DISSERTATION

COUNTER-SELECTION MARKERS FOR ALLELE REPLACEMENT IN *Burkholderia pseudomallei*

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY CAROLINA MARÍA LÓPEZ PELÁEZ ENTITLED "COUNTER-SELECTION MARKERS FOR ALLELE REPLACEMENT IN *Burkholderia pseudomallei*" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

COUNTER-SELECTION MARKERS FOR ALLELE REPLACEMENT IN *Burkholderia pseudomallei*

Burkholderia pseudomallei is a gram negative bacillus that lives in the soil of tropical regions around the planet and causes melioidosis in humans, a disease endemic in regions of Southeast Asia and Northern Australia. The United States government has classified *B. pseudomallei*, and its relative *Burkholderia mallei*, as potential bioterrorism agents. The increased interest in these complex pathogens initiated a quest to better understand the biology of these bacteria at the molecular level. Completed genome sequences of diverse strains have provided a wealth of information that opened new venues for further study. Many genetic tools have been successfully adapted for use in *Burkholderia* species, but others are yet to be discovered. The ability to introduce unmarked single nucleotide changes or other genetic modifications into the *B. pseudomallei* genome, by way of the host's natural homologous recombination pathways, has been hampered by the lack of a suitable counter-selection marker that works efficiently in different wild-type strains. Counter-selection markers allow for the positive selection of strains that have lost the marker and other unwanted sequences around them. This dissertation describes the search for a system that allows isolation of unmarked mutations and single nucleotide changes in

the *B. pseudomallei* genome. Two different systems were proven effective and provide alternative options for isolation of allelic mutants of genes of interest.

The first method uses a mutated allele of the *B. pseudomallei pheS* gene. This gene encodes for a subunit of phenylalanine tRNA synthase. A specific PheS mutant protein exhibits relaxed substrate specificity, allowing for incorporation of a toxic chlorinated phenylalanine analog into proteins resulting in death of cells expressing the mutant protein. Counter-selection based on the mutant *pheS* gene of *B. pseudomallei* allowed for the creation of *amrRAB-oprA* deletion mutants of different *B. pseudomallei* strains. The AmrAB-OprA efflux pump is responsible for intrinsic resistance to aminoglycosides and macrolides in *B. pseudomallei.* Consequently, efflux pump mutants became sensitive to selected aminoglycosides. Also, as a proof of concept experiment, a clean unmarked *purM* mutant was created. *purM* mutants are thiamine and adenine auxotrophs and have been shown to result in a strong attenuation of virulence in a mouse model of melioidosis.

A second system based on the I-*Sce*I homing endonuclease of *Saccharomyces cerevisiae* was also developed. Expression of the endonuclease in cells containing chromosomal I-*Sce*I recognition sites integrated in their chromosomes in place of counter-selection markers via homologous recombination, leads to the selection of isolates that have lost the sites and thus unwanted sequences containing them. This is because I-*Sce*I creates double-strand breaks and promotes recombination between nearby homologous sequences. As a proof of concept experiment this system was also used to create a *B. pseudomallei purM* mutant. Furthermore, by creating a temperature sensitive *fabD* mutant due to a point mutation in the *fabD* gene proved that I-*Sce*I could be used to create

point mutations. FabD is an essential enzyme of the bacterial fatty acid biosynthesis pathway.

In summary, this report describes the first counter-selection markers that work in wild-type *B. pseudomallei* strains. Availability of the markers will allow the routine generation of mutants required for studies of the biology and pathogenesis of this understudied pathogen and the related *B. mallei.*

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LIST OF ABBREVIATIONS

Δ	gene deletion
aacC1	aminoglycoside N-acetyltransferase protein encoding gene
ACC	acetyl-CoA carboxylase complex
aceK	bifunctional isocitrate dehydrogenase kinase/phosphatase protein
ACP	acyl carrier protein
AHL	acyl homoserine lactone
AIR	5'-Phosphoribosyl-5-aminoimidazole
AMP	adenosine monophosphate
amrAB-oprA	AmrAB-OprA efflux pump operon
amrR	putative transcription regulator gene
Ap	ampicillin
araA	L-arabinose isomerase enzyme encoding gene
araB	L-ribulokinase enzyme encoding gene
AraC	Transcriptional regulator of the arabinose operon in *E. coli*
araD	L-ribulose-phosphate epimerase enzyme encoding gene
ATCC	American Type Culture Collection
bla	β-lactamase-encoding gene
ble	bleomycin resistance gene
bp	base pair
bpeE	multidrug efflux pump *bpeE* gene
cAMP	cyclic adenosine monophosphate
ccdB	plasmid maintenance protein gene
CoA	coenzyme A

CS	counter-selection
dhfrII	dihydrofolate reductase II gene
DNA	deoxyribonucleic acid
DnaA	chromosomal replication initiator protein DnaA
dNTP	deoxy-ribonucleoside triphosphate
EDTA	ethylenediamine tetraacetic acid
era	*E. coli* Ras-like protein encoding gene
eradE	*E. coli era* gene with a deletion in the effector region
FabA	β-hydroxyacyl-ACP dehydratase
FabB	β-ketoacyl-ACP-synthase I
FabD	malonyl-CoA:ACP transacylase
FabF	β-ketoacyl-ACP synthase II
FabG	β-ketoacyl-ACP reductase
FabI	enoyl-ACP reductase
FabK	enoyl-ACP reductase
FabZ	hydroxyacyl-ACP dehydratase
FavV	enoyl-ACP reductase
FtsZ	cell division protein ftsZ
g	gram
galK	galactokinase enzyme encoding gene
glnQ	glutamine transporter subunit encoding gene
Gm	gentamycin
GMP	guanosine monophosphate
GST	Glutathione-S-transferase
guaA	bifunctional GMP synthase/glutamine amidotransferase protein
guaB	inositol-5-monophosphate dehydrogenase
GyrA	DNA gyrase subunit A protein
h	hour
hemH	phosphoribosylaminoimidazole-succinocarboxamide synthase

hfq	*E. coli* gene encoding host factor for RNA phage Q beta replication
IHA	Indirect hemaglutination
IMP	formyltransferase/IMP cyclohydrolase
IPTG	Isopropyl-β-D-thiogalactopyranoside
IS	insertion sequence
kb	kilobase
Km	kanamycin
kV	kilovolts
L	liter
lacZα	β-galactosidase alpha peptide encoding gene
LB	Luria Bertani
lys	lysine
MIC	minimal inhibitory concentration
min	minute
mL	mililiter
mM	milimolar
mRNAs	messenger ribonucleic acids
NAD^+	oxidized β-nicotinamide adenine dinucleotide
NADH	reduced β-nicotinamide adenine dinucleotide
$NADP^+$	oxidized β-nicotinamide adenine dinucleotide phosphate
NADPH	reduced β-nicotinamide adenine dinucleotide phosphate
NEB	New England Biolabs
ng	nanogram
nptII	aminoglycoside phosphotransferase encoding gene
oprA	outer membrane efflux protein encoding gene
oriT	origin of transfer
p	*para*
P_{BAD}	*E. coli* arabinose inducible *araBAD* promoter

PCR	polymerase chain reaction
pheS	phenylalanine tRNA synthase gene
*pheS**	phenylalanine tRNA synthase gene with substitution of alanine in position 304 for glycine
$pheS^{A304G}$	phenylalanine tRNA synthase gene with substitution of alanine in position 304 for glycine
prsA	ribose-phosphate pyrophosphokinase
P_{S12}	promoter for the *B. thailandensis* ribosomal S12 protein-encoding gene
P_{tac}	*E. coli trp-lac* hybrid promoter
purA	adenylosuccinate synthetase
purB	adenylosuccinate lyase
purD	phosphoribosylamine--glycine ligase
purE	phosphoribosylaminoimidazole carboxylase catalytic subunit
purF	amidophosphoribosyltransferase
purH	bifunctional phosphoribosylaminoimidazolecarboxamide
purL	phosphoribosylformylglycinamidine synthase
purM	phosphoribosylaminoimidazole synthase
purN	phosphoribosylglycinamide formyltransferase
R	resistant
rpsL	*E. coli* ribosomal protein of the large subunit encoding gene
sacB	*Bacillus subtilis* gene encoding levansucrase
sRNAs	small ribonucleic acids
tRNA	transfer ribonucleic acid
thiC	thiamine biosynthesis protein ThiC
thiD	phosphomethylpyrimidine kinase
thiE	thiamine-phosphate pyrophosphorylase
Tmp	trimethoprim
TS	temperature sensitive

μF	microfaraday
μg	microgram
μL	microliter
μm	micrometer
Φ	phage

CHAPTER ONE

Introduction

1.1 The genus *Burkholderia*

1.1.1 History of the genus *Burkholderia*

In 1992 some bacterial species belonging to the rRNA homology group II of the genus *Pseudomonas* were transferred to a new genus, *Burkholderia*, based on DNA-DNA homology values, cellular lipid and fatty acid composition, and phenotypic characteristics (Yabuuchi et al. 1992). The new genus became the type order of the new class *Betaproteobacteria.* The Betaproteobacteria posses 16S rRNA sequence similarities approaching 86% (Lessie et al. 1996).

This new taxonomic classification assigned the name *Burkholderia* to the new genus, to honor Walter H. Burkholder, a Cornell University bacteriologist who had described the type species of the genus, now named *B. cepacia* complex, as a causative agent of onion rot in 1950 (Burkholder 1950). The *Burkholderia. cepacia* complex includes five different genomovars (phylogenetically different genomic species which are indistinguishable phenotypically), these bacteria are now known as opportunistic human pathogens that

cause pneumonia in immunocompromised individuals with underlying diseases, especially in cystic fibrosis patients (Vandamme et al. 2003).

During its sixteen years of existence the genus has been extensively modified and expanded through detailed genotypic and phenotypic analyses which include whole-cell protein profiles, DNA-RNA hybridization, DNA-DNA hybridization, fatty-acid analyses and biochemical tests to now include more than 40 valid and proposed species of Gram negative rods (www.bacterio.cict.fr/b/*Burkholderia*.html). The genus is well defined phylogenetically, but there is a lot still to be learned from a functional perspective.

1.1.2 The bacteriology of the *Burkholderia* spp.

Bacteria in the genus are aerobic, non-spore-forming, straight or slightly curved rods of 1 to 5 μm in length and 0.5 to 1 μm in width. They are motile due to the presence of one or more polar flagella (with the exception of *B. mallei* which is nonmotile). They are all catalase positive and lactose-non-fermenters when grown on MacConkey agar (Brenner et al. 2005). Strains can grow on minimal media and they are chemoorganotrophs and can use diverse carbon and energy sources such as simple and complex carbohydrates, amino acids and alcohols (Vermis et al. 2003). Most are able to degrade glucose oxidatively and can use nitrate as the ultimate electron acceptor under anaerobic conditions (Brenner et al. 2005). Interestingly, some species are able to degrade man made organic pollutants and their potential as biodegraders is still being assessed (O'Sullivan and Mahenthiralingam 2005). Most culturable species can be grown at 30°C in the laboratory and many can also

grow at 37°C or even at 42°C (*B. mallei* cannot be grown at temperatures above 40°C) (ASM Sentinel Laboratory Guidelines).

Some members of the genus, including *B. pseudomallei*, accumulate carbon reserve granules within their cytoplasm filled primarily with poly-ß-hydroxy-butyrate, exhibiting a bipolar staining in Gram stains described as having a safety pin appearance (Coenye et al. 2001). This accumulation of energy in cellular granules may be reflective of a metabolism adapted for long term survival under nutrient or oxygen deprived conditions because the poly-ß-hydroxy-butyrate can be degraded back to acetyl-CoA generating reducing equivalents (Mandon et al. 1998).

1.1.3 Habitats of *Burkholderia* spp.

Burkholderia species can be found in a great variety of niches. The majority are soil dwelling and exhibit different types of non-pathogenic interactions with plants. Some colonize roots, stems and leaves and have been shown to fix nitrogen (Achouak et al. 1999; Viallard et al. 1998; Goris et al. 2002; Brämer et al. 2001; Zolg and Ottow 1975). Others are recognized plant pathogens (Coenye et al. 1999; Coenye et al. 2001; Ballard et al. 1970; Li et al. 1999) and yet others are endosymbionts which can live in eukaryote-associated environments (Vandamme et al. 2002; Van Oevelen et al. 2002; van Borm et al. 2002). At the same time, some species are able to occupy multiple environments and may have both pathogenic and symbiotic interactions with plants at one time, but then turn into pathogens in humans and other animals. These species are known as opportunistic pathogens and include, most prominently, the *B. cepacia* complex, *B. pseudomallei* and *B.*

mallei, but others are also reported to cause disease (Graves et al. 1997; Yabuuchi et al. 2000).

Burkholderia in general, are capable of withstanding very hostile environments. Many studied bacteria within the genus have been shown to be able to survive in acidic environments (Inglis and Sagripanti 2006), in dry soils (soil water less than 10%) (Brook et al. 1997), in antiseptic or disinfectant solutions (Oie and Kamiya 1996) or nutrient deficient environments and have been cultured from distilled water after some days (Hamill et al. 1995).

