq DNA Polymerase High Fidelity (Life Technologies Corporation, Grand Island, NY). The 694 bp PCR products were initially cloned into pGEM-T easy and confirmed by DNA sequencing using the M13F-20 and M13R primers. DNA fragments containing the correct *folA* mutations were excised by *Eco*RI digest and ligated into the pEXKm5 plasmid, resulting in plasmids pPS2959 and pPS2960 (**Table 4.1**). These plasmids were transformed into RHO3 and conjugated into Bp82, as previously described (34).

Similarly, primers P2636 & P2637 were used to amplify the *bpeT* gene and flanking regions from both Bp82.102 (C310R) and Bp82.103 (L265R) using the Phusion High-Fidelity PCR Master mix (New England BioLabs, Ipswich, MA). The 2,026 bp PCR products were purified using the GenElute DNA Extraction kit (SIGMA), and single 3' adenine base overhangs were added to the blunt product using a standard Taq DNA polymerase (New England Biolabs). The constructs were then ligated into pGEM-T easy and confirmed by DNA sequencing as described above. The plasmids containing the correct mutant *bpeT* constructs were digested with *Pvu*II and the resulting DNA fragments were ligated into *Sma*I linearized pEXKm5, resulting in plasmids pPS3177 and pPS3178 (**Table 4.1**). These plasmids were transformed into RHO3 and conjugated into Bp82.253, as previously described (34).

Merodiploids for each mutation were selected on LB media containing 80 µg/mL of adenine, 1 mg/mL of Km and 50 µg/mL of X-Gluc. Sucrose counter-selection was used as previously described (25) to resolve the merodiploids. Resolved colonies were screened by PCR and DNA sequencing, to confirm the presence of the desired chromosomal mutant allele.

4.4 Results

4.4.1 Characterization of trimethoprim resistant strains.

Following selection of Bp82 on solid media containing trimethoprim, we obtained a collection of isolates with decreased trimethoprim susceptibilities. Of these, 3 isolates were selected at random for further testing and characterization, Bp82.102, Bp82.103 and Bp82.104 (**Table 4.2**). The MICs of these isolates were above the detection limit of the assay for trimethoprim (≥ 32 µg/mL). Isolates Bp82.102 and Bp82.103 had increases in the co-trimoxazole MICs as well, but were not resistant to the combination (**Table 4.4**). In addition to the folate pathway inhibitors, we also determined the MICs of these strains for antimicrobials from several other classes. The Bp82.102 and Bp82.103 strains had increased MICs for several antimicrobials that are known substrates of the BpeEF-OprC efflux pump: acriflavine, chloramphenicol, norfloxacin and tetracycline (T. Mima and H.P. Schweizer, unpublished findings), whereas Bp82.104 did not and was more susceptible to norfloxacin than the Bp82 parent strain. While there were slight differences in MIC, there were no major changes in susceptibility of the strains to carbenicillin, erythromycin or gentamicin (**Table 4.4**).

To confirm that the BpeEF-OprC efflux pump was contributing to the increased MICs observed in strain Bp82.102 and Bp82.103, RT-qPCR was used to determine the relative expression of *bpeF* and *bpeT* mRNA in the trimethoprim resistant strains compared to the Bp82 parental strain. There was a significant increase in *bpeF* and *bpeT* mRNA expression in strains Bp82.102 and Bp82.103, but no change in Bp82.104 (**Figure 4.1**). Both Bp82.102 and Bp82.103 had similar expression profiles with an over 35 fold increase in *bpeF* mRNA and over 2 fold increase in *bpeT* mRNA compared to Bp82. These results are consistent with the MIC results (**Table 4.4**).

Table 4.4 Minimal inhibitory concentrations of trimethoprim resistant Bp82 isolates.

Minimal Inhibitory Concentrations[1] (μg/mL)									
Strain	Car	Ery	Gen	Acr	Chl	Nor	Tet	Tmp[2]	SXT
Bp82	256	256	256	32	16	16	4	0.75	0.094
Trimethoprim-selected Bp82 derivatives									
Bp82.102	512	256	256	64	128	32	8	≥ 32	1.5
Bp82.103	512	512	256	64	128	16	8	≥ 32	1.0
Bp82.104	512	128	256	32	8	4	2	≥ 32	0.125

[1]**Abbreviations:** Car, carbenicillin; Ery, erythromycin; Gen, gentamycin; Acr, acriflavine; Chl, chloramphenicol; Nor, norfloxacin; Tet, tetracycline; Tmp, trimethoprim; SXT, co-trimoxazole (**x** trimethoprim + 19**x** sulfamethoxazole).
[2] The detection limit for the Etest® method for trimethoprim is 32 μg/mL.

4.4.2 BpeT mutations cause BpeEF-OprC overexpression and resistance.

The *bpeT* gene encodes a LysR-regulator that controls expression of BpeEF-OprC. Previous work in our lab has demonstrated that mutations to this regulator can cause significant overexpression of the efflux pump (T. Mima and H.P. Schweizer, unpublished findings). To determine if mutations to *bpeT* were the cause of the observed overexpression of *bpeEF-oprC* and *bpeT*, the *bpeT* gene was sequenced in the 3 trimethoprim resistant isolates. In comparison to the Bp82 wild-type sequence, we identified 2 single nucleotide polymorphisms (SNPs) in *bpeT* resulting in amino acid substitutions: a cysteine to arginine change at position 310 in strain Bp82.102 and a leucine to arginine at position 265 in Bp82.103. Both of these mutations are in the co-inducer binding domain of BpeT. There were no *bpeT* mutations found in the Bp82.104 trimethoprim resistant strain.

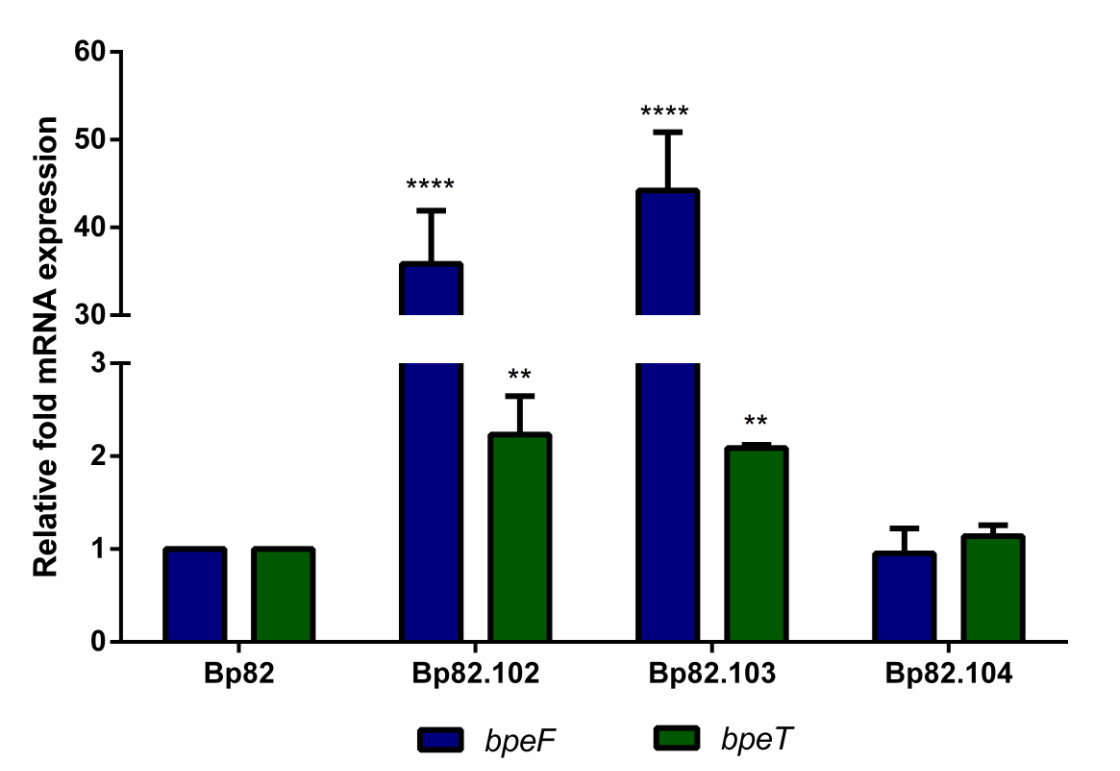

Figure 4.1 *bpeF* and *bpeT* mRNA expression is significantly elevated in some trimethoprim resistant Bp82 mutants. The relative *bpeF* (**blue**) and *bpeT* (**green**) expression was determined in 3 Bp82-derived isolates. All fold expression values are relative to the Bp82 parent strain. Error bars indicate the standard deviation between three biological replicates, which were each performed in technical triplicate. Statistical significance is indicated above (****, $p \leq 0.0001$; **, $p \leq 0.01$).

To confirm that these mutations were causing the observed overexpression of *bpeF* and *bpeT* mRNA, they were individually introduced into the Bp82 wild-type background using allele replacement techniques. The resulting strains Bp82.268 (BpeT C310R) and Bp82.269 (BpeT L265R) were tested by RT-qPCR to compare the relative expression of *bpeF* and *bpeT* mRNA. These experiments demonstrated that introduction of the L265R and C310R mutations into Bp82 resulted in a similar mRNA expression profile as that found in the original trimethoprim resistant strains (**Figure 4.2**). The expression levels of *bpeF* mRNA were all near or above 30-fold higher than Bp82, while *bpeT* expression was lower and ranged from below 1-fold to above 2-fold compared to Bp82.

The MICs for trimethoprim, sulfamethoxazole and co-trimoxazole were determined in the Bp82.268 and Bp82.269 strains. Both *bpeT* SNPs caused a greater than 4-fold increase in trimethoprim MICs (**Table 4.5**). Additionally there was an increase to the co-trimoxazole MICs and a small increase in the sulfamethoxazole MICs. These data suggest that both BpeT amino acid changes, C310R and L265R, cause overexpression of the BpeEF-OprC efflux pump, which in turn results in reduced susceptibility to trimethoprim.

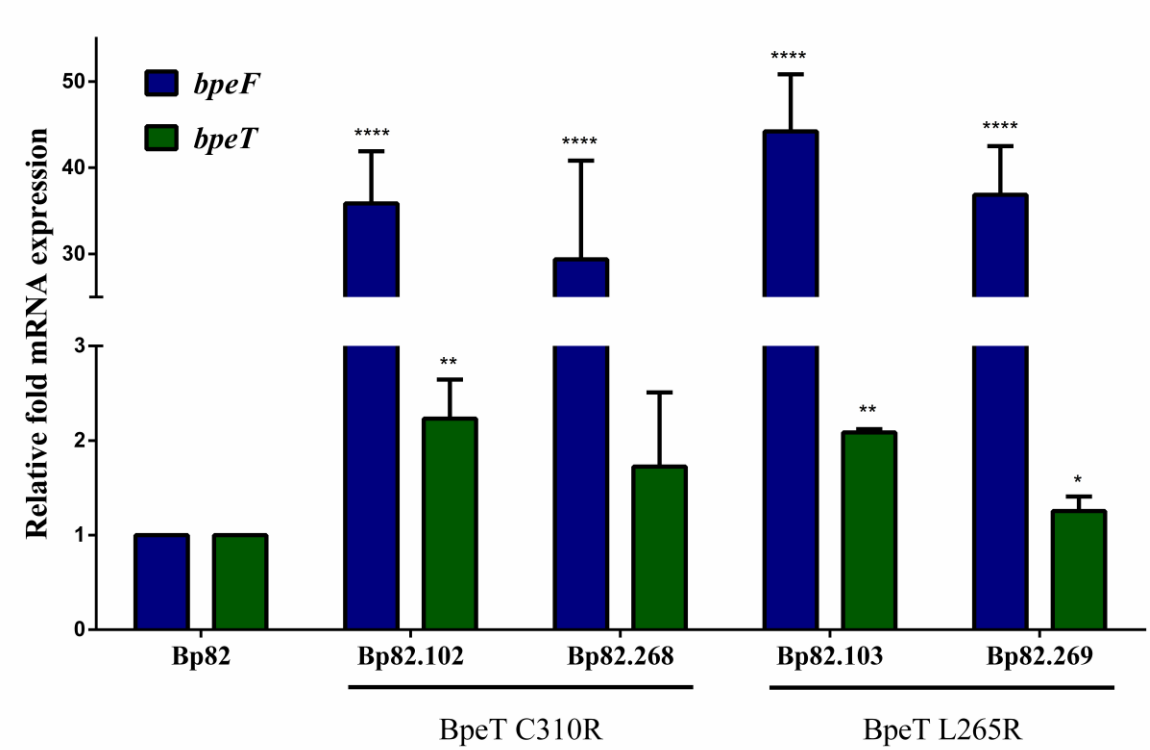

Figure 4.2 BpeT mutations cause significant increases in *bpeF* mRNA levels. The relative *bpeF* (**blue**) and *bpeT* (**green**) expression was determined in trimethoprim resistant Bp82-derived isolates (Bp82.102 and Bp82.103), and the Bp82 mutants containing either the BpeT C310R or L265R mutation (Bp82.268 or Bp82.269, respectively). All fold expression values are relative to the Bp82 parent strain. Error bars indicate the standard deviation between three biological replicates, which were each performed in technical triplicate. Statistical significance is indicated above (****, $p \leq 0.0001$; **, $p \leq 0.01$; *, $p < 0.5$).

Table 4.5 Mutations to BpeT increase trimethoprim minimal inhibitory concentrations.

MIC (μg/mL)				
Strain	BpeT mutation	Tmp	Smx	SXT
Bp82	—	0.75	4	0.094
Bp82.268	C310R	4	8	0.38
Bp82.269	L265R	4	8	0.38

Abbreviations: MIC, minimal inhibitory concentration; Smx, sulfamethoxazole; SXT, co-trimoxazole (**x** Tmp + 19**x** Sulfamethoxazole); Tmp, trimethoprim.

4.4.3 Mutations to the dihydrofolate reductase result in trimethoprim resistance.

DNA sequencing of the *folA* gene identified 2 SNPs encoding for amino acid substitutions in the dihydrofolate reductase. Strain Bp82.102 had a valine in place of phenylalanine at position 158, while strain Bp82.104 had a mutation resulting in a leucine instead of isoleucine at position 99. Interestingly, strain Bp82.102 had both a BpeT and FolA mutation. To determine if these mutations to *folA* confer trimethoprim resistance, allelic replacement was used to introduce each of these SNPs into the wild-type Bp82 background. The trimethoprim and co-trimoxazole MICs were then determined for each of these strains (**Table 4.6**).

Table 4.6 Mutations to *folA* increase trimethoprim minimal inhibitory concentrations.

MIC (μg/mL)			
Strain	FolA mutation	Tmp[1]	SXT
Bp82	—	0.75	0.094
Bp82.184	I99L	≥ 32	0.5
Bp82.183	F158V	24	0.5

Abbreviations: MIC, minimal inhibitory concentration; SXT, co-trimoxazole (**x** Tmp + 19**x** Sulfamethoxazole); Tmp, trimethoprim.
[1] The detection limit for the Etest® method for trimethoprim is 32 μg/mL.

Both *folA* mutations caused increased trimethoprim MICs, such that the mutant strains were resistant (MIC > 8 μg/mL) to the antimicrobial. The I99L conferred a trimethoprim MIC

above the limit of detection. Additionally, as a result of these mutations we observed increases in the co-trimoxaozle MIC, though the strains were not resistant (MIC > 2 μg/mL) to this combination.

Interestingly, in subsequent experiments Bp82 deletion mutants lacking either the BpeEF-OprC efflux pump or its cognate regulator, BpeT, were also selected on trimethoprim. Several of the resulting trimethoprim resistant isolates were tested, and in all of these isolates (n = 12) the FolA I99L mutation was present (data not shown).

4.4.4 Bp82 can tolerate high concentrations of sulfamethoxazole.

Following a similar experimental design, several unproductive attempts were made to isolate Bp82 derived mutants resistant to sulfamethoxazole using very high concentrations of sulfamethoxazole, up to 2 mg/mL. While we were able to obtain isolates that grew with high concentrations of sulfamethoxazole, the sulfamethoxazole MICs of most of the strains were over 10-fold lower than the concentrations used for selection (data not shown). The phenotype of these isolates suggested that *B. pseudomallei* may be able to tolerate sulfamethoxazole even at very high concentrations, though further investigation would be necessary to fully understand these findings.

4.5 Discussion

Burkholderia pseudomallei is a Gram-negative pathogen and the etiologic agent of melioidosis. While this disease is primarily confined to regions in which it is endemic, it is a significant risk to travelers and *B. pseudomallei* has the potential to be used as a biothreat agent. Current recommendations advise a lengthy eradication phase, following initial treatment, with a

combination of trimethoprim and sulfamethoxazole (7). This combination is also recommended for post-exposure prophylaxis in the event of accidental exposure.

In this current study we identified mutations to the trimethoprim drug target, dihydrofolate reductase (FolA), which conferred resistance to trimethoprim alone. Drug target alterations have been documented as effective resistance mechanisms to trimethoprim in several other instances (21, 22), however to our knowledge this is the first report of such a mutation in *B. pseudomallei*. The I99L FolA mutation has been previously described in *Burkholderia cenocepacia* (35). Additionally the equivalent mutation (I94L) was also described in mutator *E. coli* isolates selected on trimethoprim (36). Mutator strains are bacterial strains deficient in DNA repair mechanisms, often methyl-directed mismatch repair (MMR) (37) and thus have a higher than usual rate of genetic mutation. The I94L mutation was found in 8 independently selected trimethoprim resistant mutants that were derived from 4 strains with various deleted MMR genes (36). There is very little in the literature about the F158V FolA mutation, although the equivalent residue in *E. coli* (F153) has been shown to be associated with a β-bulge region. Mutation to this residue may result in a conformational change to the dihydrofolate reductase inhibiting the ability of trimethoprim to bind to the enzyme (38, 39).

In addition to these mutations, we also observed SNPs in the *bpeT* gene. BpeT is a LysR-type transcriptional regulator of BpeEF-OprC efflux pump expression which has been shown to efflux trimethoprim (18, 19) (Mima, T. and Schweizer H.P, unpublished observations). The BpeT mutations were located in the C-terminal co-inducer binding domain. BpeT mutations were found in isolates that were trimethoprim resistant and also had decreased susceptibility to several known substrates of the BpeEF-OprC efflux pump. These mutations were linked to over-expression of the BpeEF-OprC efflux pump. While we cannot confirm that the described

mutations to *folA* and *bpeT* were the only mutations acquired in these strains during the selection on trimethoprim, the introduction of each of these mutations individually into the wild-type Bp82 background allowed us to demonstrate that each mutation contributes to decreased trimethoprim susceptibility. Decreases in co-trimoxazole susceptibility were observed in these strains, but all remained susceptible to the antimicrobial combination.