To thrive in environments deprived of iron, Burkholderia synthesize different classes of siderophores which allow them to scavenge iron with high affinity from other molecules present in their environment. Most siderophores produced are hydroxamates, low molecular weight molecules containing a chelating group derived from hydroxamic acid. Strains of the *B. cepacia* complex produce salycilate, pyochelin, ornibactin and cepabactin (Nair et al. 2004; Thomas 2007; Darling et al. 1998). The genes for pyochelin production are present in *B. pseudomallei* and *B. thailandensis*, but not in *B. mallei* (Nierman et al. 2004). Additionally, *B. pseudomallei* has been shown to produce malleobactin, a hydroxamate siderophore molecule (Alice et al. 2006). Siderophores are required for virulence of many bacteria that use them as the most important means of iron acquisition inside the host. Burkholderia have been shown to possess several different putative and proven siderophore systems, as well as other systems that allow them to obtain iron from haem and ferritin, it is possible that to generate a completely virulence attenuated strain, many different systems will have to be silenced.

The fact that so many different habitats can be occupied by species of *Burkholderia*, and even by strains of the same species, may indicate that there are common regulatory mechanisms that allow these bacteria to shift between environments and challenge eukaryotic defenses. The survival and persistence, not only in the environment but also in host cells, offers a remarkable example of bacterial adaptation that warrants more detailed studies.

1.1.4 Characteristics of the genomes of *Burkholderia* spp.

The versatility of the *Burkholderia* spp. is probably related to their complex genomes, which are suited for rapid adaptation to radical changes in environmental growth conditions. Even though the genomes of these bacteria are amongst the largest described in Gram negative bacteria, there appears to be an enormous variation in genome size among these phylogenetically close organisms. For example *B. xenovorans* LB400 has a genome of more than ~9.7 Mb, while the *B. mallei* ATCC23344 genome contains only ~5.8 Mb. This can be explained, at least in part, by the different mobility elements present in their genomes consisting of bacteriophages, plasmids, transposons, IS elements and genomic islands (Lessie et al. 1996; Brown et al. 2000; Holden et al. 2004; Chain et al. 2006) that can promote movement of DNA sequences between species and strains, and in the case of IS elements can also lead to chromosomal rearrangements or deletions (Nierman et al. 2004). Also, chromosomal rearrangements and associated mutations, could provide a basis for spontaneous "pulsed" evolutionary spurts, such as those seen when these bacteria switch from the soil to the lungs of infected patients. Foreign DNA can be

detected by looking for atypical GC content areas. The amount of foreign DNA in genomic islands acquired by horizontal transfer in *B. cepacia* J2315 is estimated to approach 10% of the total genome (Holden and Parkhill 2003). The presence of these genomic islands in isolated bacteria may mean that many of them contain genes encoding pathogenic features, as is the case in other pathogens. But it is also possible that most genomic islands are just the transient evidence for the plasticity of the genomes, and do not confer any advantage for pathogenecity.

All members of the *Burkholderia* genus have multi-replicon genomes. Within the Proteobacteria multiple chromosomes are usually associated with free-living, opportunistic lifestyles. Bacteria which are obligatorily parasitic contain no plasmids and usually only a single chromosome (Moreno 1998). This is generally true also for the *Burkholderia* spp., although *B. mallei*, an obligate intracellular parasite contains two chromosomes, but it is of note that this is one of the smallest genomes within the genus. In these multipartite genomes, large plasmids can be differentiated from chromosomes because they lack housekeeping genes such as ribosomal or tRNA genes.

A conserved feature among *Burkholderia spp.* is the fact that the largest chromosome usually keeps the majority of essential "housekeeping" genes with core functions associated with central metabolism and bacterial growth, and the smaller chromosomes carry more genes associated with adaptation and survival in different habitats. This type of genomic arrangement may account for *Burkholderia* spp. nutritional versatility and adaptability. The division of genomic content would allow for high levels of homologous and illegitimate recombination. The GC content of most species is close to

69% and the regions of the genome, where the GC content is lower than average, usually represent DNA segments that have been acquired more recently, genomic islands present in several *Burkholderia* species are examples of such segments.

It can be expected that knowledge derived from the genome sequencing projects will allow us to gain further insights into functional diversity, evolution and pathogenicity mechanisms. To unravel aspects of biology and evolution and elucidate the function of some 6000 genes, in each strain, versatile genetic methods are needed.

1.1.5 Antimicrobial interactions with *Burkholderia* spp.

Burkholderia spp. are known for being intrinsically resistant to many antimicrobials, and even though most studies have been done only in bacteria that cause human disease, it is reasonable to assume that this resistance can confer some advantage to bacteria that live in competitive environments in the soil. For example, resistance to fusaric acid produced by fungi has been reported in *B. cepacia* (Utsumi et al. 1991). Also resistance to heavy metals in soil has been documented (Jiang et al. 2008). Resistance to cationic antibiotics in *B. cepacia* is attributed to ineffective binding to the outer membrane because of the low number of phosphate and carboxylate groups in its lipopolysaccharide (Cox and Wilkinson 1991).

Due to the architecture of the outer membrane of *B. cepacia*, the LPS cation-binding sites may be protected from binding by polymyxin B and aminoglycosides (Moore and Hancock 1986). Some authors have proposed that resistance to certain certain ß-lactams is due to impermeability of the porins in the membrane to those compounds, or to the fact

that permeable porins may be scarce (Aronoff 1988). Efflux pumps (Guglierame et al. 2006) and ß-lactamases (Baxter and Lambert 1994) have also been described.

B. pseudomallei and *B. mallei* have been found to be intrinsically resistant to many antibiotics but the mechanisms for resistance have been less studied than in *B. cepacia.* As with *B. cepacia*, efflux pumps and β-lactamases have also been identified in *B. pseudomallei* and *B. mallei* (Holden et al. 2004; Chan et al. 2004).

Understanding the mechanisms of resistance is important for better management of patients suffering the diseases caused by *Burkholderia* spp.

An important aspect of the intrinsic resistance to commonly used antibiotics is the fact that *B. pseudomallei* and *B. mallei* are select agents (see section 1.8) and these bacteria are highly resistant to the only antibiotic markers that are permitted, which creates challenges for genetic manipulation.

1.1.6 Production of antimicrobials and toxins

Burkholderia spp. produce a great variety of toxins and compounds that can help in pathogenicity or help suppress the growth of competitive microorganisms in nature, these products contribute to the ability of *Burkholderia* spp. to compete in hostile environments. Phytotoxins such as toxoflavin, tropolone and rhizoxin cause problems in crops but are being studied because their mechanism of action might be useful in the creation of novel antitumor (Scherlach et al. 2006; Wang et al. 2007) or antimicrobial agents (Bentley 2008). Bongkrek acid is a toxin produced by *B. cocovenenans* (Garcia et al. 1999), it was discovered as a poison in an Indonesian food preparation that uses the mold *Rhizopus*

oligosporus to ferment coconut. The contaminated coconut dish can kill its consumer because the toxin impairs mitochondrial respiration (Henderson and Lardy 1970) . This food-borne disease is still a public health concern in some places. Also known are the rhizonins, very potent hepatotoxins produced by a *Burkholderia* strain that colonizes the cytoplasm of *Rhizopus microsporus*, another fungus used frequently to ferment foods (Partida-Martinez et al. 2007).

Some antifungals and phytohormones are studied because they can improve plant health. Several strains of *Burkholderia* produce different compounds that exert beneficial effects on their plant hosts, either by antagonizing and suppressing the effects of pathogens or by promoting plant growth directly, but the deliberate release of these bacteria in the fields is still controversial as little is known about the potential risk of spreading possible human pathogens (Parke and Gurian-Sherman 2001). Nonetheless, from an agricultural perspective, many of the bacteria in the genus should be further studied for their beneficial interactions with plants.

1.1.7 Quorum sensing and biofilms

A characteristic common to all species studied is the presence of complex regulatory networks able to sense environmental changes and respond accordingly, and to send signals to other cells. Quorum sensing mechanisms and biofilm formation have been found in every *Burkholderia* where they have been sought. Biofilms are known for conferring antimicrobial resistance to the cell populations which form them (Donlan and Costerton 2002).

Quorum sensing provides a means by which bacteria can communicate with each other in a population and respond to the communicating signals by regulating a good number of their biosynthetic functions accordingly. Cell to cell communication is achieved via small autoinducer molecules, usually N-acyl-homoserine-lactones (AHLs), which induce the expression of diverse genes in a population-density-dependent manner. In the *B. cepacia* complex there are three known quorum sensing mechanisms, CepIR being the most commonly encountered (Lutter et al. 2001) and BviIR and CciIR being present in only certain strains (Conway and Greenberg 2002; Baldwin et al. 2004). The interaction of these different systems with each other is not well understood. The quorum sensing mechanisms of *B. pseudomallei* and *B. mallei* are even more complex, since several homologues of genes for AHL synthases and genes for receptors identifying the signal have been found, indicating the presence of multiple AHL signal molecules (Eberl 2006). There is still little known about the network of regulated genes that are expressed under the control of quorum sensing but it is already clear that these systems play fundamental roles in the biology and pathogenicity of *Burkholderia* (Sokol et al. 2007).

Quorum sensing has been shown to be an important way of regulating the synthesis and release of exoproducts, which are in many cases associated with virulence, as is the case with proteases, lipases, chitinases, collagenase, polygalacturonase and phospholipase C (Vial et al. 2007).

1.1.8 Select Agents

Since the terrorist attacks of September 11, 2001, and the events involving the intentional release o anthrax spores, in October of the same year, the government of the United States has created a Select Agent List that includes viruses, bacteria and toxins that could threaten the health and safety of the public, animals, and plants. Each agent on this list is federally restricted through regulation of its possession, use, and transfer. The Centers for Disease Control and Prevention (CDC) is one of the agencies that oversees the Select Agent Program, to ensure that restrictions are in place and followed. Every entity possessing a select agent must be registered with the CDC and every person handling Select Agents must complete a Federal Bureau of Investigation security risk assessment. The CDC and another governmental agency (APHIS) also determine what is categorized as a Select Agent by taking into account the following criteria: 1) the effect that exposure to the agent or toxin has on human, animal, or plant health, or on animal or plant products; 2) how the agent or toxin transfers to humans, animals, or plants and how contagious, virulent, and toxic it is; 3) the effectiveness and availability of therapy and vaccines for any resulting disease; and 4) any other criteria deemed critical such as susceptibility of specific age groups. Select Agents are grouped in categories named A, B and C. A similar A, B and C priority pathogen list is used by the National Institute of Allergy and Infectious Diseases (NIAID) to prioritize the institute's biodefense research and funding.

B. pseudomallei and *B. mallei* are Select Agents and many rules and restrictions apply to their handling. For example, all laboratory experiments must be performed in a Biosafety Level 3 (BSL3) facility approved by the CDC. Restrictions also apply to the tools allowed for genetic manipulation. In general, antibiotic selection markers that confer resistance to antibiotics with potential use in clinical settings (human and veterinary medicine) are not allowed. To date, only three antibiotic selection markers are approved and include the marker for zeocin resistance (*ble* gene), the marker for kanamycin resistance (*nptII* gene) and the marker for gentamycin resistance (*aacC1* gene). Also *B. pseudomallei* and *B. mallei* are classified as Category B agents because they are moderately easy to spread, cause moderate rates of disease and mortality, and necessitate BSL3 capabilities and disease monitoring by the CDC.

1.2 *Burkholderia pseudomallei*

1.2.1 Bacteriology of *B. pseudomallei*

The Select Agent *B. pseudomallei* is an oxidase-negative, aerobic, non-spore forming Gram negative bacillus (White 2003). It is a soil saprophyte, and an opportunistic pathogen causing disease called melioidosis in mammals, including humans. It generally resides in surface or muddy water, found in tropical and subtropical regions of the planet, most isolates have been collected from regions where melioidosis is endemic (Radua et al. 2000). It is not clear why there is an apparent preferential distribution in regions of Southeast Asia and Northern Australia.

B. pseudomallei uses a great variety of carbon sources, including sugars, with the characteristic exception of L-arabinose, which can be assimilated instead by *B. thailandensis*. This was recognized as a distinguishing feature between the two species before the taxonomic division came to place defining the two as distinct species. Unlike other members of the genus residing in the rizosphere, *B. pseudomallei* does not fixate molecular nitrogen, but it does accumulate polyhydroxybutyrate in its cytoplasm (Inglis and Sagripanti 2006). *B. pseudomallei* is also able to degrade glyphosate, a glycine analogue used as a herbicide (Peñaloza-Vazquez et al. 1995), further demonstrating its adaptability that is so characteristic of the genus.

B. pseudomallei colonies exhibit different morphotypes on agar plates, even when arising from a single clone. Several environmental conditions seem to trigger these morphotypes. A recent report suggests an association between colony morphotype switching and expression of factors that may be necessary for pathogenesis (Chantratita et al. 2007).

B. pseudomallei grows over a temperature range from 30°C to 42°C in the laboratory. Apparent loss of viability is seen after 24 h at 0°C (Inglis and Sagripanti 2006). The bacterium, like other members of the genus, can live in harsh environments. It probably survives in dry soil for prolonged periods as is evident from the persistence in endemic areas throughout the dry seasons (Thomas and Forbes-Faulkner 1981). Also, studies show the survival of the organims under nutrient deficient envirconditions (Wuthiekanun et al. 1995).