Finally, several attempts were made to investigate potential mechanisms of sulfamethoxazole resistance in *B. pseudomallei*. However, in contrast to the selection on trimethoprim, the ability to grow at high concentrations of the antimicrobial was not coupled with large changes in the sulfamethoxazole MIC. As there were slight differences in the sulfamethoxazole MICs observed in the trimethoprim resistant isolates, we determined the sulfamethoxazole MICs for a collection of efflux pump mutants derived from 1026b with varying expression levels of 3 efflux pumps: AmrAB-OprA, BpeAB-OprB and BpeEF-OprC. The MIC data did not provide any definitive answers, but suggested sulfamethoxazole may be effluxed at a low rate by the BpeEF-OprC and BpeAB-OprB pumps, as there were 2-fold changes observed between strains overexpressing and not expressing these pumps (data not shown). However, it is also possible that these changes are simply due to variable fitness of each strain caused by the expression or lack of various efflux pumps. Further studies are necessary to elucidate potential mechanisms of sulfamethoxazole resistance in *B. pseudomallei*.

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CHAPTER 5: Investigation of Adaptive Co-Trimoxazole Resistance Mechanisms in *Burkholderia pseudomallei*

This chapter describes the identification of two novel resistance determinants responsible for decreased susceptibility to trimethoprim, sulfamethoxazole and the drug combination co-trimoxazole. Here we identify and propose the name BpeS for a novel regulator of BpeEF-OprC efflux pump expression, a key contributor to decreases in both trimethoprim and sulfamethoxazole susceptibilities. We also identified mutations to a gene encoding a putative pteridine reductase, *ptr1*. Mutations to *ptr1* were found in all of the co-trimoxazole resistant isolates, suggesting a role for this gene in folate synthesis and folate pathway inhibitor resistance in *Burkholderia pseudomallei*. These findings are applied in **Chapter 6** to investigate a collection of clinical and environmental strains with decreased co-trimoxazole susceptibilities.

5.1 Summary

Burkholderia pseudomallei, the causative agent of the disease melioidosis, poses a serious threat as it is resistant to many antimicrobials. Due to the propensity for melioidosis infections to be difficult to treat, a combination therapy of trimethoprim and sulfamethoxazole, co-trimoxazole, is commonly used for the eradication phase and prophylaxis treatment of melioidosis. Co-trimoxazole resistant strains of *B. pseudomallei* have been reported in endemic regions, but to our knowledge there has been no description of the underlying resistance mechanisms. In this study we selected spontaneous co-trimoxazole resistant derivatives of the Select Agent excluded *B. pseudomallei* strain Bp82. The resulting isolates had decreased susceptibilities to co-trimoxazole and both trimethoprim and sulfamethoxazole individually.

Using reverse transcriptase quantitative PCR, we discovered that these isolates had significantly increased expression of the BpeEF-OprC efflux pump. Targeted deletion of this efflux pump confirmed that it was essential for the observed phenotype. DNA sequencing of the trimethoprim and sulfamethoxazole drug targets and known regulatory components of the BpeEF-OprC efflux pump offered no explanation for the observed resistant phenotype. However, using whole-genome sequencing, mutations to genes encoding a novel BpeEF-OprC efflux pump regulator, *bpeS*, and a pteridine reductase, *ptr1*, were observed. Repair of these point mutations restored co-trimoxazole sensitivity to the mutant strains. This is the first study to implicate sulfamethoxazole as a substrate of the BpeEF-OprC efflux pump in *B. pseudomallei*. Efflux was required but not sufficient to confer resistance to co-trimoxazole. Additionally, in the course of this study, we determined that both *bpeS* and *ptr1* are non-essential genes for growth *in vitro*.

5.2 Introduction

Burkholderia pseudomallei is a soil-borne bacterium endemic to tropical and sub-tropical regions of the world (1, 2). It is a Gram-negative opportunistic pathogen and the etiologic agent of the disease melioidosis (2-4). Melioidosis has a highly variable clinical presentation, ranging from minor wound infection to severe, often life-threatening, septicemia and pneumonia (4). *B. pseudomallei* infections are challenging to treat, primarily due to the organism's intrinsic resistance to many classes of antimicrobials (5, 6). Previous studies have suggested that relapse is very common among survivors of this disease (7, 8), which resulted in several changes in the clinical treatment of melioidosis. In order to improve the efficacy of treatment and reduce the risk of relapse, the current recommendations include a lengthy eradication phase following initial treatment (9-11). The eradication phase typically consists of 12 to 20 weeks of oral co-trimoxazole with or without doxycycline, or alternatively co-amoxiclav can be used (11). Co-

trimoxazole is a potent combination of the folate pathway inhibitors trimethoprim and sulfamethoxazole and is also recommended for post-exposure prophylaxis (10, 11). However, there have been reports of clinical isolates of *B. pseudomallei* that are resistant to co-trimoxazole (12-16). The overall frequency of co-trimoxazole resistance ranges from 2.5% to 16%. Variations in the reported frequencies may, in part, be a function of the method used for antimicrobial susceptibility testing (13, 14). While previous studies have investigated the molecular mechanisms of trimethoprim resistance in *B. pseudomallei* (**Chapter 3** and **Chapter 4**) (17), to our knowledge, there have been no studies aimed at investigating co-trimoxazole resistance in *B. pseudomallei*. Based on previous studies, we know that the BpeEF-OprC efflux pump is capable of conferring resistance to trimethoprim (17), as are mutations to the trimethoprim drug target, dihydrofolate reductase, FolA (**Chapter 4**). However, very little is known about sulfamethoxazole resistance in *B. pseudomallei*. In other species of bacteria, such as *Haemophilus influenza* (18), *Streptococcus pneumonia* (19), *Escherichia coli*, *Streptococcus pyogenes* and *Neisseria meningitides* (20), mutations to the sulfamethoxazole target, dihydropteroate synthetase (FolP), have been shown to confer resistance to sulfonamides (18, 20-22). One can then postulate that mutations to both FolA and FolP or a combination of efflux and mutations to FolP could cause co-trimoxazole resistance in *B. pseudomallei*. The focus of this work was to investigate potential co-trimoxazole resistance mechanisms in *B. pseudomallei*.

5.3 Materials and Methods

5.3.1 Bacterial Strains.

The attenuated, Select Agent excluded, Bp82 strain of *Burkholderia pseudomallei* was used for all experiments in this study (**Table 5.1**). The work done with Bp82 was performed in Biosafety Level 2 (BSL-2) facilities, as approved by the Colorado State University Institutional Biosafety Committee. Bp82 and its derived strains were grown in Lennox Luria Bertani media (LB; Mo Bio Laboratories, Carlsbad, CA) supplemented with 80 μg/mL of adenine (Ade; SIGMA, St. Louis, MO). *Escherichia coli* strains DH5α and RHO3 were used for plasmid DNA manipulation and mobilization, respectively (**Table 5.2**). *E. coli* strains were grown in LB, which was supplemented with diaminopimelic acid (DAP, SIGMA) at 400 μg/mL when cultivating RHO3. The addition of 50 μg/mL of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc; Gold Biotechnology, St. Louis, MO) allowed colorimetric screening for the presence of the *gusA* gene in both pEXKm5-containing strains and merodiploids. To induce transcription of genes controlled by the P_{tac} promoter, 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Gold Biotechnology) was added to the media. For selection of desired plasmids in *E. coli* strains, 100 μg/mL of ampicillin (Amp; SIGMA), 15 μg/mL of gentamicin (Gm; SIGMA), 35 μg/mL of kanamycin (Km; SIGMA), or 15 μg/mL of Zeocin (Zeo; Life Technologies, Carlsbad, CA) were added to the media as necessary. However, in Bp82 selection of resistance markers was performed at much higher concentrations: 2 mg/mL of Gm, 1 mg/mL of Km and 2 mg/mL of Zeo. All cultures were grown at 37°C with aeration, unless otherwise noted.

Table 5.1 Bacterial strains used in this study.

Strain	Description	Reference
DH5α	*E. coli* cloning strain (F^- Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA*1 *endA*1 *hsd*R17 (r_K^- m_K^+) *phoA supE*44 *thi*-1 *gyrA*96 *relA*1 λ^-)	(24)
RHO3	*E. coli* conjugation donor strain (F^- *thi*-1 *thr*-1 *leuB*6 *lacY*1 *tonA*21 *supE*44 *recA* Δ*asd* Δ*aphA* (chr::RP4-2-Tc::Mu) λ*pir*$^+$)	(25)
Bp82	*B. pseudomallei* Δ*purM* derivative	(23)
Bp82.191-209	Bp82 SXT^r isolates	This study
Repair of *ptr1* (V15G) and *bpeS* (K267T) SNPs in SXT^r isolates		
Bp82.202	Bp82 *ptr1* (V15G), *bpeS* (K267T)	This study
Bp82.246	Bp82.202 *ptr1* (V15G), ***bpeS* (WT)**	This study
Bp82.247	Bp82.202 ***ptr1* (WT)**, *bpeS* (K267T)	This study
Bp82.248	Bp82.202 ***ptr1* (WT)**, ***bpeS* (WT)**	This study
Bp82.204	Bp82 *ptr1* (V15G), *bpeS* (K267T)	This study
Bp82.249	Bp82.204 *ptr1* (V15G), ***bpeS* (WT)**	This study
Bp82.250	Bp82.204 ***ptr1* (WT)**, *bpeS* (K267T)	This study
Bp82.251	Bp82.204 ***ptr1* (WT)**, ***bpeS* (WT)**	This study
Knockout of *ptr1* and *bpeS* in Bp82		
Bp82.256	Bp82Δ*ptr1*::*FRT*-Km^r-*FRT*	This study
Bp82.262	Bp82Δ*ptr1*::*FRT*	This study
Bp82.263	Bp82Δ*bpeS*::*FRT*-Km^r-*FRT*	This study
Bp82.264	Bp82Δ*bpeS*::*FRT*	This study

[1]**Abbreviations:** *FRT*, Flp recombinase target; Km, kanamycin; ^{r}, resistant; SNP, single nucleotide polymorphism; SXT, co-trimoxazole (trimethoprim:sulfamethoxazole, 1:19); WT, wild-type Bp82 sequence.

5.3.2 Passive step-wise selection of co-trimoxazole resistant mutants.

Co-trimoxazole is a combination of trimethoprim (Tmp) and sulfamethoxazole (Smx) at a 1:19 ratio. For all co-trimoxazole (SXT) concentrations the formula is: **x** µg/mL SXT = **x** µg/mL Tmp + 19**x** µg/mL Smx. Spontaneous mutants of the attenuated Bp82 strain were selected by serial passage in increasing concentrations of SXT. Briefly, Bp82 was grown overnight in LB broth, then sub-cultured 1:100 into LB broth containing 0.064 µg/mL SXT. Successive subcultures were performed into fresh broth with 4-fold increases of co-trimoxazole ending at 8 µg/mL SXT. The bacterial culture was plated on LB agar containing 8 µg/mL SXT.

Table 5.2 Plasmids used in this study.

Plasmid	Descriptive Name	Relevant Properties[1]	Source
pGEM-T Easy		Amp^r; TA cloning vector	Promega (Madison, WI)
pEXKm5		Km^r; Allelic exchange vector	(25)
pEXGm5B		Gm^r; Allelic exchange vector	(26)
pFLPe2		Zeo^r; T^s, inducible expression of Flp recombinase	(27)
pPS2593	pBADSce-T	Zeo^r; inducible expression of I-*Sce*I homing endonuclease, *oriT*	B. Kvitko, unpublished
pFKm2		Amp^r, Km^r; Source of *FRT* flanked Km^r marker (*FRT*-Km^r-*FRT*)	(27)
Deletion constructs			
pPS2591	pEXKm5Δ(*bpeEF-oprC*)	Km^r; Allelic exchange vector for deletion of *bpeEF-oprC*	(17)
pPS2481	pUC18T-mini-Tn*7*T-P_{tac}	Km^r; mini-Tn*7*T-P_{tac} empty control vector	(28)
pPS2670	pPS2481-*bpeEF-oprC*	Km^r; mini-Tn*7*T-P_{tac}-*bpeEF-oprC* complementation vector	(17)
pPS2647	pEXKm5Δ*bpeT*	Km^r; Allelic exchange vector for deletion of *bpeT*	(17)
pPS2280	pUC18T-mini-Tn*7*T	Km^r; mini-Tn*7*T empty control vector	(29)
pPS2787	pPS2280-P_{bpeT}-*bpeT*	Km^r; mini-Tn*7*T-P_{bpeT}-*bpeT* complementation vector	(17)
pPS3130	pGEM-T Easy Δ*ptr1*	Amp^r; 1,236 bp Δ*ptr1* SOEing PCR product ligated into pGEM-T Easy	This study
pPS3140	pGEM-T Easy Δ*ptr1*::*FRT*-Km^r-*FRT*	Amp^r; 1,514 bp *FRT*-Km^r-*FRT* fragment from pFKm2, ligated into *Hin*dIII site of pPS3130	This study
pPS3144	pEXGm5B Δ*ptr1*::*FRT*-Km^r-*FRT*	Km^r, Gm^r; 2,784 bp *Not*I fragment from pPS3140, Δ*ptr1*::*FRT*-Km^r-*FRT*, ligated into pEXGm5B	This study
pPS3127	pGEM-T Easy Δ*bpeS*::*FRT*-Km^r-*FRT*	Amp^r; 2,641 bp Δ*bpeS*::*FRT*-Km^r-*FRT* SOEing PCR ligated into pGEM-T Easy	This study
pPS3158	pEXGm5B Δ*bpeS*::*FRT*-Km^r-*FRT*	Km^r, Gm^r; 2,670 bp *Not*I fragment from pPS3127, Δ*bpeS*::*FRT*-Km^r-*FRT*, ligated into pEXGm5B	This study
Repair of *ptr1* (V15G) and *bpeS* (K267T) SNPs in Bp82.202 and Bp82.204			
pPS3099	pGEM-T Easy *ptr1* (WT)	Amp^r; 1,560 bp *ptr1* PCR product from Bp82 ligated into pGEM-T Easy	This study
pPS3093	pEXKm5 *ptr1* (WT)	Km^r; 1,595 bp *Not*I fragment from pPS3099, *ptr1* (WT), ligated into pEXKm5	This study
pPS3097	pGEM-T Easy *bpeS* (WT)	Amp^r; 1,456 bp *bpeS* PCR product from Bp82 ligated into pGEM-T Easy	This study
pPS3090	pEXKm5 *bpeS* (WT)	Km^r; 1,490 bp *Not*I fragment from pPS3097, *bpeS* (WT), ligated into pEXKm5	This study

[1]**Abbreviations:** Amp, ampicillin; Km, kanamycin; Gm, gentamicin; Zeo, Zeocin; T^s, temperature sensitive; ^{r}, resistant; WT, wild-type Bp82 sequence.

Isolated colonies were patched onto LB agar containing 16 µg/mL SXT and subsequently stored at -80˚C for further studies.

5.3.3 Antimicrobial susceptibility testing.

Minimal inhibitory concentrations (MIC) were determined for trimethoprim, sulfamethoxazole and co-trimoxazole by the Etest® method, following manufactures guidelines (AB Biomérieux, Marcy l'Etoile, France) on Mueller Hinton II agar (Becton Dickinson, Franklin Lakes, NJ) supplemented with 40 µg/mL of Ade. Previously described MIC breakpoints were used to define susceptibility and resistance to trimethoprim, sulfamethoxazole and co-trimoxazole (17, 30). MICs for other antimicrobials were determined by standard microdilution in Mueller Hinton II broth (Becton Dickinson) supplemented with 40 µg/mL Ade, following CLSI guidelines (30). Antibiotics for microdilution MIC testing and the respective suppliers are listed: acriflavine (Acr, SIGMA), carbenicillin (Car, Gemini Bio-Products, West Sacramento, CA), chloramphenicol (Chl, SIGMA), erythromycin (Ery, SIGMA), gentamicin (Gm, SIGMA), norfloxacin (Nor, SIGMA), and tetracycline (Tet, SIGMA). Microtiter plates were incubated at 37°C in ambient air for 16-20 h before MIC determination. Etests were read at 80% inhibition, while microdilution was read at 100% inhibition of growth, as judged by eye. MICs were tested in a minimum of 3 replicates and final results were reported as the mode of the replicates.

5.3.4 Reverse-transcriptase quantitative PCR (RT-qPCR).

Expression levels of *bpeF* and *bpeT* mRNA in cultures grown to mid-log phase were analyzed as previously described (**Chapter 3**) (17). The following oligonucleotide sets were used for amplification of PCR fragments containing the genes of interest: P1516 & P1517 for 23S ribosomal RNA (rRNA), P1524 & P1525 for *bpeF*, and P1814 & P1815 for *bpeT* (**Table**

5.3). The fold expression of each gene relative to the wild-type Bp82 reference was determined by the Bio-Rad iCycler iQ™ Optical System software version 2.0 with defined amplification efficiencies for each primer set. Comparisons of the relative mRNA expression data were analyzed by two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons tests using GraphPad Prism version 6.0c for Mac OS X (GraphPad Software, La Jolla, CA). P-value < 0.05 were considered significant.

5.3.5 DNA sequencing and analysis.

DNA sequencing of specific genes was performed as previously described (**Chapter 3**) (17). Briefly, the gene encoding antimicrobial targets, *folA* (dihydrofolate reductase – trimethoprim target) and *folP* (dihydropteroate synthetase – sulfamethoxazole target), and regulatory components of BpeEF-OprC expression, *bpeT* (regulator) and *bpeT-llpE* (putative promoter region), were PCR amplified in four independent PCR reactions from genomic DNA isolated using the PureGene Core kit A (QIAGEN, Valencia, CA) using specific primer sets (**Table 5.3**). PCR reactions were pooled and sequenced at the Colorado State University Proteomic and Metabolomics core facility using gene-specific primers. Alignment of the sequencing reads and subsequent comparisons were performed using Sequencher version 5.1 (Gene Codes Corporation, Ann Arbor, MI) (31).

Table 5.3 Oligonucleotides used in this study.

Primer	Descriptive Name[1]	Primer Sequence[2]	Source
M13F-20		5'- GTAAAACGACGGCCAGT-3'	(32)
M13R		5'- AACAGCTATGACCATG-3'	(32)
P1966	*folA*-F	5'- CTTCCGGCCTCTTTTCTTTC-3'	(17)
P1967	*folA*-R	5'- GTGCTGATCGAGCAGATGAC-3'	(17)
P2323	*folP*-F	5'- CCAGATCAACGACATCATGG-3'	This study
P2324	*folP*-R	5'- CGAGCATATAGCCCGATACC-3'	This study
P1791	*bpeT*-R	5'- CGACGCATCGCGATGGAAAC-3'	(17)
P1790	*bpeT*-F	5'- ATGGACCGGCTGCAAGCCAT-3'	(17)
P2570	*bpeS*-F1	5'- GGATGACTTCGGCGCTATC-3'	This study
P2571	*bpeS*-R1	5'- CCGTTCAACCTGACCTCAAC-3'	This study
P2572	*bpeS*-F2	5'- GTCTTCCGCCAGCGCTAC-3'	This study
P2573	*bpeS*-R2	5'- AAGCCGATTCATCTGGACAC-3'	This study
P2592	*ptr1*-F1	5'- CTCGCTCACGCTGATTGC-3'	This study
P2575	*ptr1*-R1	5'- CGTCGATGCGGTCTATACG-3'	This study
P2576	*ptr1*-F2	5'- ATCGAAGCTCGGCAGGTG-3'	This study
P2577	*ptr1*-R2	5'- CGCGCCTACGAGGAGTTC-3'	This study
Construction of *bpeEF-oprC* and *bpeT* deletion strains, complementation vectors and determination of chromosomal mini-Tn*7* insertion sites			
P1989	Δ*bpeEF-oprC*-F	5'- GGAAGTACGCGGACTTCGC-3'	(25)
P1990	Δ*bpeEF-oprC*-R	5'- GCATCAACCTCGGCTACACG-3'	(25)
P479	Tn*7*L	5'- ATTAGCTTACGACGCTACACCC-3'	(29)
P1509	*BpglmS*-1	5'- GAGGAGTGGGCGTCGATCAAC-3'	(29)
P1510	*BpglmS*-2	5'- ACACGACGCAAGAGCGGAATC-3'	(29)
P1511	*BpglmS*-3	5'- CGGACAGGTTCGCGCCATGC-3'	(29)
Reverse transcription quantitative PCR experiments			
P1516	Bp23S-F	5'- GTAGACCCGAAACCAGGTGA-3'	(33)
P1517	Bp23-R	5'- CACCCCTATCCACAGCTCAT-3'	(33)
P1524	*bpeF*-F1-RT	5'- TCCGAGTATCCGGAAGTCGT-3'	(33)
P1525	*bpeF*-R1-RT	5'- GTCCTCGACACCGTTGATCT-3'	(33)
P1814	*bpeT*-RT-F	5'- GAGCTTTCAGGTCAACAACC-3'	T. Mima, unpublished
P1815	*bpeT*-RT-R	5'- GTGAGTGGAATTCGCAGAG-3'	T. Mima, unpublished
bpeS deletion			
P2618	Δ*bpeS*-F1	5'- CCTGAAGCAGCAACAGCAC-3'	This study
P2619	Δ*bpeS*-R1 (SOE)	5'- TCAGAGCGCTTTTGAAGCTAATTCGATATC GATAGCGCCGAAGTC-3'	This study
P2620	Km^r-F1 (SOE)	5'- GACTTCGGCGCTATCGATATCGAATTAGCT TCAAAAGCGCTCTGA-3'	This study
P2621	Km^r-R1 (SOE)	5'- GCTTCCTTCGCTTCGATGCGAATTGGGGAT CTTGAAGTACCT-3'	This study

Primer	Descriptive Name[1]	Primer Sequence[2]	Source
P2622	Δ*bpeS*-F2 (SOE)	5'-AGGTACTTCAAGATCCCCAATTCGCATCGA AGCGAAGGAAGC-3'	This study
P2623	Δ*bpeS*-R2	5'-CTTTCGCGTGAACGATCC-3'	This study
*ptr1*deletion			
P2640	Δ*ptr1*-F1	5'-CGAGCCGCGACGAAG-3'	This study
P2639	Δ*ptr1*-R1 (SOE)	5'-TCACTGCTTGCCGTCC**AAGCTT**AGGTGTTC GTCAGGTTGACC-3'	This study
P2642	Δ*ptr1*-F2 (SOE)	5'-GGTCAACCTGACGAACACCT**AAGCTT**G GACGGCAAGCAGTGA-3'	This study
P2641	Δ*ptr1*-R2	5'-CATCGACCACGGCACG-3'	This study

[1] **Abbreviations:** SOE, splicing by overlap extension; Km^r, kanamycin resistance.
[2] Bold letters indicate an introduced *Hin*dIII restriction enzyme recognition site.

5.3.6 Whole genome sequencing and analysis.

Previously extracted genomic DNA samples (see above) for Bp82, Bp82.202 and Bp82.204 were prepared for paired-end sequencing on the Illumina GAIIx Genome Analyzer (Illumina, Inc, San Diego, CA) using the Kapa Biosystems library preparation kit (Woburn, MA, catalog #KK8201) protocol with an 8 bp index modification. Briefly, 2 µg double-stranded DNA from each sample was sheared to an average size of 350 bp and then input into the Kapa Illumina paired end library preparation protocol. Modified oligonucleotides (Integrated DNA Technologies, Coralville, IA) that provide 8 bp indexing capability (34), were substituted at the appropriate step. Prior to sequencing the libraries were quantified with qPCR on the ABI 7900HT (Life Technologies Corporation, Carlsbad, CA) using the Kapa Library Quantification Kit (Catalog #KK4835). The libraries were sequenced to a read length of 100 bp on the Illumina GAIIx Genome Analyzer, yielding 13.2 M, 18.4 M and 20.8 M reads, respectively. The DNASTAR SeqMan NGen application (Madison, WI) was used to analyze the Illumina sequence data, using default settings. For SNP analysis, the sequence read data was aligned to the *B. pseudomallei* 1026b reference genome (NC_017831.1, NC_017832.1.) SNPs positions identified were required to have > 10X coverage depth and > 90% variant base calls.

5.3.7 Construction of gene deletion mutants in Bp82.

The *bpeEF-oprC* structural efflux pump genes and the *bpeT* gene were deleted in Bp82 derived strains using the pEXKm5 allelic exchange vector as previously described (17, 25) using plasmids pPS2591 and pPS2647, respectively (**Table 5.2**). Resulting deletion strains were complemented with specific gene(s) originating from 1026b or with an empty-mini-Tn*7* element as a control, using pPS2481, pPS2670, pPS2280 and pPS2787 (**Table 5.2**) as previously described (17).

To build the knockout construct for the *ptr1* gene, the PCR products of primers P2639 & P2640 and P2641 & P2642 (**Table 5.3**) were assembled by splicing by overlap extension (SOEing) PCR and cloned into the pGEM-T easy vector (**Table 5.2**). This plasmid was digested with *Hin*dIII (New England Biolabs, Ipswich, MA), which cut at a site introduced in the SOEing primers between the flanking DNA fragments. A Flp-recombinase target (*FRT*)-flanked kanamycin resistance marker (Km^r, *nptII*) was excised from pFKm2 as a *Hin*dIII fragment and was ligated between the cloned chromosomal DNA fragments using T4 DNA ligase (Invitrogen, Carlsbad, CA). The Δ*ptr1* construct was cloned into the pEXGm5B allelic exchange vector, moved into and then conjugated from RHO3 to Bp82. Finally, sucrose counter-selection was performed as previously described (25), with additional maintenance selection for the Km^r marker.

For deletion of the *bpeS* gene, primer pairs P2618 & P2619 and P2622 & P2623 (**Table 5.3**) were used to amplify DNA fragments immediately upstream and downstream of the gene from Bp82 genomic DNA. These products were assembled with the *FRT*-flanked Km^r maker (*nptII*) internally using SOEing PCR and primers P2619-P2622 (**Table 5.3**). This SOEing product was initially cloned into the pGEM-T easy vector background, and then sub-cloned into

the pEXGm5B allelic exchange plasmid (**Table 5.2**). This plasmid was then conjugated from RHO3 into Bp82 using previously described methods (25). Selection for the merodiploid was done on LB media containing 80 µg/mL of Ade and 1 mg/mL of Km. Several unsuccessful attempts were made to resolve the merodiploids using sucrose counter-selection (25) while maintaining selection of the Km^r marker. The I-*Sce*I endonuclease expressing plasmid pPS2593 (**Table 5.2**) was conjugated into the merodiploid and both I-*Sce*I and sucrose mediated counter-selection methods were used simultaneously (25). The exconjugates were plated on media containing 80 of µg/mL Ade, 1 mg/mL of Km, 15% of Sucrose, 2 mg/mL of Zeo and 0.5% of L-arabinose to finally obtain the Bp82Δ*bpeS*::Km^r strain, Bp82.263 (**Table 5.1**). The temperature sensitive I-*Sce*I-encoding pPS2593 was then cured by incubation at 42°C (25).

The *FRT*-flanked Km^r markers were removed from each of the knockout strains by Flp recombinase-mediated excision using the pFLPe2 plasmid, as previously described (27). pFLPe2 was subsequently cured by growth at 42˚C.

5.3.8 Allelic replacement of *bpeS* and *ptr1* mutations.

The pEXKm5 allele replacement vector was used to repair observed single nucleotide polymorphisms (SNPs) in selected resistant isolates. Primers P2640 & P2641 (**Table 5.3**) were used to amplify the *ptr1* gene and flanking regions from the Bp82 (WT) strain using the Platinum Taq DNA Polymerase High Fidelity (Life Technologies Corporation, Grand Island, NY). The PCR product was initially cloned into pGEM-T easy and confirmed by DNA sequencing as described above. The exchange construct was then moved into the pEXKm5 plasmid and conjugated from RHO3 into the desired strain (pPS3093 into Bp82.204) (**Table 5.2**) as previously described (27). Merodiploids were selected with 1 mg/mL of Km, and sucrose was

used for counter-selection (25). Resolved colonies were screened by DNA sequencing to confirm the presence of the *wild-type* allele.

Similarly, primers P2618 & P2623 were used to amplify the *bpeS* gene for allele replacement from the Bp82 (WT) strain. This construct was cloned into pGEM-T easy, confirmed by DNA sequencing and moved to the pEXKm5 plasmid. Conjugation of pPS3090 from RHO3 into Bp82.204 resulted in merodiploids that were then resolved by sucrose counter-selection and confirmed by DNA sequencing. This method allowed us to repair the *bpeS* K627T mutation in Bp82.204.

5.4 Results

5.4.1 Co-trimoxazole resistance is dependent on BpeEF-OprC mediated efflux.

Following serial passage of Bp82 in increasing concentrations of both trimethoprim and sulfamethoxazole, we obtained a collection of isolates with decreased co-trimoxazole susceptibilities, Bp82.191-Bp82.209 (**Table 5.1**). From this population, several isolates were chosen for further testing. These 6 isolates had trimethoprim and sulfamethoxazole MICs above the limit of detection (32 µg/mL and 1024 µg/mL, respectively) (**Table 5.4**). This combination of reduced susceptibility to both trimethoprim and sulfamethoxazole resulted in increased co-trimoxazole MICs, which ranged from 2 µg/mL to 6 µg/mL in contrast to the wild-type Bp82 MIC of 0.047 µg/mL (**Table 5.4**).

In addition to the MICs for the folate pathway inhibitors, we also tested the sensitivities of these strains to other classes of antimicrobials. We observed no major changes in the susceptibilities to carbenicillin, erythromycin or gentamicin. However, the MICs for acriflavine, chloramphenicol, norfloxacin and tetracycline were much higher than those of the parent strain (**Table 5.4**). The latter antimicrobials are known substrates of the BpeEF-OprC efflux pump (T.

Mima and H.P. Schweizer, unpublished findings), suggesting that the mutant strains are overexpressing this efflux pump.

Table 5.4 Antimicrobial susceptibilities of co-trimoxazole resistant Bp82 isolates.

	Minimal Inhibitory Concentration (μg/mL)									
Strain	Tmp[1]	Smx[1]	SXT	Acr	Chl[2]	Nor	Tet	Car	Ery	Gen
Bp82	0.75	4	0.047	32	4	4	1	256	128	128
Co-trimoxazole selected isolates										
Bp82.191	≥ 32	≥ 1024	4	128	≥ 128	32	2	128	128	64
Bp82.193	≥ 32	≥ 1024	3	128	≥ 128	32	4	256	128	128
Bp82.199	≥ 32	≥ 1024	2	128	≥ 128	32	16	256	256	64
Bp82.202	≥ 32	≥ 1024	6	128	≥ 128	32	4	128	128	128
Bp82.204	≥ 32	≥ 1024	4	128	≥ 128	32	4	128	128	128
Bp82.207	≥ 32	≥ 1024	2	128	≥ 128	32	4	128	128	64

Abbreviations: Car, carbenicillin; Ery, erythromycin; Gen, gentamicin; Acr, acriflavine; Chl, chloramphenicol; Nor, norfloxacin; Tet, tetracycline; Tmp, trimethoprim; SXT, co-trimoxazole.
[1] Etest® detection limits: ≥ 1024 μg/mL for Smx and ≥ 32 μg/mL for Tmp.
[2] Microdilution for chloramphenicol was not tested above 128 μg/mL.

To confirm the contribution of the BpeEF-OprC efflux pump to the observed increase in co-trimoxazole MICs, allelic exchange methods were employed to delete the *bpeEF-oprC* structural genes from each of the 6 strains. The deleted *bpeEF-oprC* genes were complemented using site-specific, single copy insertion of a mini-Tn*7* element carrying a wild-type *bpeEF-oprC* operon, as previously described (**Chapter 3**) (17). Similarly, *bpeT* deletion strains were constructed and complemented with a *bpeT* gene transcribed from the endogenous P_{bpeT} promoter. The MICs of trimethoprim, sulfamethoxazole and co-trimoxazole were determined for each of these mutants. MICs were also determined in the presence of IPTG for expression of the *bpeEF-oprC* operon from the inducible P_{tac} promoter. In all 6 isolates, deletion of the BpeEF-OprC efflux pump resulted in a significant drop in the co-trimoxazole MICs (**Table 5.5**).

Complementation resulted in increased MICs, though not to the levels observed with the original isolates (**Table 5.5**).

Table 5.5 The BpeEF-OprC efflux pump is required for increased folate inhibitor MICs.

	Minimal Inhibitory Concentration (μg/mL)											
	bpeE^{+}*F*$^{+}$-*oprC*$^{+}$			Δ(*bpeEF-oprC*)			Δ(*bpeEF-oprC*):: mini-Tn*7*T-P_{tac}-*bpeEF-oprC*			Δ(*bpeEF-oprC*):: mini-Tn*7*T-P_{tac}-*bpeEF-oprC* + 1 mM IPTG		
Strain	Tmp[1]	Smx[1]	SXT	Tmp	Smx	SXT	Tmp	Smx	SXT	Tmp	Smx	SXT
Bp82.191	≥ 32	≥ 1024	4	1.5	8	0.125	1.5	12	0.125	12	16	0.5
Bp82.193	≥ 32	≥ 1024	3	1.5	6	0.094	1	6	0.094	6	8	0.5
Bp82.199	≥ 32	≥ 1024	2	1.5	6	0.125	1.5	12	0.125	8	24	0.75
Bp82.202	≥ 32	≥ 1024	6	1.5	6	0.125	1.5	16	0.125	12	24	0.5
Bp82.204	≥ 32	≥ 1024	4	2	8	0.125	1.5	12	0.125	6	16	0.5
Bp82.207	≥ 32	≥ 1024	2	1.5	12	0.125	1.5	16	0.125	8	24	0.5

Abbreviations: IPTG, isopropyl-1-thio-β-D-galactopyranoside; Smx, sulfamethoxazole; SXT, co-trimoxazole (x Tmp + 19x Smx); Tmp, trimethoprim.
[1] The detection limit for the Etest® assay is ≥ 32 μg/mL for Tmp and ≥ 1024 μg/mL for Smx.

Similarly the absence of the *bpeT* gene resulted in lower trimethoprim, sulfamethoxazole and co-trimoxazole MICs, while complementation resulted in slight increases in most of the strains (**Table 5.6**). In contrast, the trimethoprim and sulfamethoxazole MICs of strains Bp82.199 and Bp82.204 appear unaffected by deletion of the *bpeT* gene. However, since these results are above the detection limit of the Etest® strips, it is impossible to determine if there was any change to these MICs. In Bp82.199 and Bp82.204 there was a slight drop in the co-trimoxazole MICs from 2 μg/mL to 1 μg/mL and from 4 μg/mL to 1 μg/mL, respectively, indicating that the mutant strains are sensitive to the combination (**Table 5.6**). These results would suggest a difference in the mechanisms of resistance; while all of the isolates are dependent on presence of the BpeEF-OprC efflux pump a sub-population appears to be maintain

resistance to trimethoprim and sulfamethoxazole independent of the BpeT transcriptional regulator.

Table 5.6 BpeT is required for increased folate inhibitor MICs in most of the tested strains.

	Minimal Inhibitory Concentration (μg/mL)											
	bpeT^{+}			Δ*bpeT*			Δ*bpeT::* mini-Tn*7*T			Δ*bpeT::* mini-Tn*7*T-P_{bpeT}-*bpeT*		
Strain	Tmp[1]	Smx[1]	SXT	Tmp	Smx	SXT	Tmp	Smx	SXT	Tmp	Smx	SXT
Bp82.191	≥ 32	≥ 1024	4	4	16	0.38	4	16	0.38	≥ 32	32	0.75
Bp82.193	≥ 32	≥ 1024	3	2	12	0.25	2	8	0.19	8	24	0.38
Bp82.199	≥ 32	≥ 1024	2	≥ 32	≥ 1024	1	≥ 32	≥ 1024	1	≥ 32	≥ 1024	1
Bp82.202	≥ 32	≥ 1024	6	4	24	0.5	6	12	0.38	8	32	0.75
Bp82.204	≥ 32	≥ 1024	4	≥ 32	≥ 1024	1	≥ 32	≥ 1024	1	≥ 32	≥ 1024	2
Bp82.207	≥ 32	≥ 1024	2	3	16	0.38	4	16	0.25	≥ 32	32	0.75

Abbreviations: Smx, sulfamethoxazole; SXT, co-trimoxazole (x Tmp + 19x Smx); Tmp, trimethoprim.

[1] The detection limit for the Etest® assay is ≥ 32 μg/mL for Tmp and ≥ 1024 μg/mL for Smx.

5.4.2 *bpeF* mRNA is overexpressed in co-trimoxazole resistant isolates.

RT-qPCR was used to determine the relative expression levels of *bpeF* and *bpeT* mRNA in the 6 co-trimoxazole resistant isolates compared to the parental strain, Bp82. Remarkably, each of the strains were expressing *bpeF* mRNA at over 100 times that of Bp82 (**Figure 5.1**). There was no significant difference in *bpeF* expression between the different co-trimoxazole resistant isolates. Based on previous work by T. Mima, we know that overexpression of BpeT will result in overexpression of BpeEF-OprC (T. Mima and H. P. Schweizer, unpublished results). Small increases in *bpeT* mRNA expression were observed in all 6 isolates relative to Bp82 (**Figure 5.2**), however these increases were not statistically significant.