1.2.2 Genome of *B. pseudomallei*

B. pseudomallei strain K96243 was sequenced, annotated and published by the Sanger Institute in 2004 (Holden et al. 2004). According to the NCBI website there are now three additional completed genomes awaiting publication, and 16 are being assembled. The K96243 strain was isolated from a patient from Thailand. Two circular chromosomes comprise the genome. Chromosome 1 is 4.07 Mb in length and encodes 3399 predicted proteins and 69 RNAs. Chromosome 2 is 3.17 Mb in length and encodes 2329 predicted proteins and only 12 RNAs. As in other *Burkholderia* spp. the larger chromosome carries most of the house keeping genes and RNAs, while the second chromosome has a variety of genes encoding accessory functions and genes with no known orthologs that may be important for its adaptation to different environments (Holden et al. 2004). The fact that chromosome 1 has a greater proportion of conserved genes, and that the gene order is well conserved when comparing it to other bacteria, could imply that the two chromosomes have two different ancestral origins.

The genome of this organism carries 16 genomic islands (6% of its genome) (Holden et al. 2004), suggesting extensive horizontal transfer. Also a recent study showed that the presence of these genomic islands can vary amongt different strains of *B. pseudomallei*, but no correlation between the presence of a specific island and the virulence of the isolate was demonstrated (Tumapa et al. 2008). The study only included a few representative islands and strains. It is possible that other studies will be able to identify striking genomic characteristics between clinical and environmental isolates. Phylogenetic studies show

that *B. pseudomallei* is more closely related to *B. mallei* than to *B. thailandensis*, and that *B. pseudomallei* has evolved more recently than other members of the genus (Ou et al. 2005).

In a study aimed at comparing the genomes of 4 different *Burkholderia* species (*B. pseudomallei*, *B. mallei*, *B. cepacia* and *B. thailandensis*) it was found that regions in the genome more likely to undergo rearrangements are richer in species-specific genes and conserved genes with point mutations. Since similar findings were also reported in *Pseudomonas* and *Shigella*, it seems likely that genome rearrangements contribute to functional diversity of the species by providing foci for divergence of shared genes and deletion or acquisition of unique genes (Cheng and Currie 2005).

Another study corroborating the finding that the genome is very prone to changes, compared genomic, transcriptional and proteomic factors between two different isolates and found that many transcriptome differences were due to genes that were present only in one of the strains, and that many of the differences at the proteomic level were due to different protein isoforms (Ou et al. 2005).

1.2.3 Melioidosis

B. pseudomallei causes a disease in humans called melioidosis. Melioidosis was described by Alfred Whitmore in 1911, who found the bacillus in clinical samples from heroin addicts in Burma (now Myanmar) who had been infected with what is now known as *B. pseudomallei* (Cheng and Currie 2005). The disease is called melioidosis because its clinical symptoms are similar to those of glanders, a predominantly equine disease caused

by *B. mallei.* The word melioidosis is composed of the greek "melis" (distemper of asses) and "eidos" (resemblance).

Melioidosis is endemic in Southeast Asia and Northern Australia. It causes 20% of the community acquired septicemias in Northern Thailand, where overall mortality is around 50% (Stevens et al. 2005). In Australia, where medical resources are much better, the disease causes the majority of the fatal cases of community acquired pneumonia in Darwin, with a mortality rate of 20% (Stevens et al. 2003). In endemic areas, *B. pseudomallei* is found in wet soil and rice paddies, where farmers are at greatest risk for acquiring the infection (Suputtamongkol et al. 1994). In Australia, melioidosis is not a very significant public health problem, but the disease does persist in livestock, which has important economic consequences (Thomas 1981). There are also sporadic reports of infections in many other regions around the world including Africa, Indian subcontinent and Central and South America (Dance 1991). The world distribution of melioidosis is shown in **Figure 1.1**.

The route of transmission is not very clear. It is thought that most cases arise from direct contact with contaminated water and soil, but only 6% of cases have a clear history of the source of inoculation (Dance 2000). Aerosols in the environment during rainy seasons have been proposed to cause pneumonia by inhalation, since melioidosis has been shown to be seasonal (Faa and Holt 2002). Also, Vietnam veterans flying in helicopters had a higher rate of melioidosis, undescoring the potential of aerosols as sources of infection (Howe et al. 1971). In experiments, animals can be infected by different routes, but there is no proof that these routes of infection exist outside of the laboratory setting. A few

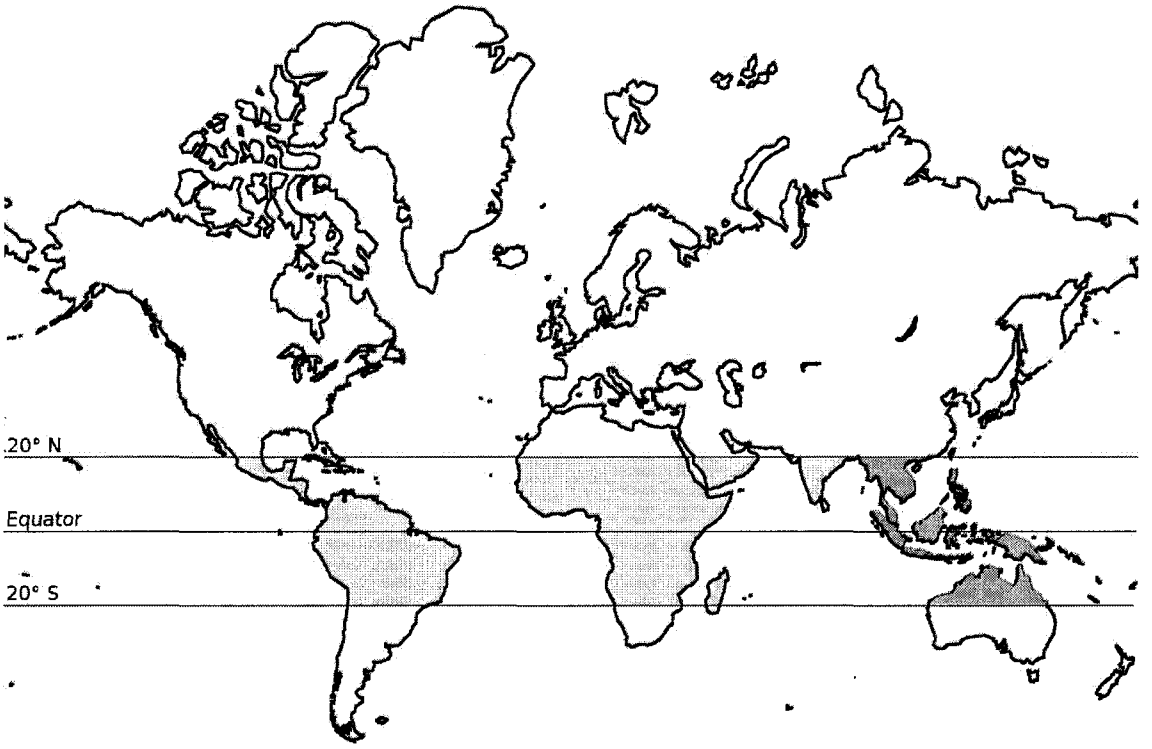


Figure1.1 World map of melioidosis endemic areas

The map shows areas where melioidosis is endemic (in dark gray), and areas where *B. pseudomallei* is possibly found (in light gray). Melioidosis is endemic in countries of Southeast Asia and Northern Australia, where most cases are reported. Sporadic cases of melioidosis are reported in tropical regions around the world, but subtropical regions, immediately above and below the tropics, are believed to be also appropriate habitats for *B. pseudomallei.*

sporadic cases have been reported that resulted from iatrogenic accidents, person to person transmission and animal to person transmission. In Northeast Australia more than 80% of the inhabitants have antibodies against *B. pseudomallei* acquired during early childhood (Dance 2000), but repeated exposure does not lead to protective immunity. This means that the bacterium does not cause overt disease in all individuals exposed, but it seems that it is able to persist somewhere in the body to cause the disease later in life triggered by unknown mechanisms (just like *M. tuberculosis* does). There are reports of Vietnam veterans who were exposed to the bacterium and acquired an overt disease many years post-exposure (Sanford and Moore 1971).

B. pseudomallei can infect a wide range of host species including camels, horses, sheep, cattle, swine, kangaroos, koalas, deer, dogs, cats and marine mammals. Its clinical picture in animals varies as much as it does in humans. Infection associated with emaciation, weakness, edema, lymphangitis of limbs, respiratory signs occasionally with nasal discharge and generalized signs of colic, and always with huge economical burden (Thomas 1981).

In humans the epidemiology of melioidosis is characterized by subtle differences amongst patients in Thailand and Australia. Males older than 45 are affected most commonly, but in general the disease affects mostly adults with predisposing factors generating an altered immune response such as diabetes mellitus, chronic renal failure, alcoholism and granulomatous disease, probably because a neutrophil defect is important for pathogenesis (Cheng and Currie 2005). In Thailand only one fifth of the cases occur in children and most of them do not have identifiable risk factors (Wiersinga et al. 2006). It is

not known whether the cases in children represent differences in genetic host factors or in bacterial virulence. There is a higher incidence of genitourinary infection in Australia, with prostatic abscess in almost 20 percent of men. In contrast, children in Thailand present acute supurative parotiditis in almost 40% of cases. (Cheng and Currie 2005). At the same time a central nervous system involvement, although very rare, seems to be unique to Australian patients. No association was found with specific strains, it is possible that the cases happen because some host factor allows for CNS invasion, though it is not clear if the exclusivity of the localization in Darwin is true or is just the result of better documentation (Currie et al. 2000).

Whether an individual acquires the disease upon exposure seems to be the result of a combination of factors expressed as much by the host as by the pathogen.

Clinical presentation of the disease varies. Most patients present with a pneumonia which can be localized or disseminated and lead to empyema or abscess formation. From the lungs the disease can disseminate hematogenously to distant sites in the body and organ abscesses are common. In cases where the route of infection is likely to be a skin abrasion, bacteremia can also ensue with dissemination throughout the body. The most severe progression is to septic shock, which kills many patients even under the best possible medical care (White 2003), but many patients develop a chronic disease with protean symptoms.

Confirmation of diagnosis requires isolation of the bacterium due to the lack of commercially available. Morover, no studies have confirmed the utility of experimental tests. Different media preparations have been proposed but most laboratories rely on

Ashdown's agar for cultures. This medium contains crystal violet, neutral red and gentamycin to distinguish *B. pseudomallei* from other pathogens (Ashdown 1979). Immunofluorescence detection of lipopolysaccharide is a fast method but requires specialized laboratory equipment and reagents. Indirect hemagglutination (IHA) remains the most widely used antibody detection method, but its results are difficult to interpret, because people in endemic areas have a background of circulating antibodies against *B. pseudomallei* (Cheng and Currie 2005).

Treatment of melioidosis varies between countries. There are differences in bacterial susceptibilities amongst strains, and it is difficult to eradicate the microbe during treatment (Jenney et al. 2001). In general, treatment requires an initial phase of intravenous antibiotic ceftazidime, meropenem, or imipenem/cilastatin for at least two weeks (ceftazidime is the agent of choice because of its lower price). A second phase is aimed at clearing the organism from the body. This maintenance phase with oral trimethoprim sulphamethoxazol or amoxicillin clavulanate lasts at least three months. Even after 20 weeks of treatment relapses can occur (White 2003), showing how inadequate current regimens are.

B. pseudomallei is intrinsically resistant to many antibiotics. Many factors have been implicated in this but not many experimental studies have approached the issue. Beta-lactamases have been found in the genome and some have been cloned (Niumsup and Wuthiekanun 2002; Cheung et al. 2002). Efflux pumps mediate resistance to a wide range of antibiotics as reported by different groups. For example, AmrAB-OprA effluxes macrolides and aminoglycosides (Moore et al. 1999).

There are currently no vaccines available for humans. However, since the bacterium is intracellular candidates should be able to elicit both a humoral and T-cell mediated response. Various groups are trying to create vaccine candidates (Druar et al. 2008).

1.2.4 Virulence factors

B. pseudomallei is capable of invading many cell types including epithelial and phagocytic cells (Harley et al. 1998). Three different type III secretion systems (TTSS) are encoded by the chromosomes of this organism, two of which are similar to plant pathogenic TTSSs, while the third is similar to the Salmonella pathogenicity island, all of which may contribute to pathogenicity. One of the first virulence factors to be studied experimentally in *B. pseudomallei* was a TTSS called the *Burkholderia* secretion apparatus (or *bsa* system), that allows the bacterium to escape the phagosome, replicate in the cytosol and spread to other cells (Stevens et al. 2002). Once in the cytoplasm the bacteria induces formation of actin tail protrusions that get phagocytized by other cells, thereby leading to cell dissemination. BimA is a bacterial protein shown to be required for the polymerization of actin (Stevens et al. 2005). BopE (homologous to SopE of Salmonella) is another type three secreted protein which promotes cell invasion (Stevens et al. 2003). BipD (homologous to SipB of Salmonella) is necessary for assembly of the translocation apparatus that delivers effector proteins necessary for invasion. BipD mutants have been shown to be attenuated in murine models (Druar et al. 2008).

The ability to invade different cell types is in part a result of its resistance to several host antimicrobial peptides, resistance to complement, lysosomal defensins, and cationic

peptidases; and of its interference with the induction of inducible nitric oxide synthase (iNOS) which in normal circumstances would play a role in killing of intracellular bacteria (Wiersinga et al. 2006).