The *bpeF* mRNA expression levels were also determined in mutant derivatives of the 6 isolates, which lack *bpeT* (**Figure 5.1**). There was a significant decrease in *bpeF* expression in

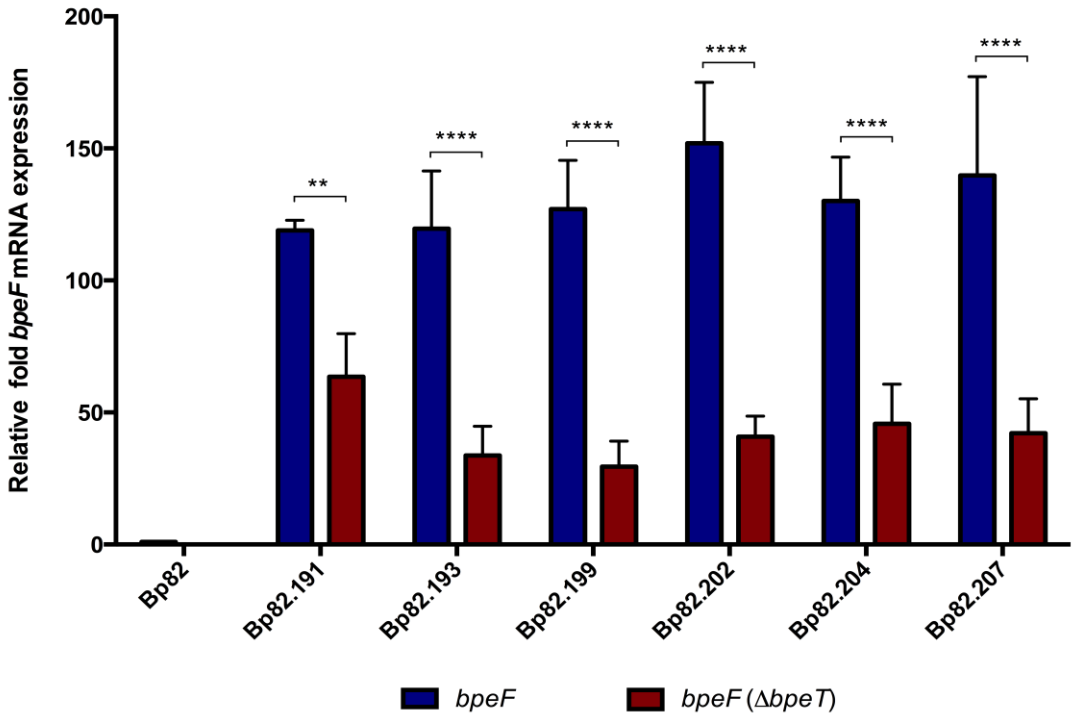

Figure 5.1 *bpeF* mRNA levels are significantly elevated in co-trimoxazole resistant Bp82 mutants. The relative *bpeF* expression was determined in 6 Bp82 derived-isolates (**blue**) and mutant derivatives lacking the BpeT regulator (**red**) under normal growth conditions. All fold expression values are relative to the Bp82 parent strain. Error bars indicate the standard deviation between three biological replicates, which were each performed in technical triplicate. The *bpeF* mRNA expression levels were very high in the strains compared to the parent Bp82 strain. However, there was a significant drop in *bpeF* expression in the absence of *bpeT*. Statistical significance is indicated above (****, $p \leq 0.0001$; **, $p \leq 0.01$).

the absence of *bpeT*. However, relative to Bp82, these isolates still overexpress *bpeF* by at least 30 fold. These results suggest that mutations in these resistant strains may have affected another regulator of *bpeEF-oprC* expression causing enhanced expression of the *llpE-bpeEF-oprC* operon even in the absence of the BpeT transcriptional regulator. Of note, reduced *bpeF* mRNA expression was observed in strains Bp82.199 and Bp82.204 in the absence of *bpeT*. This result is intriguing, as in all of the other strains decreased BpeEF-OprC efflux was linked with increased susceptibility to the folate pathway inhibitors (**Table 5.6**). While there were decreases in

Bp82.199 and Bp82.204 MICs, as discussed above, they were noticeably higher than those of the other strains tested.

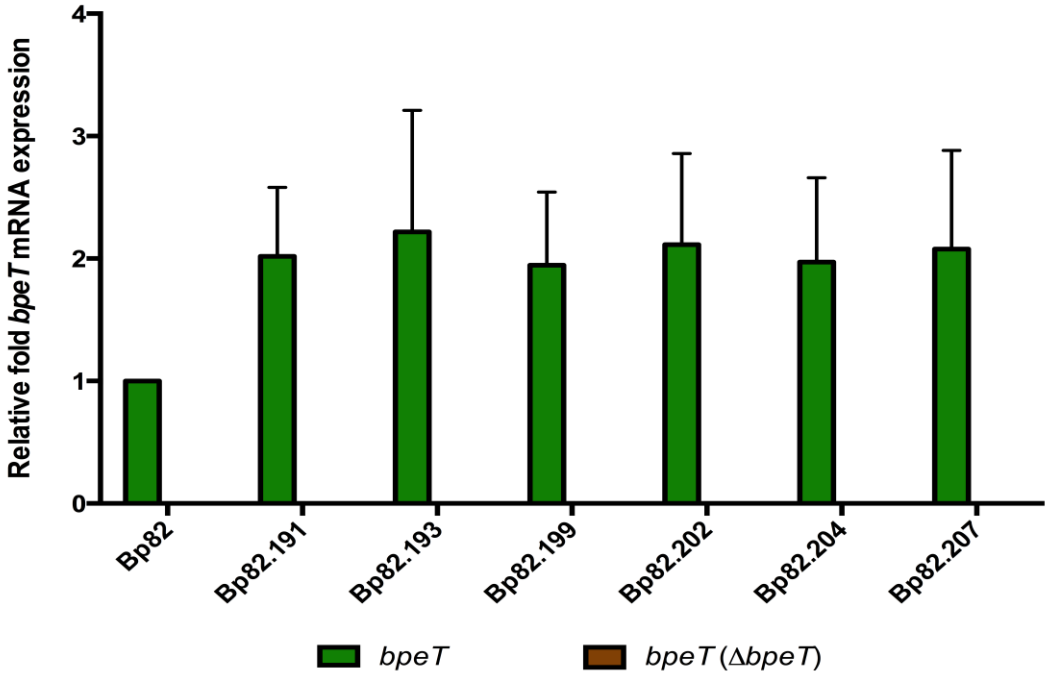

Figure 5.2 *bpeT* mRNA levels are marginally elevated in co-trimoxazole resistant Bp82 mutants. The relative *bpeT* expression was determined in 6 Bp82 derived-isolates under normal growth conditions (**green**). All fold expression values are relative to the Bp82 parent strain. Error bars indicate the standard deviation between biological replicates, which were each performed in technical triplicate. There was no significant change in *bpeT* expression in these strains, and no *bpeT* mRNA was detected in the Δ*bpeT* strains (**brown**), as expected.

5.4.3 DNA sequencing of suspected co-trimoxazole resistance determinants.

PCR amplification and Sanger sequencing methods were used to investigate mutations, which could account for the overexpression of BpeEF-OprC in these isolates. We sequenced *bpeT* and the intergenic region containing predicted promoters for both *bpeT* and the *llpE-bpeEF-oprC* operon in the 6 strains of interest. However, there were no mutations to the nucleotide sequence of these regions. Similar to previous work by Podnecky *et al*. (17) (**Chapter 3**) these data suggest involvement of an additional unidentified regulator of BpeEF-OprC efflux pump expression. While expression of the BpeEF-OprC efflux pump appears to be the major

determinant of co-trimoxazole resistance, we also sequenced the genes encoding the trimethoprim and sulfamethoxazole drug targets, dihydrofolate reductase (*folA*) and dihydropetorate synthetase (*folP*). We found no mutations in *folA* or *folP*, confirming that drug target modification is not responsible for the co-trimoxazole resistance in the tested isolates.

5.4.4 Whole genome sequencing reveals mutations in *bpeS* and *ptr1*.

Comparison of whole genome sequences of the co-trimoxazole resistant mutants to the Bp82 parent revealed two single nucleotide polymorphisms (SNPs) found in both Bp82.202 and Bp82.204. The first SNP is a thymine to guanine transversion in the carboxy-terminal effector binding domain of a LysR family transcriptional regulator encoded by a gene designated *BP1026B_I1290*, which was renamed *bpeS* (**Figure 5.3-A**). This mutation results in a lysine to tyrosine amino acid substitution at position 267 of BpeS. BpeS was found to have high sequence homology to the known BpeEF-OprC LysR transcriptional regulator, BpeT. A key region in these regulators, the N-terminus of the protein containing the DNA binding domain, is 90% identical over the first 60 amino acids. This suggests that these two proteins likely bind to similar regulatory sequences.

The second SNP is an adenine to cytosine transversion in a pteridine reductase 1 encoding gene designated *BP1026B_II0040*, *ptr1* (**Figure 5.3-B**). The mutation encodes a valine to glycine amino acid change at position 15 in the N-terminus NADPH binding domain of Ptr1. Pteridine reductases are most notable for conferring resistance to methotrexate in parasites (35). Pteridine reductases are also homologs of an enzyme, FolM, in bacteria that can function as a dihydrofolate reductase similar to *folA* (36). The *folM* gene is commonly found to cluster genetically with *folE*, a gene necessary for initiation of pterin synthesis (37, 38). This is true in *B. pseudomallei*, where *folE* is immediately upstream of *ptr1* (**Figure 5.3**).

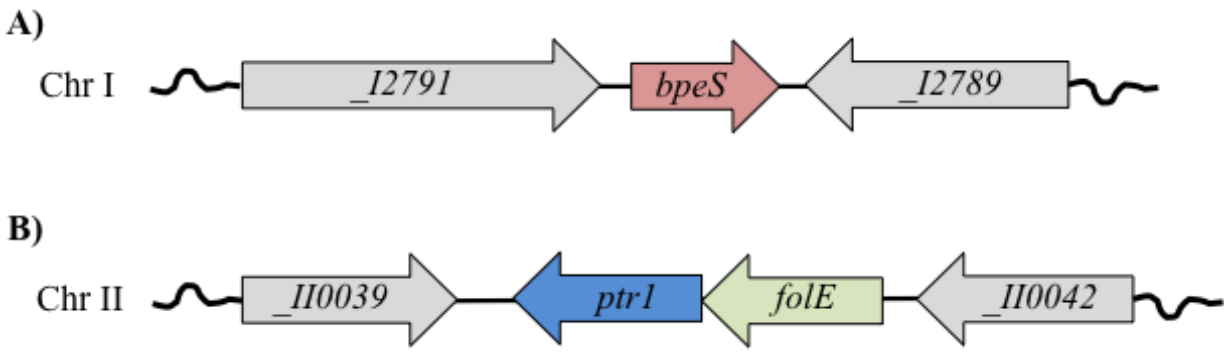

Figure 5.3 Genomic location and organization of the *bpeS* (A) and *ptr1* (B) regions of *B. pseudomallei* 1026b. Sequence coordinates are taken from the GenBank entries for the 1026b strain (accession numbers NC_017831.1 and NC_017832.1). Gene annotations are as follows: *_I2791 (BP1026B_I2791*), peptidase; ***bpeS*** (*BP1026B_I1290*), LysR-type transcriptional regulator; *_I2789 (BP1026B_I2789*), two-component regulator histidine sensor kinase; *_II0039 (BP1026B_II0039*), serine O-acetyltransferase; ***ptr1*** (*BP1026B_II0040*), pteridine reductase 1; *folE* (*BP1026B_II0041*), GTP cyclohyrolase; and *_II0042 (BP1026B_II0042*), LysR-type transcriptional regulator. The *bpeS* transcriptional regulator is distal from the *llpE-bpeEF-oprC* operon, which is located on Chromosome II. The *ptr1* gene is located immediately downstream of the *folE* gene, which is essential for pterine synthesis (37).

Both the BpeS K267T and Ptr1 V15G mutations were confirmed in Bp82.202 and Bp82.204 by targeted Sanger sequencing. Additionally, DNA sequencing of these genes showed that all of the co-trimoxazole resistant isolates in this study had the BpeS K267T mutation, and all had the Ptr1 V15G mutation with one exception. Strain Bp82.193 did not have the Ptr1 V15G mutation, but instead has a single base deletion at position 203 causing a frame shift mutation starting at amino acid 67. The resulting frame shift causes early termination of the protein following the 92nd residue. Bp82.193 is phenotypically similar to the 5 other strains in all other aspects, suggesting that the V15G mutation in Ptr1 may also disrupt function.

5.4.5 Mutations in *bpeS* and *ptr1* cause co-trimoxazole resistance.

To determine the effect of the identified mutations in *bpeS* and *ptr1*, the SNPs were repaired individually and in combination in strains Bp82.202 and Bp82.204. MIC testing of these strains indicated that loss of either SNP individually resulted in antimicrobial susceptibility;

demonstrated by the reduced MICs for trimethoprim, sulfamethoxazole and co-trimoxazole (**Table 5.7**).

Table 5.7 Antimicrobial susceptibilities of genetically repaired co-trimoxazole isolates.

	Minimal Inhibitory Concentration (μg/mL)		
Strain	Tmp[1]	Smx[2]	SXT
Bp82	0.75	4	0.047
Co-trimoxazole resistant isolates			
Bp82.202	≥ 32	≥ 1024	6
Bp82.204	≥ 32	≥ 1024	4
Co-trimoxazole resistant isolates with wild-type *bpeS*			
Bp82.246	0.5	4	0.064
Bp82.249	0.5	4	0.064
Co-trimoxazole resistant isolates with wild-type *ptr1*			
Bp82.247	≥ 32	32	0.75
Bp82.250	≥ 32	32	0.75
Co-trimoxazole resistant isolates with wild-type *bpeS* and *ptr1*			
Bp82.248	0.19	1.5	0.032
Bp82.251	0.19	1.5	0.032

Abbreviations: Smx, sulfamethoxazole; SXT, co-trimxoazole; Tmp, trimethoprim,
[1] The detection limit for the Etest® method for trimethoprim is ≥ 32 μg/mL.
[2] The detection limit for the Etest® method for sulfamethoxazole is ≥ 1024 μg/mL.

Repair of the *bpeS* gene caused a greater drop in MIC, where the susceptibility to each drug was reduced to that of the parental Bp82 strain. Repair of the *ptr1* gene had a lesser effect, but still reduced the MICs of sulfamethoxazole from the detection limit of 1024 μg/mL to 32 μg/mL and co-trimoxazole from 4-6 μg/mL to 0.75 μg/mL. Surprisingly, the repair of both SNPs resulted in MICs below those of the original Bp82 parent strain.

In addition to measuring the MICs of the repaired mutant strains, RT-qPCR was used to examine changes in *bpeF* mRNA expression (**Figure 5.4**).

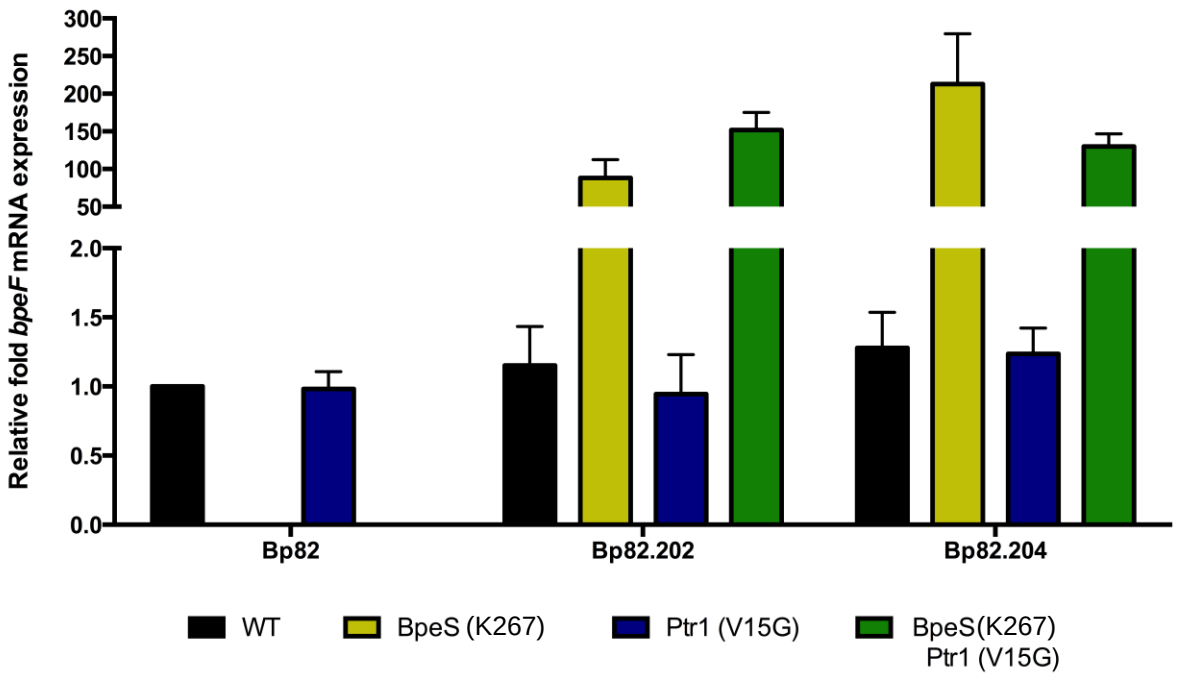

Figure 5.4 The BpeS (K267T) mutation is responsible for increased *bpeEF-oprC* expression. The relative *bpeF* mRNA expression was determined in Bp82 derived-isolates grown in LB medium. All fold expression values are relative to the Bp82 parent strain. Error bars indicate the standard deviation between three biological replicates, which were each performed in technical triplicate. Isolates containing the BpeS K267T mutation had very high expression, whereas all other isolates had expression similar to the parental strain Bp82.

In each of the mutant strains that contained the BpeS K267T mutation, there was close to 100 fold increased expression of *bpeF* relative to Bp82. There was no change to the gene expression with the introduction of the Ptr1 V15G mutation, as was expected. This evidence clearly indicates that BpeS plays a major role in regulation of BpeEF-OprC expression.

5.4.6 *bpeS* and *ptr1* are non-essential in Bp82.

In order to further investigate the *bpeS* and *ptr1* genes in *B. pseudomallei*, allelic exchange methods were used to delete each of these genes individually in the Bp82 strain background. Successful deletion of these genes indicates the neither are essential for *in-vitro* growth. This was especially interesting for the *ptr1* deletion, as it is unclear what function Ptr1

plays in the folate synthesis pathway of *B. pseudomallei*. However, this result could suggest that, similar to previous findings, Ptr1 could provide redundancy to this essential pathway (36).

Table 5.8 Deletion of *ptr1* causes decreased antimicrobial susceptibilities.

		MIC (μg/mL)		
Strain	Deletion	Tmp	Smx	SXT
Bp82	—	0.5	4	0.094
Bp82.262	Δ*ptr1*	1.5	12	0.125
Bp82.264	Δ*bpeS*	0.5	4	0.094

Abbreviations: MIC, minimal inhibitory concentration; Smx, sulfamethoxazole; SXT, co-trimoxazole (**x** Tmp + 19**x** Smx); Tmp, trimethoprim.

Deletion of the *ptr1* gene in Bp82 (Bp82.262) resulted in 3-fold increases to the trimethoprim and sulfamethoxazole MICs and a small increase in the co-trimoxazole MIC (**Table 5.8**). These data suggest that mutations that inactivate Ptr1 function may contribute, to but are sufficient alone, to confer resistance. In contrast, there were no MIC changes in Bp82 lacking *bpeS* (**Table 5.8**).

5.5 Discussion

Major folate pathway inhibitors are known to be efficacious against *B. pseudomallei.* A combination of trimethoprim and sulfamethoxazole, co-trimoxazole, is commonly part of the treatment for melioidosis. Unfortunately, co-trimoxazole resistant isolates have been identified in the clinical setting further complicating the treatment of this notoriously antimicrobial resistant organism. Resistance to co-trimoxazole is complex, as strains resistant to either trimethoprim or sulfamethoxazole alone remain susceptible to the combination. Typically resistance to trimethoprim and sulfamethoxazole is the result of mutant FolA and FolP proteins insensitive to the respective drug (18, 22). Additionally, overexpression of the *folA* target gene can lead to resistance (39). However, mutations in the drug targets were not observed in this study.

We instead identified several mutations that contribute to co-trimoxazole resistance in laboratory-selected mutants of Bp82. Efflux has been previously shown to mediate resistance to both trimethoprim and sulfamethoxazole (40). In *B. pseudomallei* clinical and environmental isolates overexpression of the BpeEF-OprC efflux pump has been previously shown to confer resistant to trimethoprim and to low-level increases in co-trimoxazole MICs (17). The BpeS mutation K267T identified in this study resulted in high-level expression of the BpeEF-OprC efflux pump, even in the absence of the BpeT regulator. Interestingly, when this mutation is repaired or the efflux pump deleted, the MICs of both trimethoprim and sulfamethoxazole drop. This is the first evidence that sulfamethoxazole may also be a BpeEF-OprC pump substrate. This is alarming, as efficient efflux of both drugs could confer co-trimoxazole resistance. However, it is important to note that, while increased expression of BpeEF-OprC led to increased co-trimoxazole MICs, according to the CLSI cutoffs (30), the strains remained sensitive to this drug combination. These findings suggest that efflux by BpeEF-OprC alone may not be sufficient to confer clinically significant co-trimoxazole resistance in *B. pseudomallei*.

Pteridine reductases are short-chain dehydrogenase/reductase (SDR) enzymes that have been previously shown to confer resistance to methotrexate, a dihydrofolate reductase inhibitor, in trypanosomatids (35, 36). The genetically similar *folM* gene from *E. coli* encodes an enzyme with dihydrofolate reductase activity in the absence of *folA* (36). We identified two mutations to *prt1* in the *B. pseudomallei* co-trimoxazole resistant isolates in this study. A frame shift mutation causing early termination of the protein most likely results in a non-functional enzyme. A recent study showed increased trimethoprim MICs in *E. coli* strains lacking FolM (41). The authors argue that while this result was unexpected, absence of the FolM dihydrofolate reductase may prompt increased expression and activity of FolA resulting in decreased trimethoprim

susceptibilities (41). However, we observed slight increases to both the trimethoprim and sulfamethoxazole MICs. It is possible that the acquired mutations to *ptr1* in the selected *B. pseudomallei* isolates may have a similar effect on both the *folA* and *folP* genes. Further studies to investigate the relative expression of each of these genes and the role of Ptr1 in folate synthesis, would lead to a better understanding of the relationship of Ptr1 to folate inhibitor resistance.

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CHAPTER 6: The BpeEF-OprC Efflux Pump is a Major Contributor to Co-Trimoxazole Resistance in *Burkholderia pseudomallei* Clinical Isolates

This chapter applies methods used in the preceding chapters to look for previously described resistance mechanisms for trimethoprim (**Chapter 3** and **Chapter 4**) and sulfamethoxazole (**Chapter 4** and **Chapter 5**) in a collection of clinical isolates from Thailand with a range of co-trimoxazole MICs. This work demonstrates that while complex, the clinical isolates can be grouped phenotypically based on antimicrobial resistance profiles and expression of the BpeEF-OprC efflux pump.

6.1 Summary

Burkholderia pseudomallei is a saprophyte capable of causing the disease melioidosis. *B. pseudomallei* is naturally resistant to many antimicrobials, which limits and complicates the treatment of melioidosis. Co-trimoxazole, a potent combination of trimethoprim and sulfamethoxazole is typically used for the eradication phase treatment of this infection. However, co-trimoxazole resistant isolates have been identified in endemic regions in the clinical setting. These resistant isolates are concerning as there are currently limited treatment options for this broadly antimicrobial resistant bacterium. The purpose of this study was to characterize a collection of clinical isolates from Thailand and determine the mechanisms responsible for co-trimoxazole resistance. We found that the BpeEF-OprC efflux pump is essential for co-trimoxazole resistance in the isolates used in this study. Deletion of the *bpeEF-oprC* operon resulted in a co-trimoxazole sensitive and trimethoprim resistant phenotype; however the *bpeT*

gene was only necessary for resistance in some of the strains. Reverse-transcriptase quantitative PCR experiments showed that *bpeEF-oprC* is greatly overexpressed in co-trimoxazole resistant strains, though in some isolates *bpeT* is not necessary for this overexpression. A single amino acid substitution in the BpeS LysR-type regulator may be the cause of BpeT independent constitutive overexpression of *bpeEF-oprC*; however DNA sequence comparisons did not suggest explanations for *bpeEF-oprC* overexpression in other strains.

6.2 Introduction

Burkholderia pseudomallei is a pathogen endemic to soils and ground water in tropical and sub-tropical regions of the world (1, 2). *B. pseudomallei* is known to cause an uncommon but often-serious disease, melioidosis (2-4). *B. pseudomallei* is notorious for its resistance to a wide-range of antimicrobials (5, 6), resulting in limited options for the treatment of melioidosis. Trimethoprim and sulfamethoxazole are folate pathway inhibitors that are synergistic and effective against *B. pseudomallei*. The co-trimoxazole combination is typically given for a minimum of 12-20 weeks following the initial phases of treatment (7, 8). Additionally co-trimoxazole is recommended for post-exposure prophylaxis in the event of accidental laboratory exposure or intentional release of the agent (7). *B. pseudomallei* is currently listed as a Tier 1 Select Agent by the United States government. The potential for its use as a bioweapon and further increases to the antimicrobial resistance of this organism is cause for concern. Naturally occurring resistance to co-trimoxazole is found at rates ranging from 2.5% to 16% in endemic regions (9, 10). With limited alternatives for efficacious therapeutics, improved understanding of the mechanisms of resistance is vital to allow for the development of novel therapeutics.

Previously we have investigated trimethoprim resistance in clinical, environmental and laboratory-selected *B. pseudomallei* isolates (**Chapter 3** and **Chapter 4**). These studies have

shown that a resistance nodulation and cell division (RND) efflux pump, BpeEF-OprC, can confer resistance to trimethoprim alone, as do mutations to the drug target dihydrofolate reductase, FolA. The BpeEF-OprC efflux pump also appears to efflux sulfamethoxazole and contributes to co-trimoxazole resistance (**Chapter 5**). Mutations to the sulfamethoxazole target, dihydropteroate synthetase (FolP), have been shown to confer resistance to sulfonamides in other bacteria (11-14), however no such mutations have been reported in *B. pseudomallei*. Finally, mutations observed in the *ptr1* gene, encoding for a putative pteridine reductase, were found to contribute to sulfamethoxazole and co-trimoxazole resistance. Previous studies have suggested that a homologue of this gene, *folM*, found in *Escherichia coli* can act as a dihydrofolate reductase and contribute to methotrexate, or potentially trimethoprim, resistance (15). However it is unclear what role Ptr1 plays in folate synthesis in *B. pseudomallei*. The purpose of this study was to determine which, if any, of the above mechanisms contribute to co-trimoxazole resistance in clinical *B. pseudomallei* isolates.

6.3 Material and Methods

6.3.1 Bacterial strains.

The *Burkholderia pseudomallei* strain 1026b (16) was used as a prototype strain for experiments with clinical *B. pseudomallei* isolates. A collection of 14 clinical isolates from Thailand (isolated between 1993-2009) was used in this study (**Table 6.1**). Additionally a collection of 9 clinical and environmental strains from Thailand and 3 clinical and environmental isolates from Australia used by Podnecky *et al*. (**Chapter 4**) (17) was further examined in the current study. All procedures involving *B. pseudomallei* clinical isolates were performed in Select Agent approved Biosafety Level 3 (BSL-3) facilities at the Rocky Mountain Regional Biosafety Laboratory at Colorado State University using Select Agent compliant procedures and protocols.

Table 6.1 Clinical isolates used in this study.

Thai Clinical *B. pseudomallei* Isolates		
Strain	**Specimen**	**Isolation**
1026b	**blood**	**1993**
1130	**pus**	**1993**
1374	**pus**	**1995**
1468	**sputum**	**1995**
1553	**blood**	**1996**
1641	**sputum**	**2000**
2131	**pus**	**1998**
2259	**pus**	**1999**
2411	**pus**	**2001**
2431	**blood**	**2000**
2444	**urine**	**2000**
2517	**blood**	**2000**
2703	**throat swab**	**2001**
5041	**sputum**	**2008**
5242	**pus**	**2009**

Additionally, the Select Agent excluded *B. pseudomallei* 1026b-derived strain; Bp82 (**Table 6.2**) was used for several experiments performed at Biosafety Level 2 (BSL-2), as approved by the Colorado State University Institutional Biosafety Committee. *B. pseudomallei* was grown in Lennox Luria-Bertani (LB; MO BIO Laboratories, Carlsbad, CA) broth or LB agar at 37°C unless otherwise noted. LB was supplemented with 80 μg/mL of adenine (Ade; SIGMA, St. Louis, MO) and with or without 80 μg/mL of thiamine (Thi; SIGMA) for cultivation of Bp82 and its derivatives. *E. coli* strains DH5α and XL1-Blue were used for genetic manipulation of plasmid DNA and the RHO3 strain was used as a conjugation donor strain for mobilization of plasmids (**Table 6.2**). *E. coli* strains were grown in LB media at 37°C, which for cultivation of RHO3 was supplemented with 400 μg/mL of diaminopimelic acid (DAP; SIGMA). For selection of plasmids in *E. coli* strains, 100 μg/mL of ampicillin (Amp; SIGMA, St. Louis, MO), 15 μg/mL of gentamicin (Gm; SIGMA) or 35 μg/mL of kanamycin (Km; SIGMA) was added to the media as necessary, while selection for the Km resistance marker in *B. pseudomallei* was performed on media containing 1 mg/ml of Km. Colorimetric screening for the presence of the *gusA* gene in pEXKm5-containing strains and merodiploids was done on media containing 50 μg/mL of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc; Gold Biotechnology, St. Louis, MO). Induction of genes controlled by the P_{tac} promoter was

Table 6.2 Bacterial strains and plasmids used in this study.

Strain	Description	Reference
DH5α	*E. coli* general cloning strain (F$^-$ Φ80*lacZ*ΔM15 Δ(*lac*Z*YA-argF*)U169 *recA*1 *endA*1 *hsd*R17 (r_K^- m_K^+) *phoA supE*44 *thi*-1 *gyrA*96 *relA*1 λ$^-$)	(18)
RHO3	*E. coli* conjugation donor strain (F$^-$ *thi*-1 *thr*-1 *leuB*6 *lacY*1 *tonA*21 *supE*44 *recA* Δ*asd* Δ*aphA* (chr::RP4-2-Tc::Mu) λ*pir*$^+$)	(19)
XL1-Blue	*E. coli* competent strain for QuikChange mutagenesis (*recA*1 *endA*1 *gyrA*96 *thi*-1 *hsdR*17 *supE*44 *relA*1 *lac* [F' *proAB lacI*^{q}ZΔM15 Tn10 (Tet^r)]	Agilent Tech. Santa Clara, CA
Bp82	1026bΔ*purM* (Select Agent excluded *Burkholderia pseudomallei* strain)	(20)
Bp82.265	Bp82 *folP* (A120V)	This study

Plasmid	Descriptive Name	Relevant Properties[1]	Source
pGEM-T Easy		Amp^r; TA cloning vector	Promega (Madison, WI)
pEXKm5		Km^r; Allelic exchange vector	(19)
pTNS3		Amp^r; Tn*7* transposase expression vector	(19)
pPS2591	pEXKm5Δ(*bpeEF-oprC*)	Km^r; Allelic exchange vector for deletion of *bpeEF-oprC*	(17)
pPS2481	pUC18T-mini-Tn*7*T-P_{tac}	Km^r; mini-Tn*7*T-P_{tac} empty control vector	(21)
pPS2670	pPS2481-*bpeEF-oprC*	Km^r; mini-Tn*7*T-P_{tac}-*bpeEF-oprC* complementation vector	(17)
pPS2647	pEXKm5Δ*bpeT*	Km^r; Allelic exchange vector for deletion of *bpeT*	(17)
pPS2280	pUC18T-mini-Tn*7*T	Km^r; mini-Tn*7*T empty control vector	(22)
pPS2787	pPS2280-P_{bpeT}-*bpeT*	Km^r; mini-Tn*7*T-P_{bpeT}-*bpeT* complementation vector	(17)
pPS3129	pGEM-T Easy-*folP*	Amp^r; 1,351 bp *folP* PCR product ligated into pGEM-T Easy	This study
pPS3143	pGEM-T Easy-*folP* (A120V)	Amp^r; pPS3129 following QuikChange II mutagenesis with P2644 & P2645 to c362t	This study
pPS3145	pEXKm5-*folP* (A120V)	Km^r; 1,384 bp *Not*I pPS3143 fragment (FolP A120V) ligated into pEXKm5	This study

[1] **Abbreviations:** Amp, ampicillin; Gm, gentamicin; Km, kanamycin; ^{r}, resistant; Tet, tetracycline.

accomplished by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Gold Biotechnology) to the media, as needed.

6.3.2 Antimicrobial susceptibility testing.

The minimal inhibitory concentrations (MIC) for trimethoprim, sulfamethoxazole and co-trimoxazole were determined by Etest® method (AB Biomérieux, Marcy l'Etoile, France) on Mueller Hinton II agar (Becton Dickinson, Franklin Lakes, NJ). The MIC breakpoints used previously to define trimethoprim, sulfamethoxazole and co-trimoxazole resistance in *B. pseudomallei* (17) were used in this study. Co-trimoxazole (SXT) is a combination of trimethoprim (Tmp) and sulfamethoxazole (Smx) at a 1:19 ratio. SXT concentrations are based on the formula: **x** μg/mL SXT = **x** μg/mL Tmp + 19**x** μg/mL Smx.

6.3.3 Reverse-transcriptase quantitative PCR (RT-qPCR).

The mRNA expression levels of *bpeF* and *bpeT* were assessed in cultures grown to mid-log phase with or without trimethoprim induction (1 h incubation with 32 μg/mL of trimethoprim; trimethoprim stock solution was made in dimethylacetamide at a concentration of 100 mg/mL), as previously described (17). Relative expression was determined by the Bio-Rad iCycler iQ™ Optical System software version 2.0 with specific primer set efficiencies. Analysis of the data was performed in GraphPad Prism version 6.0c for Mac OS X (GraphPad Software, La Jolla, CA) using a two-way analysis of variance (ANOVA) on log transformed relative expression data followed by Tukey's multiple comparisons test for comparison across strains or Sidak's multiple comparisons test for comparison across condition. P-values < 0.05 were considered significant.

6.3.4 DNA sequencing and analysis.

DNA sequencing of specific genes was performed as previously described (**Chapter 3**) (17). Briefly, the genes encoding antimicrobial targets, *folA* and *folP*, *ptr1* (pteridine reductase), key BpeEF-OprC efflux related genes, as well as other pertinent DNA regions including the regulators *bpeT* and *bpeS*, the *bpeT-llpE-bpeE* intergenic region (containing putative promoters) and *bpeF* (cytoplasmic membrane transporter) were PCR amplified in 4 independent PCR reactions using the Platinum Taq DNA Polymerase High Fidelity (Life Technologies Corporation, Grand Island, NY) with specific primer sets (**Table 6.3**). PCR reactions were pooled and sequenced using gene-specific primers. Additionally, internal primers were used for *ptr1* and *bpeS* sequencing. Alignment of the sequence reads and comparisons were performed using Sequencher version 5.1 (Gene Codes Corporation, Ann Arbor, MI) (25) and ClustalW2 (26).

6.3.5 Markerless deletion and complementation of *bpeEF-oprC* and *bpeT*.

Deletion of large portions of the coding regions for the *bpeEF-oprC* and *bpeT* genes were performed individually on clinical strains of interest using the pEXKm5-based allelic exchange method, as previously described (17, 19). The deletion mutants were complemented with their respective mini-Tn*7* constructs from pPS2670 and pPS2280 (**Table 6.2**). Deletion mutants containing the empty mini-Tn*7* constructs were used as controls, as previously described (17).

Table 6.3 Oligonucleotides used in this study.