Quorum sensing has been implicated in regulation of putative virulence factors such as siderophores, phospholipase C and biofilm formation (Chan et al. 2007). Several different quorum sensing systems have been discovered in *B. pseudomallei*, and different N-acyl-homoserine lactone molecules have been detected (Ulrich et al. 2004). The PmlI-PmlR system regulates the secretion of a metalloprotease thought to be important for virulence. Mutants defective in the quorum sensing system showed decreased virulence in a murine melioidosis model (Valade et al. 2004). Finally, another quorum sensing system termed BpsI-BpsR, which regulates the expression of a protein that protects *B. pseudomallei* from the oxidative burst (DpsA), also leads to attenuation in experimental melioidosis when mutated (Lumjiaktase et al. 2006).

Other possible virulence factors include a capsular polysaccharide, that inhibits phagocytosis, and other polysaccharides, which are needed for full virulence in murine models (Sarkar-Tyson et al. 2007), the lipopolysaccharide (DeShazer et al. 1998), flagella (Boonbumrung et al. 2006), efflux pumps (Chan and Chua 2005), type IV pilin associated proteins like PilA (Boddey et al. 2006), a siderophore (Loprasert et al. 2000) and exoproducts (Ashdown and Koehler 1990). *B. pseudomallei* also carries a number of small sequence repeats which may promoter antigenic variation, similar to what was found with the *B. mallei* genome (Nierman et al. 2004).

Forty five transcriptional regulators of the Ara C family have been predicted to exist in *B. pseudomallei* by *in silico* analysis. Members of this family are known positive regulators of virulence factors in other bacteria. The analysis showed more than two thirds of the regulators in chromosome 2 and, in many cases, it seems that the targets are putative virulence factors in *B. pseudomallei* including TTSSs, capsule exopolysaccharide and pyochelin, but also many genes of unknown function. This analysis confirms the functional division of the two chromosomes having most of the genetic factors important for adaptability in chromosome 2 (Lim 2007).

A novel model of infection has recently been proposed for studies of virulence. The larvae of the wax moth (*Galleria mellonella*) was used to test attenuated and virulent strains of *B. pseudomallei* and *B. mallei* (compared to nonpathogenic related bacteria as controls), the results concluded that the model was effective in identifying virulent strains. After *in silico* predictions that led to designation of putative virulence operons, the authors created targeted gene mutations and tested the mutants in the new model, identifying three new genes are important for virulence of *B. pseudomallei* and *B. mallei* (Schell et al. 2008).

As with other microorganisms, it is important to keep in mind that *B. pseudomallei* is an opportunistic pathogen whose virulence mechanisms may just be a side effect of other mechanisms allowing it to conquer the competitive soil environments. Keeping in mind the importance of previous and ongoing studies addressing its pathogenecity from the microorganism's perspective, it is fair to say that much work is still needed to try to understand the role that the host immune system plays in infection. The metabolic activity

of *B. pseudomallei* in its natural habitat (whether inside eukaryotic cells or in polymicrobial biofilms) is likely to differ from the metabolism exhibited under rapid-growth laboratory culture conditions.

1.3 *B. mallei* and *B. thailandensis*

1.3.1 Bacteriology of *B. mallei*

B. mallei, the causative agent of glanders, it is a Gram negative, nonmotile, obligate intracellular coccobacillus. *B. mallei* grows over a temperature range from 30°C to 39°C in the laboratory. The natural reservoir is the horse which acquires infections quite easily, although other solipeds can also be infected (the normal body temperature of horses is 38°C) (Neubauer et al. 2005).

1.3.2 *B. mallei* genome features

The type strain ATCC 23344 is an isolate from a Chinese patient in Burma who had glanders in 1944. The genome of this strain has been sequenced and fully annotated (Nierman et al. 2004). Three more strains have been sequenced and six more are being assembled by the J. Craig Venter Institute. Comparison of the genomes of *B. mallei* and *B. pseudomallei* indicate that *B. mallei* is a recently evolved clone of *B. pseudomallei* (Godoy et al. 2003). The genome of this bacterium, with its 5.8 Mb in length, is probably the smallest among the *Burkholderia* genomes and this is reflected in its lack of adaptability

to different environments. The bacterium evolved by gene deletion and lost genes necessary for persistence in the environment (Nierman et al. 2004).

The genome of *B. mallei* carries a large number of insertion sequences as compared to the related *B. pseudomallei*, and a large number of simple sequence repeats that may function in antigenic variation of cell surface proteins that may have a role in the escape from host immune defenses (Fushan et al. 2005).

1.3.3 Infections caused by *B. mallei*

Infection with *B. mallei* can cause a subcutaneous disease known as farcy, or it can cause a disseminated condition known as glanders. Even though this bacterium is rarely associated with human disease, and only sporadic cases are reported around the world, it has been designated a Category B Select Agent by the CDC because: 1) it has been used as a biowarfare agent (during the first World War, for example, the German army used the bacterium to infect the enemy's horses (Wheelis 1998)), 2) it is possible to acquire the infection by inhaling aerosols, 3) the resulting disease is very incapacitating, difficult to diagnose and often fatal, and 4) there is currently no vaccine for humans.

Glanders was widespread during the 19th century, but it was eradicated from the United States, Europe and many other places around the world due to strict programs to extinguish the bacterium by slaughtering animals who showed infection or seropositivity. In 1891 a mallein skin test was developed that allowed for rapid identification of animals exposed to the bacterium (Bros 1893) . However, the disease is still prevalent in a few

countries around the world. Cases are reported mainly for regions in Asia, North Africa and Eastern Europe (Whitlock et al. 2007).

Transmission to many species of mammals, including camels, bears, cats, dogs and humans can also occur. The route of infection is most likely by inhalation, but ingestion of infected meat can also lead to infection (Krauss et al. 2003).

In humans, glanders only occurs sporadically, typically in those with close and frequent contact with infected animals, such as veterinarians and animal caretakers, or in laboratory personnel (Srinivasan et al. 2001). It is thought that the route of transmission affects the clinical outcome. If the bacterium is inhaled there is a necrosis of the tracheobronchial tree, and febrile pneumonia with pustular skin lesions. If the bacterium enters through a skin breach, the presentation is usually that of multiple abscesses and signs of sepsis. Both initial pictures can lead to bacteremia and sepsis, and can be lethal if not treated (Srinivasan et al. 2001).

Diagnosis of infection is difficult, it requires a high level of suspicion and the differential diagnoses include melioidosis, tuberculosis, anthrax and syphilis. Isolation of the bacterium from pustules, nasal secretion or saliva is required for a definite diagnosis, although serology can aid in the establishment of a diagnosis based on clinical picture (Amemiya et al. 2007).

Treatment of glanders has not been studied because of the lack of human cases. The bacteria have been reported to be susceptible to aminoglycosides and macrolides (unlike *B. pseudomallei* which is resistant to these) (Heine et al. 2001). Treatment with ceftazidime,

trimethoprim or imipenem for at least one month has been recommended (Srinivasan et al. 2001).

1.3.4 *B. mallei*'s virulence determinants

Virulence factors have been proposed based on genomic comparisons with other microorganisms. The only factors proven to be important for virulence are those shared by many other pathogens and include an exopolysaccharide capsule, type three secretion systems and lipopolysaccharide (Schell et al. 2008). Other factors include a type six secretion gene cluster (Schell et al. 2007) and a quorum sensing system, BmaR3-BmaI3 (Duerkop et al. 2008). Putative factors without experimental evidence such as hemolysin, proteases, and rhamnolipid have been proposed in *B. mallei* because they are present in *B. pseudomallei.*

1.3.5 Bacteriology of *B. thailandensis*

Gram negative soil saprophyte, similar to *B. pseudomallei* but distinguishable, in the lab, by the fact that it can assimilate arabinose, while *B. pseudomallei* cannot. *B. thailandensis* became a separate species in 1998, when significant differences in the 16S rRNA sequences of *B. pseudomallei* isolates that had striking phenotypic features were found (Brett et al. 1998).

1.3.6 *B. thailandensis* genome features

The genome of the type strain E264, originally isolated from a rice field sample in Thailand, has been completely sequenced. The genome contains two chromosomes of 3.8

and 2.9 Mb. The similarities between the architectures of the chromosomes of *B. pseudomallei* and *B. thailandensis* are striking given the fact that *B. pseudomallei* is much more closely related to *B. mallei* (Kim et al. 2005). Genomic islands can be found throughout the genome encompassing regions of divergent GC content or containing phage related genes that amount to almost 5% of the total DNA. Most genomic islands are different from those found in *B. pseudomallei*, although the relative locations of the islands are conserved between the two species (the loci may represent landmarks for horizontal acquisition of sequences) (Yu et al. 2006).

1.3.7 *B. thailandensis* as a model organism

B. thailandensis has been found to coexist with *B. pseudomallei* in the soil in Thailand but rarely causes disease and is >105-fold less virulent than *B. pseudomallei* in Syrian hamsters and mice (Brett et al. 1998). On exceedingly rare occasions, there have been reports of *B. thailandensis* isolates being cultured from patients presenting symptoms of disease (Lertpatanasuwan et al. 1999; Glass et al. 2006), therefore the bacterium cannot be classified as avirulent, but it is clear that human infections are extremely rare and experimental work under BSL2 conditions poses no risks to workers. This feature and the fact that broad similarities exist between *B. pseudomallei* and *B. thailandensis* (Yu et al. 2006), have led scientists to use *B. thailandensis*, under certain circumstances, as a model organism for studying aspects of *B. pseudomallei* biology. The model has its limitations, but poses no restrictions imposed by Select Agent regulations.

It is interesting that until recently *B. thailandensis* had only been found inhabiting the soil in regions of Southeast Asia. Extensive sampling in Australia, where *B. pseudomallei* is also endemic, had failed to isolate any *B. thailandensis*. Recently a *B. thailandensis*-like bacterium was isolated in Northern Australia (Gee et al. 2008). Sequencing of the isolate should aid in understanding the evolutionary steps that took place and allowed *B. pseudomallei* to develop virulence mechanisms against animals that may be different in or absent from *B. thailandensis*.

In the search for differences that allow identification of genes required for virulence in *B. pseudomallei* and *B. mallei*, investigators found that the arabinose assimilation operon that allows *B. thailandensis* to assimilate L-arabinose, is not present in either *B. pseudomallei* or *B. mallei* (Moore et al. 2004). When the operon was cloned into a *B. pseudomallei* strain and expressed, it was observed that the virulence of the resulting strain was diminished, and that genes important for virulence were down-regulated. Based on these observations, the authors proposed that loss of the arabinose assimilation operon probably contributed to the stronger virulence of *B. pseudomallei* and *B. mallei* as compared to the relatively avirulent *B. thailandensis*. L-arabinose, or a metabolite of it, probably has down-regulating effects on expression of genes important for virulence, since the ability to assimilate L-arabinose is not necessary for survival in vivo, it is possible that loss of the operon conferred an advantage to pathogenic *B. mallei* and *B. pseudomallei* to colonize hosts.

1.4 Targeted mutagenesis systems and *Burkholderia*

1.4.1 Importance of reverse genetics methods in *Burkholderia* spp.

With the advent of a great amount of genetic information contained in each new genome sequenced, there is a great opportunity to learn more about the many aspects of the biology of the *Burkholderia* species. Encoded by the genome is the sequence of instructions to produce every virulence associated gene, every enzyme in each of the various metabolical pathways that allow these bacteria to exist, every regulator driving the production machinery. Detailed characterization of the ecology and pathogenesis of these bacteria at the molecular level is now possible. *Burkholderia* spp. are known for their ability to colonize and successfully thrive in very diverse and harsh environments. The availability of their genome sequences provides opportunities for discovery in virtually every field in the life sciences.

Reverse genetics techniques are important in this setting, for they may allow access to the study of every single piece of sequence. In reverse genetics the sequence of a gene is known and its function is determined by creating defined mutations and observing the resulting phenotypes under specific conditions.

The genomes of *B. pseudomallei* K96243 (4.07 Mb plus 3.17 Mb) and *B. mallei* ATCC 23344 (3.51 Mb plus 2.32 Mb) have been fully annotated (Holden et al. 2004; Nierman et al. 2004). Of the 5535 putative ORFs found in *B. mallei* only 3173 have an assigned function. Much of the annotation data generated is based on the correlation of results amongst these

and other sequenced genomes, most conclusions regarding the function of each gene have been inferred from the accumulated data and await experimental confirmation, but in many cases there are no clues as to what the role of the open reading frames may be.

1.4.2 Choice of targeted mutagenesis method

Several methods for achieving chromosomal mutagenesis exist. Some of the most effective ones include transposon mutagenesis, gene silencing using antisense RNA and gene disruption by allelic exchange. The choice of method depends on various factors as they all entail different advantages and disadvantages. Random mutagenesis procedures allow one to identify multiple new genes involved in a certain process, while directed mutagenesis can be used to test the hypothesis that a specific gene product is involved in a specific function.

The methods used to generate mutations or to silence genes in a genome wide scale are very efficient for identifying interesting genes. The existing methods work fairly well in *Burkholderia* spp. or at least seem to be readily adaptable. The main advantage of the transposon mutagenesis and mRNA expression inhibition systems is their potential to be used on a genome wide scale. Nevertheless, directed mutagenesis techniques, where the gene of interest is directly modified, are still needed to allow for confirmation of gene function.