Primer	Descriptive Name	Primer Sequence	Source
M13F-20		5’- GTAAAACGACGGCCAGT-3’	(23)
M13R		5’- AACAGCTATGACCATG-3’	(23)
T7		5’- TAATACGACTCACTATAGGG-3’	
P1966	*folA*-F	5’- CTTCCGGCCTCTTTTCTTTC-3’	Chapter 3
P1967	*folA*-R	5’- GTGCTGATCGAGCAGATGAC-3’	Chapter 3
P2182	Upstream-*folA*-F	5’- CTGTATCGGCTGATGGTGTC-3’	Chapter 3
P2183	Upstream-*folA*-R	5’- AGGCCTTCCTCGTACAGTTG-3’	Chapter 3
P2323	*folP*-F	5’- CCAGATCAACGACATCATGG-3’	Chapter 5
P2324	*folP*-R	5’- CGAGCATATAGCCCGATACC-3’	Chapter 5
P1791	*bpeT*-R	5’- CGACGCATCGCGATGGAAAC-3’	Chapter 3
P1790	*bpeT*-F	5’- ATGGACCGGCTGCAAGCCAT-3’	Chapter 3
P2142	*bpeT-bpeE*-F	5’- TCTGAATGATCGTCGTCACC-3’	Chapter 3
P2143	*bpeT-bpeE*-R	5’- AATCGGTGATCGTCTTCGAC-3’	Chapter 3
P2109	*bpeF*-F1	5’- GCATCTCGTGCCGATGAC-3’	Chapter 3
P2110	*bpeF*-R1	5’- CGAACTCGTCCTCGTTCTG-3’	Chapter 3
P2094	*bpeF*-F2	5’- ACATGACGTATCTGCGCAAC-3’	Chapter 3
P2095	*bpeF*-R2	5’- CATCGCGAACTGCTTGTAGA-3’	Chapter 3
P2096	*bpeF*-F3	5’- AACGTCGAGCGCAACATC-3’	Chapter 3
P2097	*bpeF*-R3	5’- CGTTGATCTGGTAGCTCGTG-3’	Chapter 3
P2098	*bpeF*-F4	5’- GCGGCTTCAAGATGCAG-3’	Chapter 3
P2099	*bpeF*-R4	5’- ACCACACCCATGATGAACG-3’	Chapter 3
P2100	*bpeF*-F5	5’- AGGGCGACAACAACATCTTC-3’	Chapter 3
P2101	*bpeF*-R5	5’- GGCCTTCAGGTTCTGGTTC-3’	Chapter 3
P2570	*bpeS*-F1	5’- GGATGACTTCGGCGCTATC-3’	Chapter 5
P2571	*bpeS*-R1	5’- CCGTTCAACCTGACCTCAAC-3’	Chapter 5
P2572	*bpeS*-F2	5’- GTCTTCCGCCAGCGCTAC-3’	Chapter 5
P2573	*bpeS*-R2	5’- AAGCCGATTCATCTGGACAC-3’	Chapter 5
P2592	*ptr1*-F1	5’- CTCGCTCACGCTGATTGC-3’	Chapter 5
P2575	*ptr1*-R1	5’- CGTCGATGCGGTCTATACG-3’	Chapter 5
P2576	*ptr1*-F2	5’- ATCGAAGCTCGGCAGGTG-3’	Chapter 5
P2577	*ptr1*-R2	5’- CGCGCCTACGAGGAGTTC-3’	Chapter 5
Reverse transcription quantitative PCR experiments			
P1516	*Bp*23S-F	5’- GTAGACCCGAAACCAGGTGA-3’	(24)
P1517	*Bp*23S-R	5’- CACCCCTATCCACAGCTCAT-3’	(24)
P1524	*bpeF*-F1-RT	5’- TCCGAGTATCCGGAAGTCGT-3’	(24)
P1525	*bpeF*-R1-RT	5’- GTCCTCGACACCGTTGATCT-3’	(24)
P1814	*bpeT*-RT-F	5'- GAGCTTTCAGGTCAACAACC-3’	Chapter 3
P1815	*bpeT*-RT-R	5'- GTGAGTGGAATTCGCAGAG-3’	Chapter 3

Primer	Descriptive Name	Primer Sequence	Source
Construction of *bpeEF-oprC* and *bpeT* deletion strains, complementation vectors and determination of chromosomal mini-Tn*7* insertion sites			
P1989	Δ(*bpeEF-oprC*)-F	5'- GGAAGTACGCGGACTTCGC-3'	(19)
P1990	Δ(*bpeEF-oprC*)-R	5'- GCATCAACCTCGGCTACACG-3'	(19)
P479	Tn7L	5'- ATTAGCTTACGACGCTACACCC-3'	(22)
P1509	*BpglmS*-1	5'- GAGGAGTGGGCGTCGATCAAC-3'	(22)
P1510	*BpglmS*-2	5'- ACACGACGCAAGAGCGGAATC-3'	(22)
P1511	*BpglmS*-3	5'- CGGACAGGTTCGCGCCATGC-3'	(22)
Mutagenic PCR oligonucleotides			
P2644	*folP* (A120V)-QC-F	5'-TCGCCGCGGGCGTCGATCTGATCAAC-3'	This study
P2645	*folP* (A120V)-QC-R	5'-GTTGATCAGATCGACGCCCGCGGCGA-3'	This study

Deletion mutants and mini-Tn*7* containing strains were confirmed by PCR using specific primers (**Table 6.3**). The BpeEF-OprC complementation construct contains the 1026b *bpeEF-oprC* operon controlled by the P_{tac} inducible promoter. Expression of *bpeEF-oprC* was induced by the addition of 1 mM IPTG to the media.

6.3.6 Allelic replacement of the *folP* mutation in Bp82.

The pEXKm5 allele replacement vector was used to introduce point mutations into the wild-type Bp82 strain. Primers P2640 & P2641 (**Table 6.3**) were used to amplify the *folP* gene from 1026b genomic DNA isolated using the PureGene Core Kit A (QIAGEN, Valencia, CA). This PCR product was ligated into the pGEM-T Easy vector (Promega, Madison, WI) resulting in pPS3129 (**Table 6.2**). The A120V mutation was introduced into the *folP* gene using primers P2644 & P2645 (**Table 6.3**) designed using the QuikChange Primer Design software (Agilent Technologies, Santa Clara, CA). The QuikChange® II Site-Directed Mutagenesis kit (Agilent Technologies) was used for mutagenesis and transformation into XL1-Blue competent *E. coli*. Introduction of the desired mutation was confirmed by DNA sequencing of plasmid pPS3143 using primers M13R and T7 (**Table 6.3**). Merodiploids were selected on LB media containing 80 µg/mL of Ade, 1 mg/mL of Km and 50 µg/mL of X-Gluc. The merodiploids were resolved by

sucrose counter-selection, as previously described (19). Resolved merodiploids were screened for the FolP A120V mutation by PCR amplification and DNA sequencing of *folP* using specific primers (**Table 6.3**).

6.4 Results

6.4.1 Identification of co-trimoxazole resistant clinical isolates.

Trimethoprim, sulfamethoxazole and co-trimoxazole MICs were determined for each of the 14 clinical and environmental isolates obtained from the Mahidol University collection. Using the interpretation criteria from the Clinical Laboratory Standards Institute (CLSI), as described by Podnecky *et al*. (17), 11 of the 14 isolates were resistant (MIC > 8 μg/mL) to trimethoprim alone (**Table 6.4**). Only strains 2444, 1130 and 1641 were susceptible to trimethoprim. The majority of the strains were susceptible to sulfamethoxazole, while strains 1374, 1468, 5041 and 5242 had MICs at or above the 1024 μg/mL limit of detection (**Table 6.4**). A total of 4 of the 14 tested isolates were co-trimoxazole resistant. Each of these strains (1374, 1468, 5041 and 5242) had MICs above the limit of detection for both trimethoprim and sulfamethoxazole alone. Strains 1641 and 1130 had the lowest MICs for all of the antimicrobials tested. We observed that strain 1641 appeared to be infected with a lysogenic phage, as plates struck for confluency frequently showed zones of lysis, which made Etest MIC determination difficult and likely inaccurate.

6.4.2 *bpeEF-oprC* is overexpressed in co-trimoxazole resistant strains.

Relative *bpeF* mRNA levels were determined for each of the 14 clinical isolates compared to the 1026b strain using RT-qPCR (**Figure 6.1**). There was significant overexpression of *bpeF* mRNA

Table 6.4 Antimicrobial susceptibilities of *B. pseudomallei* clinical isolates.

Minimal Inhibitory Concentration (μg/mL)[1]			
Strain	Tmp[2]	Smx[2]	SXT
1374	≥ 32	≥ 1024	4
1468	≥ 32	≥ 1024	4
5041	≥ 32	≥ 1024	4
5242	≥ 32	≥ 1024	3
1553	≥ 32	192	2
2703	≥ 32	128	1.5
2411	≥ 32	64	1.0
2259	≥ 32	32	0.75
2517	≥ 32	24	0.75
2431	≥ 32	24	0.5
2131	12	32	0.38
2444	8	16	0.38
1130	4	4	0.064
1641	0.5	3	0.032

Abbreviations: Smx, sulfamethoxazole; SXT, co-trimoxazole (**x** Tmp + 19**x** Smx); Tmp, trimethoprim.
[1] Red lettering indicates antimicrobial resistance: Tmp > 8 μg/mL, Smx > 256 μg/mL, and SXT > 2 μg/mL.
[2] The detection limits for Etest® are 32 μg/mL for Tmp and 1024 μg/mL for Smx.

observed in strains 1374, 5041 and 5242 grown in LB medium ($p \leq 0.0001$). Strain 1468 did not overexpress *bpeF* under these conditions, but produced nearly 6 times as much *bpeF* mRNA as 1026b when induced with trimethoprim. These 4 strains had the highest *bpeF* expression and the highest MICs for sulfamethoxazole and co-trimoxazole (**Table 6.4**).

None of the other 10 clinical isolates had significantly increased expression of *bpeF* under normal growth conditions, but interestingly strain 1130 had significantly decreased expression of *bpeF* compared to 1026b ($p \leq 0.0001$). All of the strains showed some increase in *bpeF* expression when treated with trimethoprim, though this difference was statistically significant only in roughly half of the strains (**Figure 6.1**).

There was a large change is *bpeF* expression when strain 1641 was induced with trimethoprim, however the MICs of this strain to each of the 3 drugs tested were the lowest of all the isolates.

6.4.3 *bpeT* is differentially expressed in strains 5242 and 1641.

We also determined the relative *bpeT* mRNA expression level for each of the 14 clinical isolates compared to 1026b both with and without induction with trimethoprim (**Figure 6.2**). Interestingly, there was no significant overexpression of *bpeT* in the clinical strains under non-inducing growth conditions, expect for strain 5242 ($p < 0.05$). The *bpeT* mRNA levels in strain 5242 were influenced by induction with trimethoprim (**Figure 6.2**). Strain 1641 was the only other strain with a significant increase of *bpeT* expression in the presence of trimethoprim. The inducible expression of *bpeT* in strains 5242 and 1641 may account for the observed overexpression of *bpeF* under these conditions (**Figure 6.1**), as previous work has shown that increased *bpeT* mRNA results in increased *bpeEF-oprC* expression (T. Mima and H.P. Schweizer, unpublished results). However, there are significant differences between the *bpeT* (**Figure 6.2**) and *bpeF* (**Figure 6.1**) mRNA expression levels of these 2 strains relative to one another, which may explain the very large differences in their respective MICs (**Table 6.4**).

Using previously published methods (17, 19), the *bpeEF-oprC* efflux pump structural genes were deleted in each of these isolates, and mutants were complemented with a mini-Tn*7* element carrying the 1026b-derived *bpeEF-oprC* operon under control the inducible P_{tac} promoter. MICs were determined for trimethoprim, sulfamethoxazole and co-trimoxazole in each of the

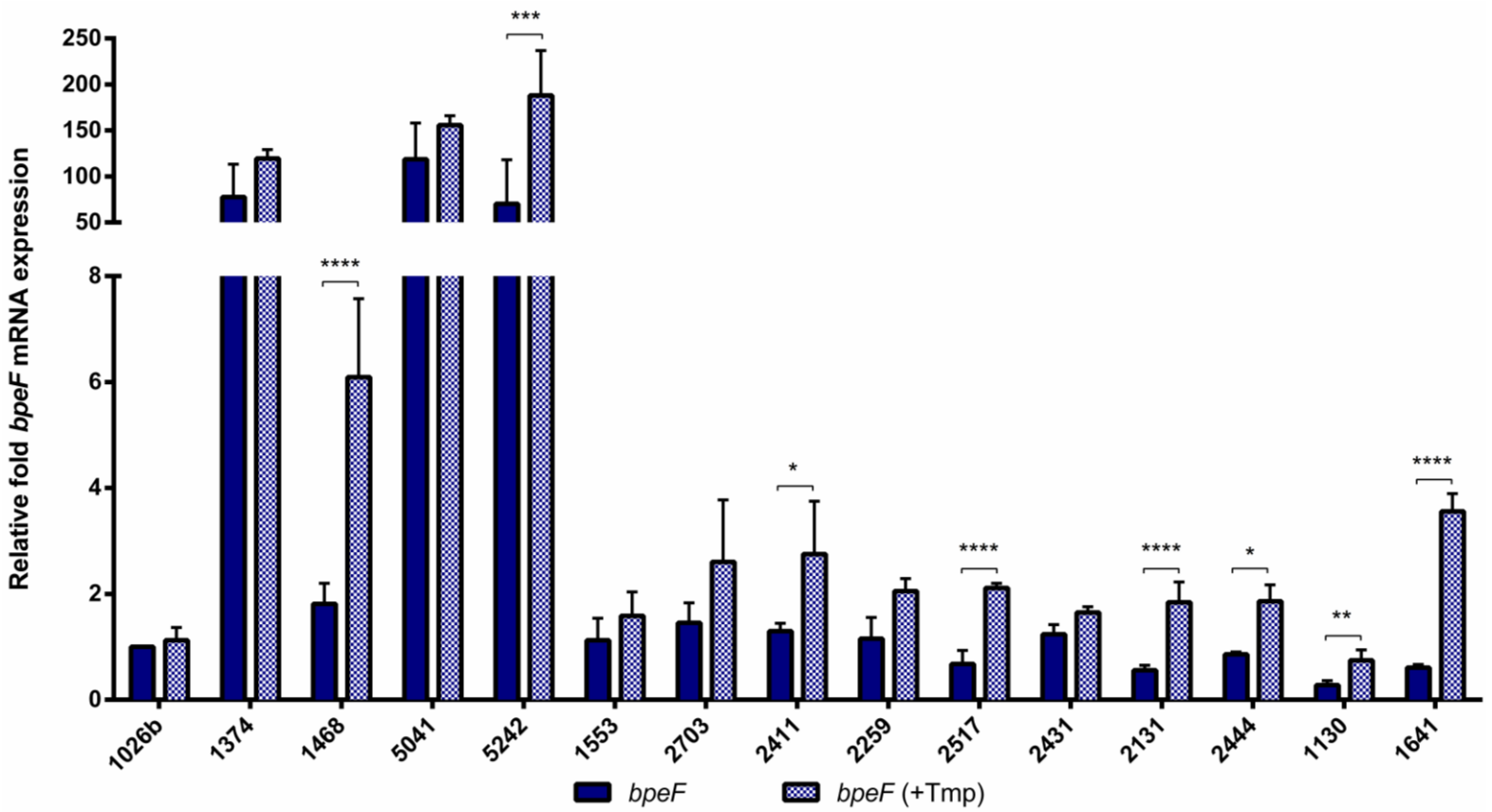

Figure 6.1 Relative expression of *bpeF* mRNA in clinical isolates from Thailand. The relative *bpeF* mRNA levels were determined in 14 clinical isolates from Thailand. The relative expression was assessed both under non-inducing growth conditions (**solid** bars) and following induction with 32 µg/mL of trimethoprim for 1 h (+Tmp; **stippled** bars). Fold expression was determined relative to the uninduced 1026b control strain. Error bars show the standard deviation between biological replicates, each of which were tested in technical triplicate. Statistical analysis by two-way ANOVA and Tukey multiple comparisons test showed significantly increased *bpeF* expression in strains 1374, 5041 and 5242. Statistical differences dependent on induction with trimethoprim are indicated above (****, $p \leq 0.0001$; ***, $p \leq 0.001$; **, $p \leq 0.01$; *, $p < 0.05$).

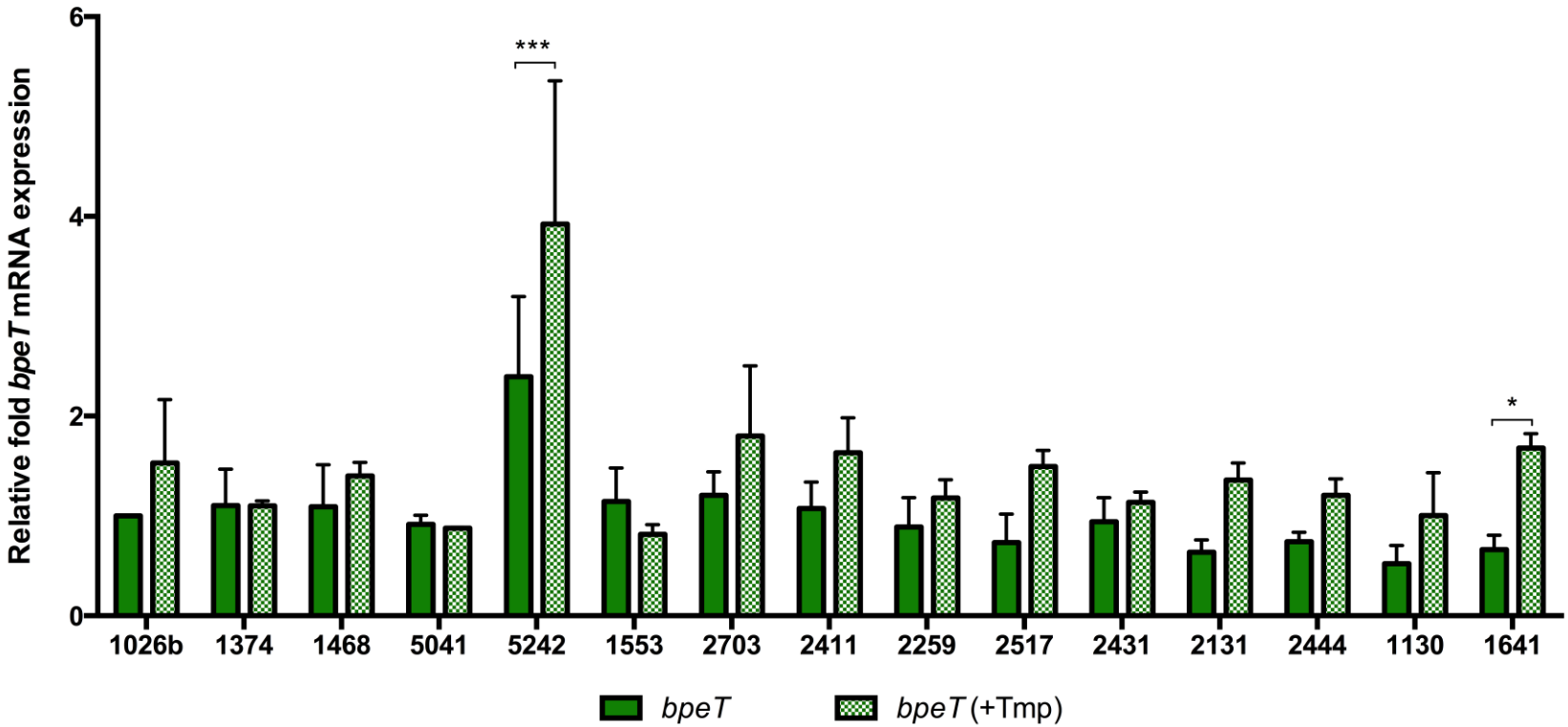

Figure 6.2 Relative *bpeT* mRNA levels were determined in 14 clinical isolates from Thailand. The relative expression was assessed both under non-induced growth conditions (**solid** bars) and following induction with 32 µg/mL of trimethoprim for 1 h prior to RNA harvest (+Tmp; **stippled** bars). Fold expression was determined relative to the uninduced 1026b control strain. Error bars show the standard deviation between biological replicates, each of which were tested in technical triplicate. Statistical analysis by two-way ANOVA and Tukey multiple comparison test showed significantly increased *bpeT* expression in strain 5242. Statistical differences resulting from induction with trimethoprim are indicated above (***, $p < 0.001$; *, $p < 0.05$).

strain derivatives (**Table 6.5**). The co-trimoxazole MICs for the 4 resistant isolates were 0.25 µg/mL or lower in the absence of *bpeEF-oprC* and increased when the complemented strains were induced with IPTG. These findings suggest that BpeEF-OprC is required for co-trimoxazole resistance in these strains.