1.4.3 Transposon mutagenesis techniques

Transposons are mobile genetic elements that can insert themselves in almost any region of the chromosome, and pools of single insertion mutants can be obtained fairly easily. Since each mutation is marked by its transposon and the selection marker, PCR can be used to recover the DNA flanking the transposon. Different protocols have been developed allowing for identification of genes required for different processes studied in *Burkholderia* spp.

Novel genes required for intracellular survival of *B. pseudomallei* were identified using simple transposon mutagenesis, where a pool of mutants were screened for the inability to form plaques on cell monolayers, indicative of reduced intercellular spreading (Pilatz et al. 2006). A more refined method of signature tagged mutagenesis, where individual transposons are tagged with short unique sequence tags and individual mutants are arranged according to the tag they contain, was used to identify genes necessary for infection in a mouse model (Cuccui et al. 2007). Signature tagged mutagenesis has also been used to identify genes necessary for phenol degradation and for colonization of the pea rhizosphere in *B. vietnamiensis*, so as to provide better knowledge of the underlying genetic mechanisms important for biotechnological fitness of environmental *Burkholderia* (O'Sullivan et al. 2007).

Transposon mutagenesis has proven very useful but has some drawbacks inherent to each specific transposon used. It is difficult, for example, to prove that an insertion has occurred in each one of the possible genes in the genome and, in general, there is always

the possibility that the integration of the transposon within a gene does not inactivate the gene product, leading to false negative results.

1.4.4 Silencing of chromosomal genes

Gene silencing using anti-sense RNA has been extensively used in eukaryotes, and even though it has not been used nearly as much in prokaryotes there are reports of its usefulness, and it seems quite possible that the methodology can be adapted for use in *Burkholderia* spp. as an alternative way of studying gene function.

Naturally occurring antisense RNAs are small, diffusible untranslated transcripts that pair to target RNAs in a complementary fashion. In the bacterial cases studied these antisense RNAs regulate gene expression by different mechanisms. Depending on the instance, right after binding to their target, there is premature transcription termination, facilitated RNA decay, or direct or indirect inhibition of translation (Wagner and Simons 1994). So far there have been no descriptions of antisense RNAs positively regulating gene expression in bacteria, but they could exist in theory by altering mRNA conformation increasing the stability of the molecule, for example, or by achieving higher rates of translation.

One of the most representative works of the application of antisense RNA technology for the disruption/inactivation of genes makes use of a precisely regulated promoter to drive the transcription of short DNA fragments (200 bp long), derived from the fractionation of a bacterial chromosome. After transformation of bacteria with the library of antisense RNA expressing plasmids, isolates that failed to grow when the plasmid

antisense RNA was induced were identified and their DNA was sequenced. The investigators were able to demonstrate the utility of their approach to identify genes essential for survival in a way that allows for the lethal phenotype to be maintained for further characterization of the gene. Furthermore, the investigators demonstrated that bacteria containing plasmids expressing antisense RNAs, targeting pathogenicity encoding genes, could be used for *in vivo* experiments, where expression of the putative pathogenicity factor could be shut down at will (Ji et al. 2001).

1.4.5 Insertional mutagenesis techniques

One of the simplest forms of essentiality analysis in a gene-by-gene basis is done with plasmid insertional mutagenesis. This technique is available every time a non-replicative plasmid with a selectable marker is inserted in a strain capable of homologous recombination, by providing a cloned fragment with sequence homology to the region in the chromosome where one wants to integrate the plasmid (**Figure 1.2**).

Even though this approach may seem effective and simple enough, it has serious disadvantages. There is the possibility that the insertion of the plasmid within the target gene still allows for translation of an active protein fragment, giving rise to false negative results. The insertion of the entire plasmid into the chromosome may lead to unwanted effects on downstream genes, a phenomenon known as polarity, and this can also lead to wrong conclusions as to the function of the mutated gene. Finally, the integration of the whole plasmid creates two regions of homology that can lead to recombination, and therefore instability, unless selective pressure is maintained. This hinders the construction

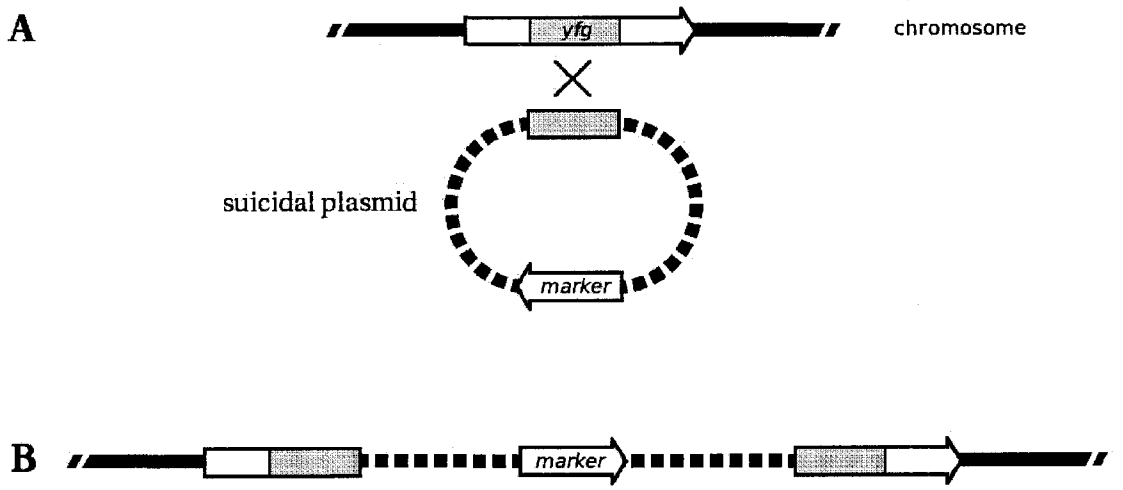


Figure 1.2 Insertional mutagenesis with a circular plasmid

Graphic representation of the process of insertional mutagenesis with a circular plasmid. A. A sequence homologous to a segment in "your favourite gene" ("*yfg*", gray box) is cloned into a non-replicative plasmid containing a selection marker. **B.** After introduction of the plasmid into the cells, selection for the marker in the backbone of the plasmid will result in cells that have the plasmid integrated via homologous recombination (indicated by X) in the chromosomal copy of *yfg*, thus inactivating it.

of several mutants in the same genome as there are only a limited amount of selectable markers available (this is particularly important when working with Select Agents as noted before). Even with all of these caveats, there are successful reports of the use of this methodology for creation and evaluation of mutant isolates of *B. pseudomallei* (Tuanyok et al. 2006; Reckseidler et al. 2001; Ling et al. 2006).

A second method to generate insertional mutants has been described for other bacteria and could be adapted eventually to work in *Burkholderia* spp. t has been commercialized and is known as "targetron" (TargeTron^{R}), it works by modifying the sequence of an RNA group II intron recognition sequence, so that the group II intron targets a desired sequence within the gene to be mutated in the chromosome and inserts itself there. The group II introns are composed of an RNA segment and a gene encoding a protein which forms a complex with the RNA. Once the RNA-protein complex has formed, the complex scans the DNA for a recognition site, complementary to a sequence in the RNA of the complex. At this site the RNA-protein complex is able to drive the insertion of the whole intron into the chromosome. After reverse transcription of the RNA segment by the protein in the complex, the host enzymes only have to seal the nicks that are left (Zhong et al. 2003). The main advantages of this system as compared to the former one, where a plasmid is integrated by homologous recombination, are 1) that the group II introns are much more efficient in integrating themselves making isolation of insertion mutants a very easy process, and 2) that the mutated chromosome is not left with two identical regions where homologous recombination can occur and therefore the targetron

insertion is very stable, which allows the researcher to remove the selection marker from the intron sequence once it has been integrated. The main disadvantages of the two technologies are similar in that it is possible that the targetron insertion will not completely inactivate the gene and polar effects are possible. The necessary adjustments to the targetron system to be able to make it work in the *Burkholderia* Select Agents include finding a good promoter to drive the expression of the reverse transcriptase protein and the intron RNA and making sure the markers in the vector are compliant with select agent rules (Choi et al. 2008).

1.4.6 Allelic exchange systems

A better system for obtaining defined unmarked mutations in the chromosome of bacteria, on a gene-by-gene basis, is that of allelic exchange (also known as gene replacement or targeted mutagenesis).

The idea underlying the system is that bacterial house-keeping mechanisms will integrate a provided DNA molecule into its genome by recombining it at a place where both molecules display sequence homology, just like it happens in the insertional inactivation experiments explained above. The difference is that in this case two recombination events are sought, one to integrate the whole molecule into the chromosome, and a second to eliminate unwanted DNA sequences (or to repair the double-stranded break in the chromosome left after one crossover event with a linear DNA molecule). Two possibilities exist for delivery of the desired change in the chromosomal sequence, a linear vector or a circular plasmid.

1.4.6.1 Allelic exchange with a linear vector

When a linear fragment is used for delivery, the ends of the fragment contain sequences homologous to the chromosome and flanking the region to be replaced. The linear fragment must necessarily contain a selection marker, since the rate of homologous recombination events after delivery is not high enough to allow one to screen for an expected phenotype (**Figure 1.3**).

Some strains of *B. pseudomallei* have been shown to be able to take up linear double stranded DNA from the environment and integrate it into their genome (Thongdee et al. 2008). The physiological state of competence has been shown to require the expression of 20 to 50 proteins in other species of bacteria studied (Thomas and Nielsen 2005), and the induction of the regulated state of competence depends on specific environmental conditions such as nutrient access, cell density or growth conditions which vary from one species to another (Lorenz and Sikorski 2000). Therefore, it is not possible to predict unequivocally if a bacterial strain will be able to develop competency or not and under what conditions. As a matter of fact, some strains of *B. pseudomallei*, including K96243, have proven to be refractory to natural transformation, excluding the use of linear fragment mutagenesis in these strains.

1.4.6.2 Allelic exchange using phage recombinant functions

A modified version of the targeted mutagenesis procedure using a linear fragment works well in other Gram-negative bacteria, specially in *E. coli* and its close relatives. It is based on the enhancement of homologous recombination capabilities of the cell by

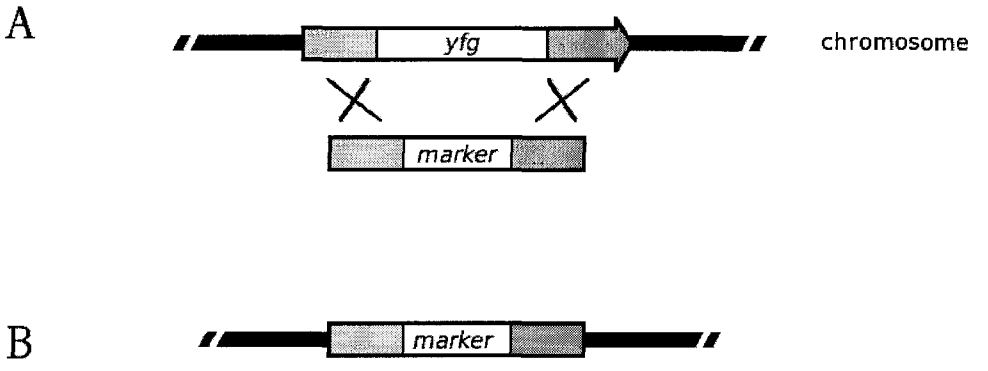


Figure 1.3 Targeted mutagenesis with a linear fragment

Schematic illustration showing targeted mutagenesis with marked linear fragments by homologous recombination. **A.** A DNA fragment is engineered so that a selection marker inserted in"your favourite gene" (*yfg*) is flanked by segments homologous to those flanking the region to be replaced in the chromosome. **B.** After introduction of the linear DNA into the cells, selection for the marker allows for isolation of cells which have replaced the wild-type copy of *yfg* with the marked mutant by undergoing two crossover events (each X represents a crossover event).

expression of the bacteriophage lambda Red system enzymes. The lambda Red system was first described when a bacteriophage lambda phage unable to do recombination was isolated (Recombinant Deficient) and the genes *exo* and *bet* were found responsible for the defect. Later a third gene was found that also affected recombination, *gam* (Shulman et al. 1970). The expression of the lambda Red system genes in *E. coli* enhances recombination of homologous sequences between linear fragments introduced by electroporation and the chromosome by many orders of magnitude, making the process extremely efficient (Murphy 1998). This system has been modified in different ways as more knowledge is gathered about the recombination pathways in *E. coli* and in the bacteriophage (Poteete 2001).

The process of homologous recombination in bacteria is not completely understood. Most studies have been done in *E. coli*, but the interactions between different pathways are not completely understood (Rocha et al. 2005). The RecA protein seems to rely on the RecBCD pathway under some circumstances and on the RecFOR under others (Fujii et al. 2006). The lambda Red system proteins act differently in different strain backgrounds of *E. coli* and their relation to other enzymes in the host machinery are not yet clear (Martinsohn et al. 2008). Homologues of *E. coli* recombination proteins can be found in all other bacteria with available sequences, but the general pathways described in *E. coli* are not always present in all bacteria and many details seem to vary among the different species. For instance the *recBCD* genes from *E. coli* have no clear orthologues in *B. pseudomallei*. For these reasons and the fact that electroporation of linear DNA in *B. pseudomallei* is inefficient, it unlikely that recombineering, by use of phage lambda Red

functions, will be easily adapted for use in *Burkholderia*, although not all host requirements have been determined and an adaptation for *P. aeruginosa* (a closer relative to *Burkholderia* spp. than *E. coli*) has already been reported (Lesic and Rahme 2008). It is more likely that analog genes carrying the same functions as the lambda Red will be found in the viruses specific for *Burkholderia* spp. (Court et al. 2002). In fact, the recombination functions of a mycobacteriophage have been used to create an analogous system in *M. tuberculosis* (van Kessel and Hatfull 2007).

1.4.6.3 Allelic exchange via circular plasmids

Apart from the "recombineering" possibilities that may exist with the bacteriophage recombination systems, the other effective way for delivering a desired change into the chromosome by homologous recombination in *Burkholderia* spp. is by the use of a non-replicative circular plasmid.

In this method, regions of homology flanking the segment to be mutated are designed and cloned into a non-replicative plasmid. The origin of replication, in the plasmid, permits replication only in specific hosts, for example *E. coli*, facilitating modification and propagation of the plasmid. However, when the recombinant plasmid is introduced into a replication restrictive host, it gets lost unless it is integrated into a chromosome via homologous sequences. In a slightly different approach a conditionally replicating plasmid can be used. For example, a temperature sensitive plasmid designed for *B. pseudomallei* could be used by selecting for integrants at the non-permissive growth temperature (Choi et al. 2008b).

Allelic replacement using non-replicative plasmids involves several steps. First, after introduction into the cytoplasm by either conjugation or electroporation, a crossover event integrating the plasmid into the chromosome is selected for using an antibiotic resistance marker encoded by the plasmid. The resulting strain, with the whole plasmid sequence integrated into the genome in one of two ways (depending on whether the crossover event occurs in one homology region or the other), is called a merodiploid. A merodiploid has two alleles of the same gene present in the chromosome, in this case the wild-type copy and the mutant copy derived from the plasmid. Second, once a merodiploid has been obtained, it is necessary to select for a second crossover event, leaving the cell with only the mutant copy of the gene in its chromosome. Crossover events are not frequent under normal circumstances, it is therefore necessary to have a way for selecting for them. This is relatively easily done in the first step of the procedure by selecting for a marker in the backbone of the plasmid. The selection for the second crossover event necessary requires a more ingenious approach, usually by use of a counter-selectable (CS) marker. It allows for the positive selection of strains that have lost the marker. When a second crossover event occurs in the merodiploid strain, each resultant cell has lost the segment of plasmid integrated into the genome and has retained a copy of one allele, either the original wild-type gene or the mutated desirable allele (**Figure 1.4**).

Counter-selection is achieved by providing the conditions under which the CS marker cannot be maintained. In theory, a high proportion of the cells isolated after counter-selection should be mutants, at least an amount easily screened for by looking for the expected phenotype(s) or by PCR screening for the mutant chromosomal region. In a

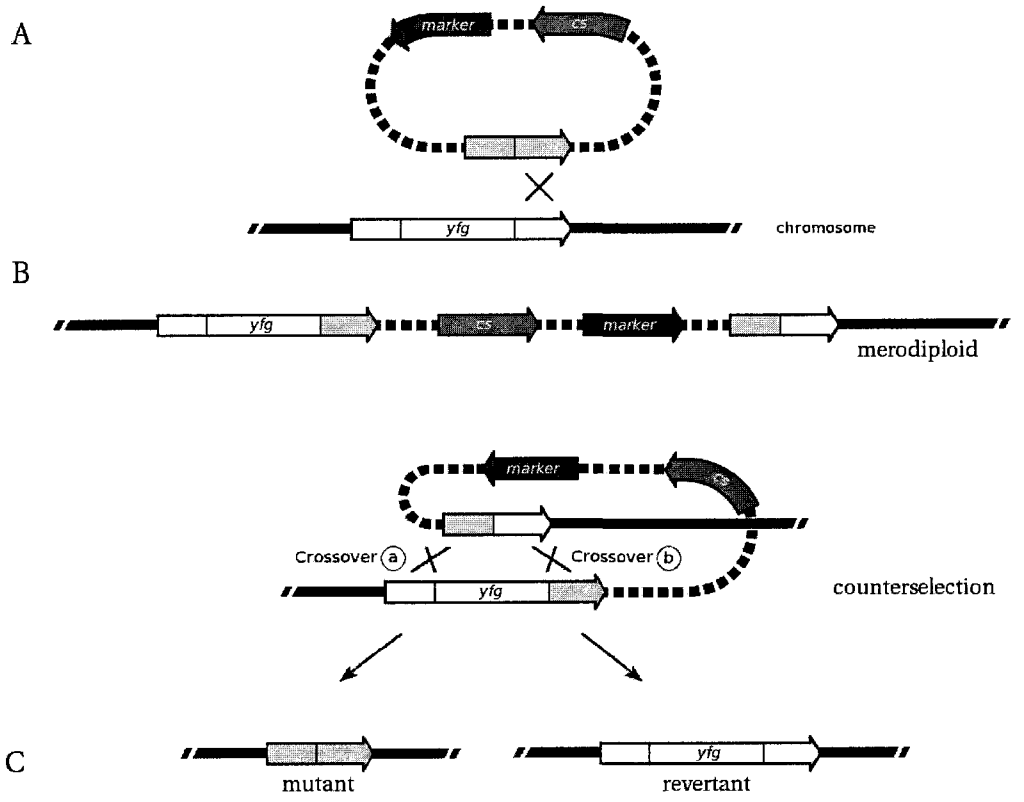


Figure 1.4 Targeted mutagenesis with a suicide plasmid

Schematic of the process of targeted mutagenesis by homologous recombination using a non-replicative plasmid. **A.** Two regions homologous to those flanking the region in the chromosome where a mutation is to be inserted are engineered together into a non-replicative plasmid which contains a selection marker and a counter-selection marker (CS) in the plasmid backbone. **B.** After plasmid transfer into the recipient cells and selection for the marker, a merodiploid is obtained. **C.** Merodiploids are subjected to counter-selection by establishing conditions in which the CS kills the cells. Depending on the site where a second crossover event occurs (a or b) the resultant strains will be mutants or will have reverted back to the original wild-type state (revertant).

model scenario, if the fragments of homology flanking the mutation are perfectly equal in size and GC content, and the introduced mutation is perfectly centered between them, then the proportion of mutants versus revertants (cells that deleted the plasmid sequence and the mutant allele) should be close to 50%, although the fitness of mutants would also influence this proportion.

1.4.7 Options for Counter-selection markers

One of the first experiments described, where targeted mutagenesis was performed via a circular plasmid, made use of a property of the antibiotic marker for tetracycline resistance, to achieve counter-selection or enrichment of cells having lost the integrated plasmid (Gutterson and Koshland 1983). Researchers first obtained merodiploids by selecting for tetracycline resistant transformants. Then to select for those that had undergone a second recombination event, they propagated the cells for a few generations in the absence of antibiotics. This allowed replication of all kinds of cell populations, including those that lost the plasmid integrant by homologous recombination. Next they added tetracycline to the growth media for a brief period to stop bacterial growth of populations sensitive to tetracycline (tetracycline is a bacteriostatic antibiotic), and then they added cycloserine to kill cells that were actively reproducing and growing (cycloserine kills cells that are growing but has no effect on the others). Finally, the surviving cells were washed and resuspended in fresh media. Most of these cells had lost the tetracycline resistance marker and were thus tetracycline sensitive. This method seems

very laborious and not awfully precise, but it yielded good results and the experimenters isolated their mutants.

Since 1983, many CS markers have been identified that allow for easier positive selection of mutants, but in many cases where genetic tools are still being developed, as is the case for most *Burkholderia* spp., the strategies for obtaining effective counter-selection are still elusive.

One of the main counter-selectable markers that has been widely used for Gram negatives and even in *M. tuberculosis* is the *Bacillus subtilis sacB* gene (Gay et al. 1983). The *sacB* gene encodes for an enzyme called levansucrase which catalyzes the hydrolysis of sucrose and the transfructosylation of sucrose to levan. It is believed that the large fructose polymer accumulates in the periplasm of Gram-negative bacteria, and leads ultimately to cell lysis by osmotic pressure (Steinmetz et al. 1983). Gram positive bacteria are supposed to be spared the toxicity as they lack a periplasmic space and the polymer gets released into the medium.

In 1991 some experimenters developed a system for chromosomal DNA replacements using a temperature sensitive plasmid and a copy of *sacB* integrated into the chromosome (Blomfield et al. 1991). Subsequently, other groups modified the idea by introducing the *sacB* gene into the replacement plasmid (Schweizer 1992; Lawes and Maloy 1995; Pelicic et al. 1997; Hoang et al. 1998).

Multiple examples exist where *sacB* counter-selection has been used in allelic replacement experiments, including *B. cepacia* complex (Komatsu et al. 2003; Zhou et al. 2003; Gronow et al. 2003).

A gene homologous to the *B. subtilis sacB* is found in many strains of *Burkholderia* spp. and its function seems to be the same. This is the reason why the use of *sacB* for counter-selection is limited to only some of the strains in *B. pseudomallei*, and even in these strains the conditions for sucrose sensitivity vary (Essex-Lopresti et al. 2005) and can be hard to establish. *B. mallei* also contains a *sacB* gene. However, when wild-type cells are grown on minimal media in the presence of sucrose, surviving colonies have deleted the gene because of recombination between nearby flanking IS elements (Holden et al. 2004).

Even though *sacB* has been used successfully in many different bacteria, the system still has flaws as evidenced by the dependence upon the growth temperature and the media composition (specifically the concentration of sodium chloride) (Blomfield et al. 1991). Also, in a few cases the *sacB* gene in a merodiploid looses its function by some unknown mechanism (Link et al. 1997), for example by inactivating mutations.

Probably the second most common CS marker is *rpsL*. This gene encodes for a protein of the small ribosomal subunit. This protein, S12, is the target of the antibiotic streptomycin and if it is altered the bacteria become resistant (Kornder 2002). Since a spontaneous mutation in the *rpsL* is easily achieved by plating bacteria on streptomycin containing media, these bacteria can be used for *rpsL* counter-selection experiments. In these strains containing a mutant *rpsL* gene in their chromosome, loss of a second copy of wild-type *rpsL* converts the cells from streptomycin-sensitive to streptomycin-resistant, because the ribosomal protein encoded by the wild-type gene is dominant to the mutant allele (Lederberg 1951).

The *rpsL* gene from *E. coli* has been proven a good CS marker in many different bacteria (Baltz and Hosted 1996; Sander et al. 1995; Johnston and Cannon 1999). *B. pseudomallei* is naturally resistant to aminoglycosides, including streptomycin, but the mechanism of resistance has nothing to do with the S12 ribosomal protein target of the drug. Instead, resistance is due to the AmrAB-OprA efflux pump that extrudes aminoglycosides (Moore et al. 1999). Investigators were able to generate an AmrAB-OprA efflux pump mutant derivative 1026b strain, which they called DD503. This strain has successfully been used in allelic replacement experiments using an *E. coli rpsL* CS marker present in plasmids designed for broad applicability in diverse gram negative bacteria (Skorupski and Taylor 1996). It has been found though, that *B. pseudomallei* DD503 is attenuated in a murine model of infection due to the *rpsL* mutation (Jeddeloh et al. 2003), therefore limiting the use of this strain. *B. mallei* is naturally susceptible to streptomycin because it lacks the AmrAB-OprA efflux pump.

Other CS markers reported in the literature have also been used with variable success in certain species. A similar approach to counter-selection to that described at the beginning of this chapter makes use of another property of tetracycline resistant bacteria. Expression of *tetAR* in *E. coli* results in alteration of the host cell membrane which interferes with tetracycline permeation and thereby renders the cell resistant to tetracycline. Investigators noticed that those bacteria were made sensitive to fusaric acid and other lipophilic chelating agents, and therefore used fusaric acid to select for bacteria that had lost the tetracycline resistance marker (Bochner et al. 1980; Maloy and Nunn 1981). Variations of this idea were used to be able to use tetracycline as a positive and

negative selection marker, but the method is not clean and a lot of background colonies arise in different strains (Nefedov et al. 2000). Tetracycline is not an approved antibiotic marker for use in *B. pseudomallei* and *B. mallei.* because of its clinical utility.

In certain cases the need for a counter-selectable marker has led researchers to modify a strain background to be able to use one of its genes as counter-selection markers. The resultant strain can then be used as a model in which one can generate mutants at will easily with the use of the marker. In the case of group B streptococci *glnQ* was shown to confer sensitivity to the glutamine analog gamma-glutamyl hydrazide to cells expressing it. The chromosomal *glnQ* gene was mutated via transposon insertion, and the new strain was used to for a successful gene replacement (Tamura et al. 2005).

In another example, researchers modified a lysine biosynthetic gene of a *B. subtilis* strain. A conditional lysine auxotroph was created by replacing the native *lys* gene promoter with a promoter for which a known repressor could be provided *in trans.* Cells expressing the repressor were lysine auxotrophs (Brans et al. 2004). The repressor gene was used as the CS marker, whose loss would allow cells to synthesize lysine when not provided in the medium.

The *E. coli galK* is one of the genes in a galactose operon which enables bacteria to use galactose as the sole carbon source. The enzyme encoded by *galK* phosphorylates galactose into galactose-1-phosphate and can also phosphorylate the analog 2-deoxy-galactose, which cannot be further metabolized and accumulates and intoxicates the cell. In strains where *galK* has been mutated, one can use the wild-type *galK* for efficient

selection both for and against its function (Warming et al. 2005). The system has also been used successfully in *Streptococcus mutans* (Merritt et al. 2007).