Table 6.5 The BpeEF-OprC efflux pump is required for co-trimoxazole resistance.

	Minimal Inhibitory Concentration (µg/mL)[1]											
	bpeE$^+$*F*$^+$*-oprC*$^+$			Δ(*bpeEF-oprC*)			Δ(*bpeEF-oprC*):: mini-Tn*7*T-P_{tac}-*bpeEF-oprC*			Δ(*bpeEF-oprC*):: mini-Tn*7*T-P_{tac}-*bpeEF-oprC* + 1 mM IPTG		
Strain	Tmp	Smx	SXT	Tmp	Smx	SXT	Tmp	Smx	SXT	Tmp	Smx	SXT
1374	≥ 32	≥ 1024	4	1.5	8	0.125	1.5	12	0.125	≥ 32	64	1
1468	≥ 32	≥ 1024	4	≥ 32	48	0.25	≥ 32	64	0.5	≥ 32	384	1.5
5041	≥ 32	≥ 1024	4	16	16	0.19	≥ 32	32	0.38	≥ 32	192	1.5
5242	≥ 32	≥ 1024	3	8	24	0.25	4	24	0.25	≥ 32	384	1.5
1553	≥ 32	≥ 1024	2	≥ 32	48	0.25	16	48	0.38	≥ 32	256	1.5
2131	12	32	0.38	1	6	0.094	1.5	6	0.125	8	64	0.5
2444	8	16	0.38	6	12	0.19	6	16	0.25	8	24	0.25

Abbreviations: IPTG, isopropyl-1-thio-β-D-galactopyranoside; Smx, sulfamethoxazole; SXT, co-trimoxazole (**x** Tmp + 19**x** Smx); Tmp, trimethoprim.
[1] The detection limits for Etest® are 32 µg/mL for Tmp and 1024 µg/mL for Smx.

Similarly, the MICs for sulfamethoxazole dropped in all of the strains lacking the *bpeEF-oprC* genes and increased in induced complemented strains. In testing the trimethoprim MICs we noted, that several of the strains remained near or at the limit of detection (32 µg/mL) in the absence of the BpeEF-OprC pump. It is not possible to determine if the trimethoprim MICs have decreased in these strains (1468, 5041 and 1553) using the Etest method. Determining the MIC by microdilution would allow for testing above 32 µg/mL, however this method has been shown to be highly variable and inaccurate in determining MICs for the bacteriostatic trimethoprim antimicrobial (N.L. Podnecky, T. Mima and H.P. Schweizer, unpublished findings). The trimethoprim MICs were shown to drop noticeably in strains 1374, 5242 and 2131 in the absence

of *bpeEF-oprC*. Interestingly, there were only very slight changes to any of the MICs for strain 2444 in the absence of the *bpeEF-oprC* genes.

6.4.4 BpeT is necessary for co-trimoxazole resistance in some strains.

Similar studies were done to examine changes to the MICs in the absence of BpeT in these 7 clinical isolates (**Table 6.6**). We observed a substantial drop in MIC for both sulfamethoxazole and co-trimoxazole in all but 2 of the strains tested (**Table 6.6**).

Table 6.6 BpeT is required for co-trimoxazole resistance in some clinical isolates.

	Minimal Inhibitory Concentration (μg/mL)[1]											
	bpeT^{+}			Δ*bpeT*			Δ*bpeT*:: mini-Tn*7*T			Δ*bpeT*:: mini-Tn*7*T-P_{bpeT}-*bpeT*		
Strain	Tmp	Smx	SXT	Tmp	Smx	SXT	Tmp	Smx	SXT	Tmp	Smx	SXT
1374	≥ 32	≥ 1024	4	≥ 32	≥ 1024	6	≥ 32	≥ 1024	6	≥ 32	≥ 1024	12
1468	≥ 32	≥ 1024	4	≥ 32	96	0.38	≥ 32	96	0.38	≥ 32	256	1.5
5041	≥ 32	≥ 1024	4	≥ 32	≥ 1024	6	≥ 32	≥ 1024	8	≥ 32	≥ 1024	12
5242	≥ 32	≥ 1024	3	6	16	0.19	8	24	0.25	≥ 32	48	0.75
1553	≥ 32	≥ 1024	2	3	8	0.125	6	16	0.19	≥ 32	32	1
2131	12	32	0.38	2	16	0.19	2	16	0.125	3	16	0.125
2444	8	16	0.38	1.5	6	0.094	1	8	0.094	3	12	0.125

Abbreviations: Smx, sulfamethoxazole; SXT, co-trimoxazole (**x** Tmp + 19**x** Smx); Tmp, trimethoprim.

[1] The detection limits for Etest® are 32 μg/mL for Tmp and 1024 μg/mL for Smx.

Complementation with the 1026b-derived P_{bpeT}-*bpeT* resulted in increases to these MICs in most of the tested strains. Strains 1374 and 5041 remained resistant to both sulfamethoxazole and co-trimoxazole in the absence of BpeT. Additionally, in these 2 strains and strain 1468 there was no detected drop in trimethoprim MIC (**Table 6.6**). The remaining 4 strains (5242, 1553, 2131 and 2444) had marked decreases to MICs for trimethoprim alone in the absence of *bpeT* (**Table 6.6**). Overall, these findings indicated that BpeT can contribute to increased trimethoprim, sulfamethoxazole and thus co-trimoxazole MICs in *B. pseudomallei* isolates.

However, BpeT is not essential for resistance to these folate-pathway inhibitors, as demonstrated in strains 1374 and 5041.

6.4.5 *bpeEF*-oprC expression in 1374 and 5041 is BpeT-independent.

In addition to determining the MICs of each of the clinical strains lacking BpeT, the expression of *bpeF* mRNA was compared among the clinical isolates. We found that in most of the strains there was a drop in *bpeF* expression (**Figure 6.3**). We however saw no change in *bpeF* expression in strains 1374 and 5041, which overexpress *bpeF* nearly 100 times that of strains 1026b, 2444 and the trimethoprim resistant 1553 and 2131 (**Figure 6.3**). The very high expression of *bpeF* in the absence of BpeT explains the unaffected trimethoprim, sulfamethoxazole and co-trimoxazole MICs and suggests that expression is BpeT-independent (**Table 6.5**).

6.4.6 Clinical isolates fall into distinct phenotypic groups.

The clinical isolates are clustered by phenotype into 5 groups based on their respective MIC and gene expression data (**Figure 6.3**). **(1)** The folate pathway inhibitor sensitive strains 1026b and 2444 do not overexpress *bpeF* or *bpeT* when grown in the absence of an inducer. Strain 2444 does however significantly increase *bpeF* expression in the presence of trimethoprim. **(2)** Strains 2131 and 1553 are resistant to trimethoprim alone and do not have high expression of *bpeF* under normal growth conditions. However, there is a significant increase in *bpeF* expression in strain 2131 when induced with high concentrations of trimethoprim. **(3)** Strain 1468 is resistant to all of the folate pathway inhibitors, but does not overexpress *bpeF* under normal growth conditions. However, it does express *bpeF* at a higher level when induced with trimethoprim, and *bpeF* expression is significantly reduced in the absence of *bpeT*.

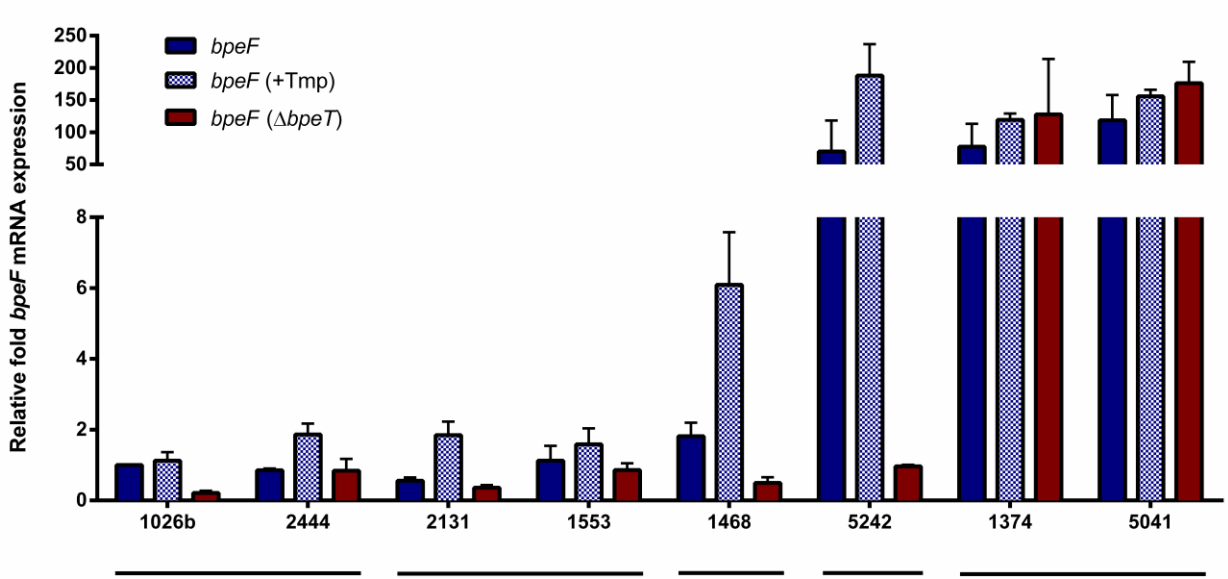

Figure 6.3 Comparison of MIC and *bpeF* expression data in 8 clinical isolates from Thailand. Comparison of resistance to the folate pathway inhibitors trimethoprim (Tmp), sulfamethoxazole (Smx) and co-trimoxazole (SXT) is shown in the table. The relative *bpeF* mRNA levels are shown both LB with (**solid blue** & **2nd row**) and without (Δ*bpeT*; **solid red** & **4th row**) BpeT and in wild-type strains following induction with 32 µg/mL for 1 h (+Tmp; **blue stippled** & **3rd row**). Relative *bpeT* mRNA expression was also compared (**5th row**, data from **Figure 6.2**). Error bars indicate the standard deviation. Two-way ANOVA with Tukey's and Sidak's multiple comparisons tests were used (****, $p \leq 0.0001$; ***, $p \leq 0.001$; *, $p < 0.05$; n.s., not significant).

(4) Strain 5242 is also resistant to all of the folate pathway inhibitors and greatly overexpresses *bpeF* mRNA under normal conditions. The addition of trimethoprim further increases the expression of *bpeF* mRNA in 5242, while the absence of *bpeT* reduces *bpeF* expression to the level seen in strain 1026b. **(5)** Finally, strains1374 and 5041 are grouped together as both are resistant to the folate pathway inhibitors and highly express *bpeF* mRNA under normal conditions, similar to strain 5242. However, these 2 strains are unique in that the high level *bpeF* expression does not increase in the presence of trimethoprim, nor does it decrease in the absence of *bpeT*. This suggests that there constitutive overexpression of the efflux pump encoding genes in these strains. In contrast, all of the other resistant strains appear have inducible phenotypes. Genetic mutations to *bpeEF-oprC* regulatory elements and other trimethoprim and sulfamethoxazole resistance determinants may explain the variety of observed phenotypes in this collection of clinical isolates.

6.4.7 Genetic mutations contributing to increased *bpeEF-oprC* expression.

DNA sequencing of clinical and environmental *B. pseudomallei* isolates was employed to identify mutations of interest. Several of the clinical isolates in this study, 1374, 5041 and 5242, significantly overexpress *bpeF* mRNA (**Figure 6.1**). DNA sequencing of the *bpeT-llpE* intergenic region containing the putative *bpeT* and *llpE-bpeEF-oprC* promoters did not identify any nucleotide changes specific to the overexpressing strains compared to 1026b and other isolates that do not overexpress *bpeF* mRNA (**Table 6.7**). However, we did observe an amino acid substitution in the putative lipase *llpE* (D293A) of strain 5041, which is the first gene in the *llpE-bpeEF-oprC* operon. Previous work has shown that the LlpE lipase is not essential for BpeEF-OprC-mediated efflux (T. Mima and H.P. Schweizer, unpublished results). We also observed a single nucleotide insertion 12 bases upstream of the *bpeE* start site in strains 1468 and

2650a. Both of these strains overexpress *bpeF* mRNA when induced with trimethoprim and are *bpeT* dependent (**Figure 6.3**, **Chapter 3**), however, based on the location of this mutation it is unlikely to have an effect on BpeEF-OprC expression or function.

The *bpeT* gene was sequenced to see if mutations to the BpeT LysR-type regulator are responsible for increased efflux pump expression in these clinical isolates. However, only synonymous mutations were found in this gene, there were no amino acid substitutions in any of the tested strains (**Table 6.7**). Additionally, we sequenced the recently described BpeS LysR-type regulator (**Chapter 5**) to determine if mutations to *bpeS* are responsible for *bpeEF-oprC* overexpression. We identified a number of mutations to this gene in both clinical and environmental isolates (**Table 6.7**). Several of the observed mutations were common among the clinical and environmental isolates, such as a nucleotide substitution 4 bp upstream of the *bpeS* start site, and the following amino acid substitutions: K88R, T178A, and L211S (**Table 6.7**). These mutations were common to isolates that overexpress *bpeF* and others that do not, suggesting that they are not involved in regulation of *bpeEF-oprC* expression. Several mutation were observed that were unique to single trimethoprim resistant strains (2677a, E0235, MSHR 305 and MSHR 668). While these mutations may contribute to trimethoprim resistance, they are not sufficient to confer resistant to the co-trimoxazole combination. However, the BpeS proline to serine substation at amino acid 28 was found to be unique to strains 1374 and 5041, which are phenotypically similar (**Figure 6.3**). Further studies are necessary to confirm that this mutations to BpeS in responsible for the constitutive overexpression of *bpeEF-oprC* in these strains.

Recent work suggests that sulfamethoxazole may be a substrate of the BpeEF-OprC efflux pump (**Chapter 5**) and the current MIC data supports this finding (**Table 6.5** & **Table 6.6**). Structural changes to the membrane associated transporter protein of an efflux pump can

alter substrate specificity. The *bpeF* gene, which encodes for the RND transporter of the BpeEF-OprC efflux pump, was sequenced. However there were no non-synonymous mutations identified (**Table 6.7**). This may suggest that sulfamethoxazole is a standard substrate of the BpeEF-OprC efflux pump, and that substantial overexpression is necessary for the observed increases in sulfamethoxazole MIC.

6.4.8 Drug target and biosynthetic pathway modifications.

The BpeEF-OprC efflux pump plays a necessary role in resistance to co-trimoxazole, but it is unclear if there are other mutations contributing to the observed resistance. To investigate this further, the *folA* and *folP* genes were sequenced in each of the co-trimoxazole clinical isolates, as well as, additional clinical and environmental isolates (Table 6.7). Interestingly, we found 2 mutations to the *folA* gene (V61A & A145T) in strains 1374, 1553 and 2444, relative to 1026b. The V61A mutation was also found in 5 other trimethoprim resistant clinical and environmental isolates (Table 6.7). However, these mutations do not likely contribute to resistance as strain 1553 is co-trimoxazole susceptible and strain 2444 is susceptible to both trimethoprim and co-trimoxazole.

DNA sequencing of the *folP* gene in the clinical isolates revealed only one mutation, FolP A120V. This mutation was found in strain 1468, which is co-trimoxazole resistant (Table 6.4), but expresses *bpeF* at a significantly lower level than the other co-trimoxazole resistant isolates in this study (**Figure 6.3**). To determine if this mutation alone contributes to increases in sulfamethoxazole MIC, this single nucleotide polymorphism (SNP) was introduced into the attenuated Bp82 strain using allelic exchange. MIC testing of Bp82.265 showed no major differences in trimethoprim, sulfamethoxazole or co-trimoxazole susceptibility, compared to the

Table 6.7 Genetic variation in clinical and environmental *B. pseudomallei* isolates.

Genetic Changes in Relevant Genes Compared to the 1026b Sequence								
Strain[1]	SXT MIC	FolA	FolP	Ptr1	BpeT	IR (*bpeT-llpE -bpeE*)	BpeS	BpeF
1374	4	V61A, A145T	-	-	-	-	t-4c, P28S, T178A	-
1468	4	-	A120V	G33D, R42H	-	**::a *bpeE* (-12)**	t-4c, T178A	-
5041	4	-	-	**Δ (54-60)**	-	LlpE D239A	t-4c, P28S, T178A	-
5242	3	-	-	-	-	-	K88R, T178A	-
1553	2	V61A, A145T	-	-	-	-	t-4c	-
2677a	1.5	-	ND	G33D, R42H	-	-	t-4c, I35T, T178A, L211S	ND
E0235	1.5	-	ND	-	-	-	**Δ179**, K88R, T178A, L211S	-
2650a	1	V61A	ND	R52C	-	**::a *bpeE* (-12)**	t-4c, T178A, L211S	ND
2665a	1	V61A	ND	-	-	-	K88R, L211S	ND
2719a	1	V61A	ND	-	-	-	t-4c, T178A	-
MSHR 465a	1	ND	ND	-	-	ND	t-4c, T178A	ND
2679a	0.75	-	ND	-	-	-	t-4c, T178A, L211S	ND
E0016	0.75	V61A	ND	-	-	-	T178A, L211S	ND
E0342	0.75	V61A	ND	R52C	-	-	t-4c, K88R, T178A, L211S	-
E0237	0.5	-	ND	-	-	-	-	ND
MSHR 305	0.5	ND	ND	R113C, D54N	-	ND	t-4c, A76T, T178A	ND
2131	0.38	-	-	-	-	-	-	-
2444	0.38	V61A, A145T	-	-	-	-	T178A	-
MSHR 668	0.38	ND	ND	R113C	-	ND	c-68a, t-25c, t-4c, T178A	ND

Abbreviations: IR, intergenic region; ND, no data; SXT, co-trimoxazole; ::, insertion; Δ, deletion.