CS markers based on toxin and antidote protein molecules, found so far mainly in *E. coli*, have also been used. For example, the Hok peptide kills cells that produce it by depolarizing the cytoplasmic membrane (Pecota et al. 2003). It has been proposed in a system to allow for the positive selection of cells that integrate DNA molecules into their chromosomes disrupting the gene (Harms et al. 2007). Similarly, the Kid protein of the *parD* system of plasmid R1 (Ruiz-Echevarría et al. 1991), has been used for positive selection since the protein kills cells that express it, but its expression can be inhibited by the antidote Kis (Gabant et al. 2000). Other toxin/antitoxin systems reported, include Phd/Doc (Magnuson and Yarmolinsky 1998) and and *mazE/mazF* (Tsilibaris et al. 2007). These could eventually be adapted for use as counter-selection markers. Nevertheless, the mode of action of these proteins varies and it is possible that they will not be active in different strain backgrounds, as seems to be the case with CcdB, a toxin described in Chapter 2 of this dissertation.

Other proposed CS markers can be found in the literature (Reyrat et al. 1998), many of which have been used only in a few bacteria. Some of these will be described in Chapter Two.

1.4.8 Alternative uses for CS markers

CS markers can be used to facilitate linear PCR fragment mutagenesis to produce scar less unmarked point mutations. The linear fragment is designed so that the mutated gene

is interrupted by insertion of a counter-selectable and a selectable marker, and that the segments flanking the insertion are both repeated segments within the gene. Under conditions of counter-selection, the repeated sequences recombine and the counter-selection and selection markers are lost, leaving a clean mutation in the chromosome. Making use of a modified strain of *B. subtilis*, investigators used the *upp* CS marker to generate point mutations by using linear PCR products (Fabret et al. 2002).

In other instances, CS markers were employed in a similar fashion for example with lambda Red recombination strategies, confirming the utility of these markers even when recombination efficiency is very high (Zhang et al. 2003).

CS markers could be used in bacterial two-hybrid system adaptations, to screen for libraries and find mutants where a given interaction does not happen. If the bait prey interaction takes place the counter-selectable marker product gets expressed and cells die, and only cells where the interaction does not happen survive (Meng et al. 2006). Similarly, CS markers may aid in identifying positive transcriptional regulators. If the promoterless marker is fused with a gene and its probable regulatory sequence, growing the cells under counter-selection conditions may lead to mutants. Some of the mutants, in theory, will arise because of mutations that inhibit the expression of the gene to be studied, and so regulatory regions and positive regulators may be identified. To screen for the responsible mutations a library of wild-type DNA can be used for complementation. This approach was used in *Rhodobacter sphaeroides* (Sabaty and Kaplan 1996).

CS markers can also be used to trap mobile elements. The *sacB* gene, for example, was used in *B. cepacia* to isolate IS elements present in the chromosome that are inducible

under specific conditions. A replicative plasmid containing the *sacB* gene was introduced into the cells and, after propagation under different selected conditions, the plasmid present in sucrose resistant derivatives was directly analyzed by agarose gel electrophoresis to find plasmids which were larger than the original plasmid. The larger plasmids were then conjugated into *E. coli* for further analysis by PCR (Ohtsubo et al. 2005).

1.5 Justification and goals of dissertation research

1.5.1 **Justification of research**

Studies in *Burkholderia* have intensified in the last few years. Despite the fact that diseases caused by these organisms have been known for many centuries, most of the attention recently brought about to these bacteria comes from the fact that two members of the genus have been classified as Select Agents. Little is relatively known about these bacteria, when comparing the wealth of knowledge accumulated with decades of research on other microorganisms. However, a better understanding of the biology of the members of the genus will have important consequences in different fields of study. Not only will humans be able to respond better, in the case of a terrorist attack; but better prevention, diagnostic and treatment strategies will be available to those more prone to acquiring a disease caused by these organisms. In the biotechnology field, different biological and metabolic features will be available for applications in biocontrol, bioremediation and plant growth promotion. Microbiologists will find answers to questions related to

evolution, host-pathogen interactions, determinants of survival and determinants of pathogenicity. Important tools allowing for crucial discoveries in all organisms where they are applied are those which allow researchers to isolate specific mutants of any given strain. In many organisms and utilizing proper approaches, the powerful homologous recombination machinery of the host, can be exploited, allowing for the isolation of target mutations in the chromosomes of cells. Counter-selectable markers, used to select for cells that have lost a given DNA fragment, provide one of the simplest and most effective means to obtaining targeted mutations.

1.5.2 Goals and specific aims of research

At the onset of this dissertation project, no effective counter-selection markers had been identified for isolation of unmarked mutations in wild-type *B. mallei* or *B. pseudomallei.* The main goal of this dissertation, therefore, was to find counter-selection markers that could be used in all *B. pseudomallei* strains for isolation of specific mutants.

Three specific aims were pursued to achieve this goal:

1. To search for and engineer a counter-selectable marker for *Burkholderia pseudomallei.*
2. To incorporate the found marker in a system and test it to accomplish targeted mutagenesis in different strains.
3. To demonstrate that the system is useful for acquisition of markerless deletions and scarless mutations into the *B. pseudomallei* chromosome.

CHAPTER TWO

Counter-selection Candidates

2.1 Introduction

The wealth of genomic information available for many *Burkholderia* spp., especially for the Select Agents *B. pseudomallei* and *B. mallei*, allow for comparative analyses to pinpoint candidates for different gene functions. Once these open reading frames of interest have been identified, reverse genetics approaches, in which these newly discovered genes are inactivated, and the associated phenotypes searched to assign every gene to a functional category, become very useful. Systems to engineer specific mutations into the genome of *Burkholderia* species, based on the homologous recombination machinery of the host, and that are applicable to all possible strains, rely on delivery of the mutated sequences by circular plasmids and, because of the almost inevitable formation of merodiploids, necessitate the use of a CS marker.

Ideal CS markers will work in all strains and not only in specially engineered mutants for use. In order to allow for the selection of the first crossover event leading to merodiploid formation, the CS marker should not exhibit any toxic effects until a specific

condition is established. Ideally, the CS marker should be encoded by a short sequence to keep the construction of the delivery vector simple and it should function when incorporated in single-copy into the chromosome. The CS marker should also allow for counter-selection under defined conditions that should only inhibit growth of cells carrying the marker. The rate of spontaneous mutations leading to the inactivation of the CS marker should be low enough to allow the rate of spontaneous crossover events to be much higher. It is of note that counter-selection (which results in the selection for loss of a gene or inactivation of its product) is, in general, less efficient than positive selection (which implies the acquisition of a specific given property). This is because the intended recombination is only one of several solutions for the counter-selection pressure; any mutation that abolishes the expression of the CS marker will also allow its host to survive under counter-selection conditions.

This chapter describes briefly some of the different candidates that were chosen and tested for their ability to inhibit growth of the bacteria harboring them. None of these first prospective markers proved to be good enough for counter-selection in *Burkholderia* spp.. Many genetic tools for *Burkholderia* have been developed recently, for example, at the beginning of these studies little was known about the promoters which could be used to drive expression of proteins in these bacteria. Evidence about which markers for selection would be effective was also scarce. *P. aeruginosa*, a closely related species belonging to a different genus, was thought at some point to constitute a valid surrogate strain (Kumar et al. 2006), since genetic manipulations in this bacterium were much more developed it was used in preliminary studies. *P. aeruginosa* was later replaced by *B. thailandensis* which

can be used at BSL2. The approaches used for testing the candidates changed many times throughout the course of these studies, as more genetic tools became available for manipulating *Burkholderia* spp.

The approaches employed for identification of CS markers included the identification of candidates seemingly worth testing based on featured characteristics described in the literature. In each instance a marker was judged to be a promising candidate, experiments were then designed to test it.

The first obvious candidates for counter-selection were the already described *sacB* and *rpsL*. Preliminary attempts at constructing the right vectors for trial were abandoned when it became clear neither of them would be very useful. First, the publication of the genome sequence of *B. pseudomallei* K96243 identified a chromosomal *sacB* gene homolog. In additionstudies demosntrated that the intrinsic streptomycin resistance of *B. pseudomallei* was due to the presence of the AmrAB-OprA efflux pump (Moore et al. 1999; Holden et al. 2004), which is present and expressed in all tested strains. Though this marker is applicable in an AmrAB-OprA efflux pump mutant carrying a chromosomal *rpsL* mutation, such mutants exhibit reduced fitness and are attenuated in the murine melioidosis model (Jeddeloh et al. 2003)

In the next paragraphs, you will find a short description of the rationale for choosing each one of the candidates tried, along with a brief explanation of the method used to test it and a discussion of the possible reasons why each one of these candidates did not work for counter-selection in *Burkholderia.*

2.2 GATA-1

GATA-1 is a zinc finger protein, which acts as a transcriptional regulator in mammalian cells. The protein plays a fundamental role in hematopoiesis, by driving commitment and differentiation of erythroid cells. The GATA-1 monomer binds to the consensus sequence (A/T) GATA (A/G), present in the promoter and enhancer regions of virtually all erythroid specific genes (Welch et al. 2004). Investigators studying the molecular properties of the mouse protein in *E. coli* found that induced expression of the DNA binding domain was toxic to the bacteria. The authors made use of this finding to create a cloning vector with positive selection for clone inserts (Truder et al. 1996). The active domain sequence was fused to an inactive glutathione-S-transferase (GST) gene, interrupted by in-frame insertions of multiple cloning sites. The same authors also showed how the zinc-finger domain could bind to the *E. coli* origin of replication at DnaA protein binding sites. This finding led them to propose that the GATA-1 zinc-finger is toxic to *E. coli* cells because it competes for DnaA-binding sites at the origin of replication (failure of DnaA to bind at the origin would inhibit initiation of replication). However, no definite proof for this hypothesis was provided. Nonetheless, because the explanation for the toxicity of GATA-1 could well be the binding at DnaA sites at the origin, and since the sequences of these DnaA sites in other bacteria are well conserved, the hypothesis that the same mechanism would render the zinc-finger toxic in other bacteria seemed reasonable.

The cloning vector designed by Truder and colleagues was used as a source of the GATA-1. The multiple cloning sites were deleted and the GST fusion was ligated back

together, using linker tailing to maintain the frame. Elimination of all restriction sites was done to facilitate subsequent attempts at subcloning the GATA-1 marker. The zinc-finger was then subcloned into a mini-Tn*7* vector (Choi et al. 2005) to express GATA-1 from a single copy in the chromosome, under the control of the inducible P_{tac} promoter, see **Figure 2.1.** and **Figure 2.2.** Results showed that GATA-1 was still toxic for *E. coli* cells, but had no effect when expressed in *P. aeruginosa.* This result discouraged any further attempts to obtain a vector for trial in *Burkholderia,* as it showed that the toxicity exhibited by the zinc-finger in *E. coli* was probably species-specific.

2.3 *ccdB*

CcdB is the toxin of the *ccd* toxin-antitoxin module of the F plasmid addiction system (Aguirre-Ramírez et al. 2006). These plasmid addiction systems are known to kill cells that have lost the plasmid present in the parent strain during propagation. The systems rely on the differential stability of the factors involved. *ccdB* and *ccdA* genes are present on the plasmid and are constitutively expressed. CcdB, the produced toxin, is stable in the cytoplasm; while CcdA, the antitoxin, is very unstable. Once the plasmid has been lost the antitoxin is rapidly degraded, and the more stable toxin is free to act on its target. CcdB acts on *E. coli* gyrase GyrA, an essential DNA topoisomerase. CcdB has been shown to poison the *E. coli* gyrase by stabilizing the cleavage complex, blocking the cellular polymerases and leading to double-strand DNA breaks, which ultimately lead cell death. This mechanism of toxicity is reminiscent of that causing cell death by quinolones (Dao-