[1] *B. pseudomallei* isolates are ordered by decreasing co-trimoxazole MIC.

Red text indicates resistance to co-trimoxazole (SXT MIC > 2 μg/mL). Lower case font indicates nucleotide, upper case indicates amino acid. Bold type font indicates nucleotide insertion or deletion. Underline indicates nucleotide changes in non-coding regions.

parental strain (**Table 6.9**). These data suggest that FolP A120V does not contribute to co-trimoxazole resistance.

Table 6.8 FolP A120V mutation does not alter relevant antimicrobial susceptibilities.

		MIC (μg/mL)		
Strain	FolP	Tmp	Smx	SXT
Bp82	WT	0.5	4	0.094
Bp82.265	A120V	0.5	4	0.094

Abbreviations: MIC, minimal inhibitory concentration; Smx, sulfamethoxazole; SXT, co-trimoxazole (**x** Tmp + 19**x** Smx); Tmp, trimethoprim.

In addition to drug target modification, previous studies have suggested that mutations to a gene encoded pteridine reductase (Ptr1) may contribute to increased trimethoprim MICs in *E. coli* (15, 27) or sulfamethoxazole and co-trimoxazole resistance in *B. pseudomallei* (**Chapter 5**). DNA sequencing of this gene in these clinical isolates revealed 2 mutations leading to amino acid substitutions in strain 1468 (G33D and R42H) and a 7 nucleotide deletion resulting in a frame shift mutation in strain 5041. The G33D and R42H mutations were also observed in the trimethoprim resistant 2677a strain. However, the frame shift mutation is unique to strain 5041 and causes amino acid substitutions starting at amino acid 20 and early termination of the protein after 90 residues. Previous studies have suggested that absence of a functional Ptr1 homolog, FolM, may result in overexpression of the FolA trimethoprim drug target causing increases to the trimethoprim MIC (15). A similar frame shift mutation was described in a recent study investigating co-trimoxazole resistance in laboratory induced mutants (**Chapter 5**). MIC testing of a Bp82Δ*ptr1* strain had slight (3-fold) increases in both trimethoprim and sulfamethoxazole MICs (**Chapter 5**), suggesting that the pteridine reductase mutation in 5041 may contribute to increased co-trimoxazole MICs but alone would not likely confer resistance. Further study is

needed to elucidate the role of Ptr1 in folate synthesis in *B. pseudomallei* and understand the role it may play in decreased co-trimoxazole susceptibility.

6.5 Conclusions

Co-trimoxazole, a potent combination of trimethoprim and sulfamethoxazole, is often used for melioidosis treatment and prophylaxis. This is the first study to investigate the mechanisms responsible for co-trimoxazole resistance in clinical *B. pseudomallei* isolates. Clinical isolates are difficult populations to work with as the genetic diversity between the isolates is high and there are often multiple explanations for observed phenotypes and convoluted observations. Despite this complexity, we were able to group these isolates into 5 unique populations based on antimicrobial resistance and BpeEF-OprC efflux pump expression phenotypes. However, characterization of a larger population of isolates is necessary to determine if there are other naturally occurring factors responsible for folate pathway inhibitor resistance in *B. pseudomallei* isolates.

We observed that the BpeEF-OprC efflux pump was necessary for co-trimoxazole resistance. Overexpression of this pump was observed in all of the co-trimoxazole resistant isolates relative to those that were co-trimoxazole sensitive. This RND efflux pump has previously been reported to be responsible for trimethoprim resistance (**Chapter 3**) (17), co-trimoxazole resistance in laboratory induced mutants (**Chapter 5**), and resistance to other clinically relevant antimicrobials, including chloramphenicol and doxycycline (T. Mima and H. P. Schweizer, unpublished results). Previous work with laboratory selected co-trimoxazole resistant *B. pseudomallei* isolates suggests that overexpression of the BpeEF-OprC efflux pump was necessary but not sufficient for co-trimoxazole resistance (**Chapter 5**). Due to the genetic diversity of the strains, it is unclear if BpeEF-OprC efflux is the only contributing factor for co-

trimoxazole resistance in these clinical isolates. While mutations to the antimicrobial targets FolA and FolP do not appear to contribute to resistance, mutations to Ptr1 may contribute to the increased co-trimoxazole MIC but alone are not sufficient for co-trimoxazole resistance.

BpeEF-OprC regulation is complex and mutations to the known LysR-type regulators BpeT and BpeS have been shown to cause increased expression of *bpeEF-oprC*. BpeEF-OprC expression is largely dependent on BpeT, however we identified 2 isolates that do not require BpeT for high level expression. Further research is needed to better understand the mechanism of action for these regulators and other components contributing to expression of BpeEF-OprC. Repression of BpeEF-OprC efflux pump expression or supplementation of treatment with a specific efflux pump inhibitor would likely reduce or prevent the development of co-trimoxazole resistance in *B. pseudomallei* and thus improve the efficacy of melioidosis treatment.

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CHAPTER 7: Concluding Remarks

Burkholderia pseudomallei is a pathogen capable of causing the severe disease melioidosis. This bacterium is of great concern in endemic regions; though its global distribution appears to be expanding, this is likely a result of improved surveillance and awareness (1), but may also be due to increased foreign travel and a changing climate (2-4). Additionally, *B. pseudomallei* is of concern as it is naturally resistant to many antimicrobials and is currently listed as a Tier 1 Select Agent. Elucidation of the mechanisms of antimicrobial resistance will provide useful information for the development of improved and novel therapeutics.

While *B. pseudomallei* is intrinsically resistant to numerous antimicrobials, it is generally susceptible to co-trimoxazole, a combination of the folate pathway inhibitors trimethoprim and sulfamethoxazole. Co-trimoxazole is the primary recommended therapy for the eradication phase and prophylaxis treatment of melioidosis with few alternatives. Co-trimoxazole resistance is relatively rare, but of great concern. The main purpose of this work was to explore and characterize the molecular mechanisms responsible for antimicrobial resistance to the clinically relevant folate pathway inhibitors, trimethoprim and sulfamethoxazole, in *B. pseudomallei*. In the course of several research studies described in this dissertation, significant contributions were made to improve our understanding of the mechanisms contributing to trimethoprim and sulfamethoxazole resistance in clinical and environmental isolates, as well as other potential mechanisms that could arise due to acquired mutations. These mechanisms include:

(i) Trimethoprim and sulfamethoxazole efflux by BpeEF-OprC.

The *B. pseudomallei* BpeEF-OprC efflux pump was previously shown to efflux trimethoprim when expressed in *Pseudomonas aeruginosa* (5). This efflux pump was

found to be responsible for trimethoprim, sulfamethoxazole and/or co-trimoxazole resistant isolates throughout this study, as briefly described below:

In **Chapter 3,** the BpeEF-OprC efflux pump was found to be essential for trimethoprim resistance in all of the clinical and environmental isolates tested. The trimethoprim resistant strains overexpress *bpeEF-oprC* in the presence of trimethoprim. This trimethoprim-induced overexpression requires expression of the BpeT LysR-type regulator. However, it remains unclear what regulatory component is responsible for the observed inducible expression, as there were no significant mutations observed in *bpeT* or the *bpeT* and *llpE-bpeEF-oprC* promoter regions.

In **Chapter 4**, Bp82 was passively selected on media containing trimethoprim. Unique mutations to the BpeT regulator (L265R and C310R) were found in strains that were trimethoprim resistant and overexpressed *bpeF* mRNA more than 30 fold higher than the parent strain. Introduction of these mutations into the wild-type Bp82 strain confirmed that each of the *bpeT* mutations resulted in overexpression of the BpeEF-OprC efflux pump and conferred resistance to trimethoprim. There also was a decrease in the co-trimoxazole susceptibility and a slight decrease in sulfamethoxazole susceptibility. Antimicrobial susceptibility testing of efflux pump mutant derivatives of the 1026b strain suggest that sulfamethoxazole is an efflux substrate of both the BpeAB-OprB and BpeEF-OprC efflux pumps. Though overexpression of these pumps caused decreased susceptibility, the strains remained well below resistant levels.

A similar technique was used in **Chapter 5** to investigate the mechanisms of co-trimoxazole resistance. The observed resistance in co-trimoxazole selected isolates of Bp82 was dependent on BpeEF-OprC, as deletion of the structural genes for this efflux

pump caused increased susceptibilities to trimethoprim, sulfamethoxazole and co-trimoxazole. The strains expressed *bpeF* mRNA over 100 times more than the parent strain, and deletion of *bpeT* caused a significant reduction of *bpeF* mRNA expression. In the absence of BpeT there was a significant increase of the susceptibility in most of the strains; it is unclear why some of the strains remain resistant to trimethoprim and sulfamethoxazole individually. Overexpression of the BpeEF-OprC efflux pump was found to be a result of mutation to a previously undescribed LysR-type regulator, BpeS. Repair of the BpeS mutation resulted in *bpeF* mRNA expression levels comparable to the Bp82 parental strain.

Finally in **Chapter 6**, the BpeEF-OprC efflux pump was shown to be essential for co-trimoxazole and sulfamethoxazole resistance in clinical isolates from Thailand. The clinical strains that are co-trimoxazole resistant overexpress *bpeEF-oprC* either under normal growth conditions or when induced with trimethoprim. Of the tested strains two had very high level expression that was BpeT-independent and both strains had a notable mutation to BpeS that warrants further investigation.

(ii) Acquired mutations to the trimethoprim drug target, dihydrofolate reductase.

In **Chapter 4**, adaptive resistance to trimethoprim alone was studied in Bp82. Trimethoprim resistant Bp82 isolates either had mutations to BpeT causing BpeEF-OprC overexpression (as described above), or one of two mutations to FolA (I99L and F158V). The I99L mutation has been previously described in a *Burkholderia cenocepacia* strain J2314 (6), and is equivalent to the I94L FolA mutation previously reported in trimethoprim resistant *Escherichia coli* (7). This FolA mutation was also found in 12 more trimethoprim resistant Bp82 isolates lacking either the BpeT regulator or the

BpeEF-OprC efflux pump structural genes. The FolA F158V mutation, to my knowledge, has not been previously described, but in *E. coli* a mutation to the equivalent residue (F153S) may cause alteration of a nearby β-bulge catalytic domain (8). The introduction of each FolA mutation into the Bp82 background caused decreased susceptibility to both trimethoprim and co-trimoxazole. One of the selected Bp82 isolates had mutations to both FolA and BpeT, however despite over 30 fold increase in BpeEF-OprC expression, this strain remained co-trimoxazole sensitive. It would be expected that drug target modification of both the trimethoprim and sulfamethoxazole targets would result in a co-trimoxazole resistant strain. However, no significant mutations to FolP were found to contribute to resistance in these studies.

(iii) Modification of the folate biosynthetic pathway.

In **Chapter 5**, the Bp82 strain was passively selected in the presence of co-trimoxazole. This resulted in two mutations to previously uncharacterized genes that were identified by whole genome sequencing; a mutation to BpeS (described above) and a V15G mutation to Ptr1. The *ptr1* gene of *B. pseudomallei* is annotated as a pteridine reductase, a gene originally identified in trypanosomatids responsible for resistance to a DHFR inhibitor, methotrexate (9, 10). Repair of the *ptr1* mutation caused a significant increases in sulfamethoxazole and co-trimoxazole susceptibilities. Interestingly, deletion of *ptr1* from Bp82 caused decreased trimethoprim and sulfamethoxazole susceptibility, but did not result in resistance to either drug. These data suggest that mutations to *ptr1* may partially contribute to both trimethoprim and sulfamethoxazole resistance, though it is unclear what role Ptr1 plays in the folate synthesis pathway in *B. pseudomallei*.

In trypanosomes overexpression of Ptr1 confers resistance to methotrexate (11, 12). Similarly, overexpression of FolM (a Ptr1 homolog) in *E. coli* caused decreased susceptibility of the strain to trimethoprim (9). FolM was shown to act as a dihydrofolate reductase (9) and Ptr1 can fulfill similar function (13, 14). Thus, it is perplexing that *ptr1* deletion in *B. pseudomallei* actually reduces drug susceptibility. This has been reported in *E. coli* where decreased susceptibility was reported in strains lacking FolM (15). The authors suggested a compensatory overexpression of FolA may contribute to the observed phenotype. In this context, it is possible that Ptr1 inactivation or deletion results in overexpression of both FolA and FolP, causing decreased susceptibilities to both trimethoprim and sulfamethoxazole. Alternatively, the absence of *ptr1* may induce other modifications to either improve the efficiency of the folate synthesis pathway or bypass the inhibited enzymatic steps of this process.

The research in this dissertation has contributed to our understanding of the mechanisms for folate pathway inhibitor resistance in *B. pseudomallei*. We were able to conclusively show that the BpeEF-OprC efflux pump is a key resistance determinant for folate pathway inhibitor resistance in *B. pseudomallei*. Combination therapy is generally thought to be superior to mono-therapy for preventing the emergence of antimicrobial resistance, as it is assumed that mutations resulting in resistance to both drugs individually are required and would be infrequent.

In most cases the BpeEF-OprC efflux pump was insufficient alone to confer co-trimoxazole resistance; however, if this efflux pump can effectively expel both antimicrobials, resistance could emerge rapidly. Much work is needed to understand the regulation of BpeEF-OprC expression. It appears that there are more regulatory components than have currently been identified, as differential expression could not be explained by mutations to currently known

regulators, including the newly described BpeS LysR-type regulator. It would also be prudent to determine whether sulfamethoxazole is a natural substrate of BpeEF-OprC or if it is effluxed only under certain conditions. As a way to prevent treatment failure, further characterization of the BpeEF-OprC efflux pump and investigation of potentially effective efflux pump inhibitors may prove useful, as deletion of *bpeEF-oprC* in all co-trimoxazole resistant strains tested resulted in co-trimoxazole susceptibility. The efflux pump inhibitor would have to be specific to limit toxicity to eukaryotic cells; for example a compound that specifically blocks the association of the BpeF transporter and the antimicrobial(s) (16).

Future work is also necessary to investigate the folate synthesis pathway in *B. pseudomallei*. The role of Ptr1 remains unclear, but the elucidation of its function as a resistance determinant could provide useful information for improvement or development of novel folate pathway inhibitors. This could include investigation of the expression levels of *folA, folP* and *ptr1* under various conditions to determine if *folA* and/or *folP* overexpression is in fact responsible for decreased susceptibility. Additionally biochemical analyses, similar to those previously described (17), could be used to study Ptr1.

Antimicrobial resistance is no longer an emerging problem; it is a current problem. This is true especially in organisms that are already intrinsically resistant to many antimicrobials. Determination and monitoring of the mechanisms of resistance will assist clinicians in adapting antimicrobial therapies to improve outcome and reduce the emergence of resistance. Also these studies may provide useful information to those who are developing improved treatment options for multi-drug resistant organisms, including one particularly remarkable organism: *Burkholderia pseudomallei*.

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LIST OF ABBREVIATIONS

a	adenine
A	alanine
ABC	ATP binding cassette superfamily
Acr	acriflavine
Ade	adenine
AHL	*N*-acyl-homoserine lactone
Amp	ampicillin
ANOVA	analysis of variance
APHIS	U.S. Department of Agriculture – Animal and Plant Health Inspection Service
ATP	adenosine triphosphate
B.	*Burkholderia*
BLAST	Basic Local Alignment Search Tool
bp	base pairs
BSL	Biosafety Level
°C	degrees Celsius
c	cytosine
C	cysteine
Car	carbenicillin
CDC	U.S. Dept. of Health and Human Services – Centers for Disease Control and Prevention
cDNA	complementary DNA
CLSI	Clinical Laboratory Standards Institute
CFU	colony forming units
Chl	chloramphenicol
CRE	carbapenem-resistance *Enterobacteriacea*
C_t	threshold cycle
D	aspartic acid
DAP	diaminopimelic acid
DHFR	dihydrofolate reductase
DHPPP	6-hydroxymethl-7,8-dihydropterin pyrophosphate
DHPS	dihydropteroate synthase
DNA	deoxyribonucleic acid
DMSO	dimethyl sulphoxide
Dox	doxycycline
E.	*Escherichia*
e.g.	for example
ELISA	enzyme-linked immunosorbent assay
EPI	efflux pump inhibitor
Ery	erythromycin
et al.	and others

FRT	Flp Recombinase Target
g	guanine
G	glycine
Gen	gentamicin
GTP	guanosine triphosphate
GCHY-I	GTP cyclohydrolase I
h	hour(s)
H	histidine
HIV	human immunodeficiency virus
I	isoleucine
IHA	indirect hemagglutination assay
Imp	imipenem
IPTG	isopropyl-1-thio-β-D-galactopyranoside
K	lysine
kb	kilobase(s)
Km	kanamycin
L	leucine
LB	Lennox Luria-Bertani
LD	lethal dose
LD_{50}	lethal dose 50%, dose necessary to kill 50% of an experimental population
LPS	lipopolysaccharide
LPSN	List of Prokaryotic Names with Standing Nomenclature
MATE	multi-drug and toxic compound extrusion family
MDR TB	multi-drug resistant tuberculosis
MFP	membrane fusion protein
MFS	major facilitator superfamily
mg	milligram
µg	microgram
MHA	Mueller Hinton II Agar
MHB	Mueller Hinton Broth (cation-adjusted)
MIC	minimal inhibitory concentration
mL	milliliter
µL	microliter
mM	millimolar
µm	micrometer
mRNA	messenger ribonucleic acid
MRSA	methicillin-resistant *Saphylococcus aureus*
N	asparagine
ND	not done / no data

Nor	norfloxacin
OD	optical density
OMP	outer membrane protein
P	proline
P.	*Pseudomonas*
pABA	*para*-aminobenzoic acid
pABGlu	*para*-aminobenzoyl-glutamate
PBP	pencillin-binding protein
PCR	polymerase chain reaction
pH	potential hydrogen
^{r}	resistance
R	arginine
RNA	ribonucleic acid
RND	resistance-nodulation and cellular-division family
rRNA	ribosomal RNA
RT-qPCR	reverse transcriptase quantitative polymerase chain reaction
s	seconds
S	serine
SCID	severe combined immunodeficiency
SDR	short-chain dehydrogenase/reductase
SMR	small multi-drug resistance family
SOE	splicing by overlap extension
Smx	sulfamethoxazole
SNP	single nucleotide polymorphism
SXT	co-trimoxazole (trimethoprim + sulfamethoxazole)
t	thymine
T	threonine
Tet	tetracycline
Tmp	trimethoprim
tRNA	transfer RNA
V	valine
VBNC	viable but non-culturable
VRSA	vancomycin-resistant *Staphylococcus aureus*
WHO	World Health Organization
XDR TB	extensively drug resistant tuberculosis
X-Gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid
Zeo	Zeocin