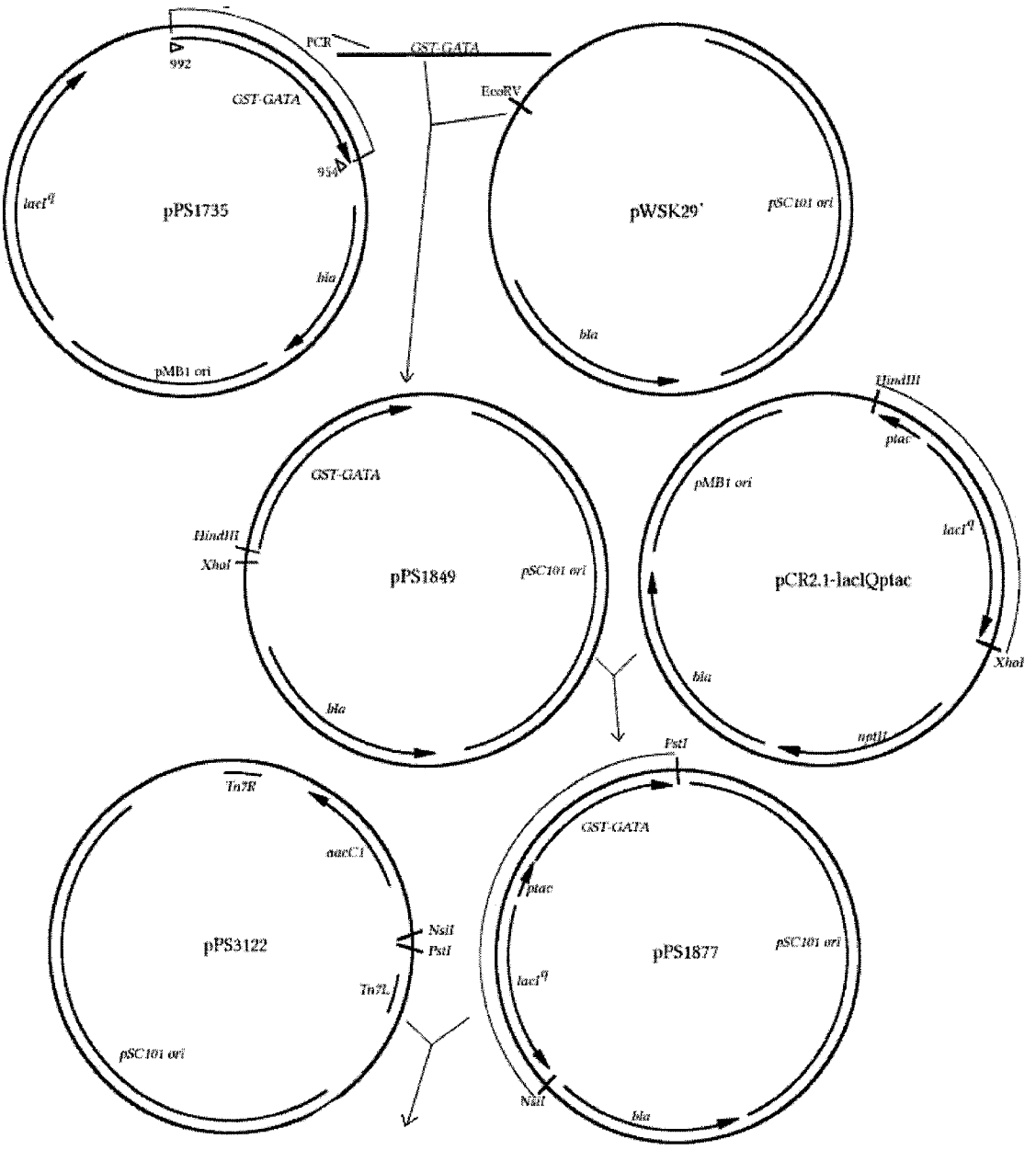


Figure 2.1 Cloning strategy for GATA vectors (part I)

Plasmid maps not drawn to scale. See text description under Materials and Methods section 2.7.3 Plasmid construction.

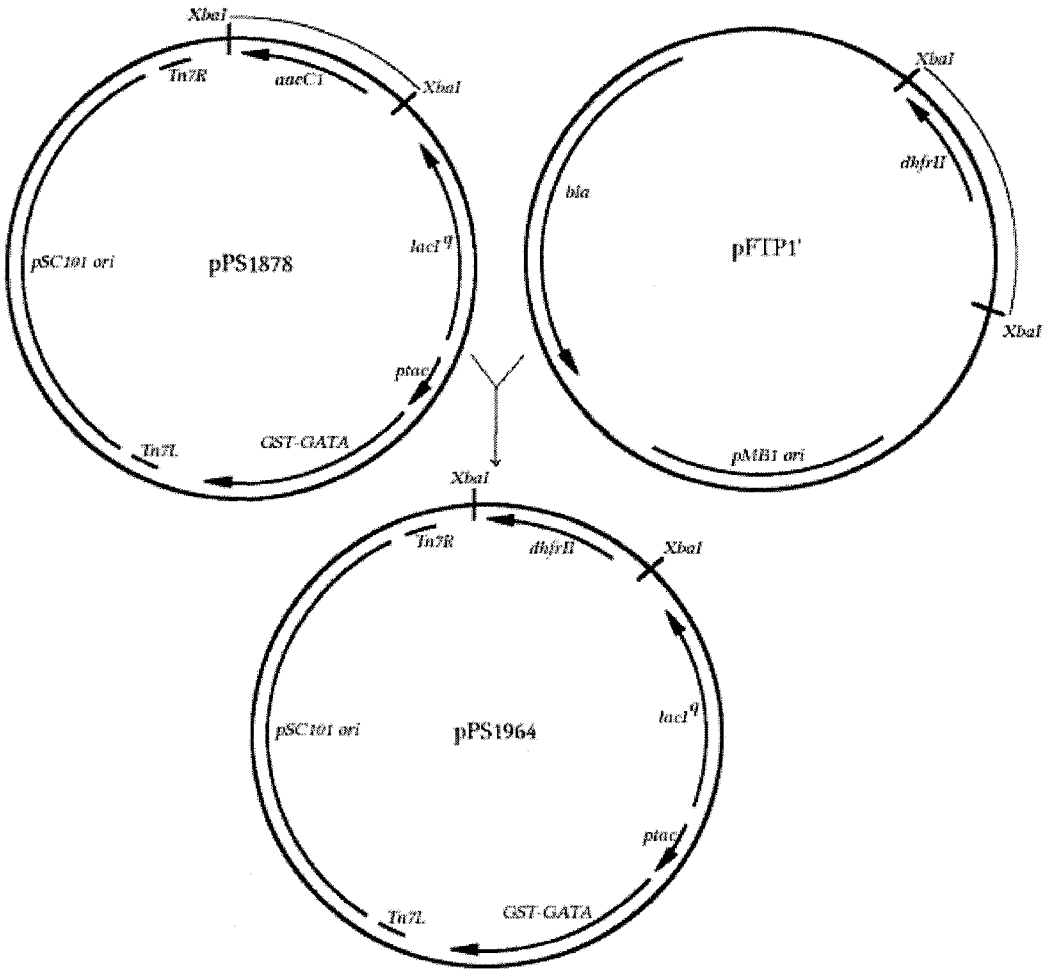


Figure 2.2 Cloning strategy for GATA vectors (part II)

Plasmid maps not drawn to scale. See text description under Materials and Methods section 2.7.3 Plasmid construction. Final vector pPS1964 was used to express GATA from a single copy integrated into the chromosome of *P. aeruginosa* by way of the mini-Tn7 system.

Thi et al. 2005). The toxicity of the protein CcdB has been exploited in the construction of very effective positive cloning systems in *E. coli* (Gabant et al. 2000). The Gateway System^{TM} provides an example where the *ccdB* sequence is disrupted by cloned inserts. In this method, the traditional restriction and ligase reactions are replaced by site- specific recombination sites and recombinases. The recombinants are selected by inactivation (deletion) of the *ccdB* gene by the insert of interest (Walhout et al. 2000). The hypothesis for testing the *ccdB* gene as a counter-selectable marker for *Burkholderia* was that the gene would inhibit the growth of the bacteria when its expression was placed under control of the inducible P_{tac} promoter. The *ccdB* gene was taken from the entry clone vector of the Gateway cloning system. The gene was amplified and subcloned into a replicative vector under control of the P_{tac} promoter. See **Figure 2.3**. However, when expressed it was not toxic in *B. thailandensis*. The reasons for this are not clear. It is possible that the gyrase proteins of different bacteria exhibit different susceptibilities towards the CcdB toxin. Even though the gyrases are known to be well conserved among bacteria, the *E. coli* K12 and the *B. pseudomallei* K96243 gyrase A subunits share an amino acid sequence identity of 59% and 75% similarity. It is known that quinolones act in a similar manner to that of CcdB, by interacting with bacterial gyrases, but different species of bacteria exhibit different susceptibilities to quinolones. It is also possible that the P_{tac} promoter did not achieve production of sufficient amounts of CcdB, although this promoter was shown be active in *Burkholderia* (Choi et al. 2008).

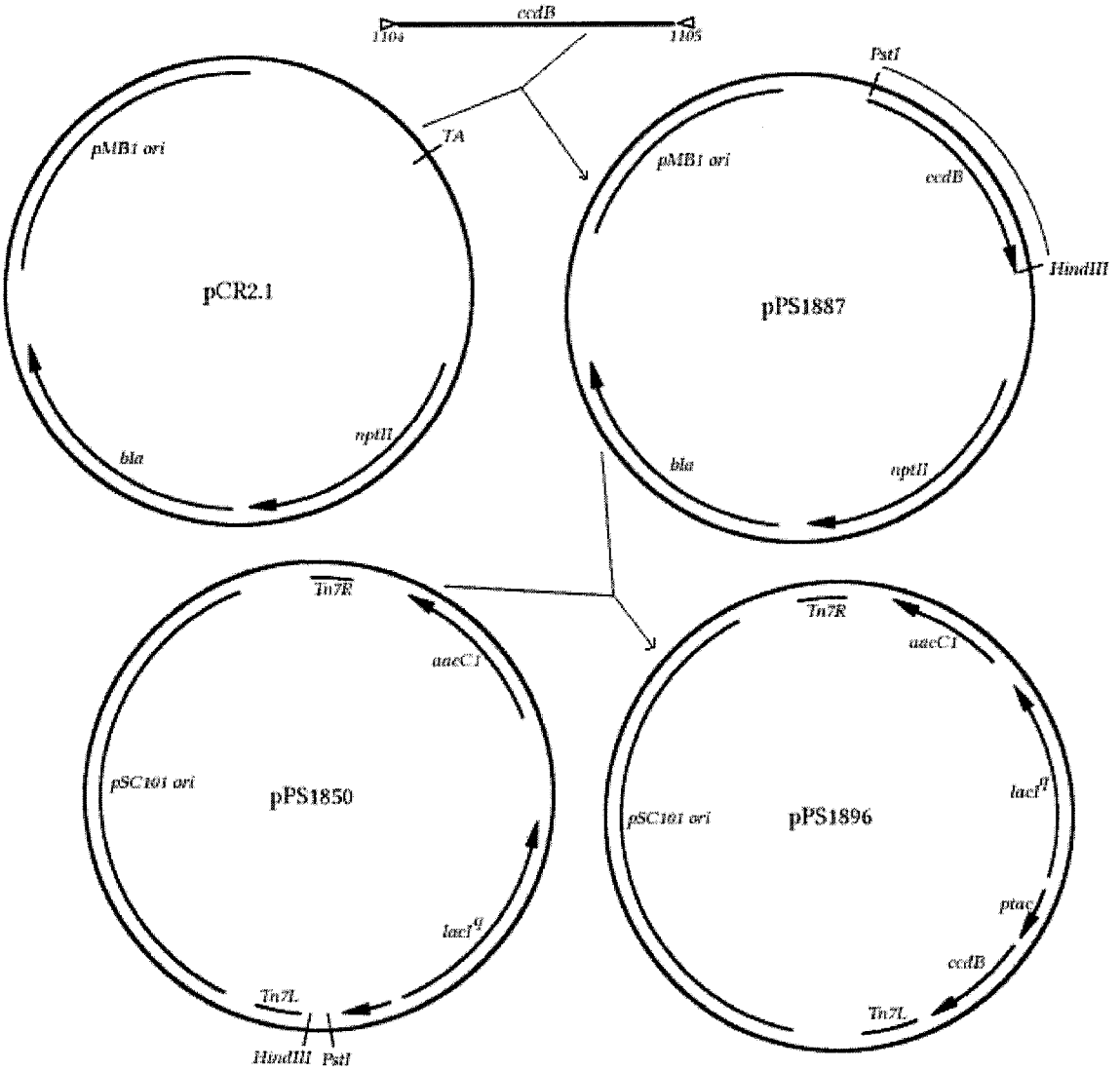


Figure 2.3 Cloning strategy for ccdB expression vector

Plasmid maps not drawn to scale. See text description under Materials and Methods section 2.7.3 Plasmid construction. Final low copy number plasmid pPS1896 was used to test toxicity of *ccdB* in *P. aeruginosa.*

2.4 *hfq*

The RNA-binding Hfq is required for the function of many small RNAs. In bacteria small RNAs (sRNAs) are used to control diverse cellular processes. A large class is involved in regulation of gene expression primarily at post-transcriptional levels through base-pairing with cognate mRNAs, and these sRNAs require Hfq for binding (Lee and Feig 2008). The inactivation of the *hfq* gene of *E. coli* causes a wide variety of phenotypes and alters the expression of many proteins. In a study aimed at determining the interactions of the endoribonuclease RNaseE (another protein with important roles in RNA metabolism in *E. coli*) and Hfq, the authors found that overexpression of Hfq caused inhibition of cell division (Takada et al. 2005). A homolog of the *hfq* gene can be found in most bacteria, including *B. pseudomallei.* Even though the mechanism for the observed cell division inhibition was not clear from the studies described in the literature, other than it being related to low levels of FtsZ in the cells (FtsZ is a conserved protein involved in the initiation of cell division), it seemed possible that induced overproduction of Hfq in other bacteria would cause similar growth inhibition. The hypothesis was that regulated overexpression of *hfq* would cause inhibition of cell growth in *Burkholderia.* The *hfq* gene from *B. thailandensis* E264 was amplified from chromosomal DNA, including a region upstream of the start codon, which had consensus to a ribosome binding site. The gene was subcloned into a vector for expression under control of the P_{lac} promoter. See **Figure 2.4.** Induced expression from the multicopy plasmid did inhibit growth to a certain extent in *E. coli* as seen by replica patching of cells onto induced and uninduced plates, and by a

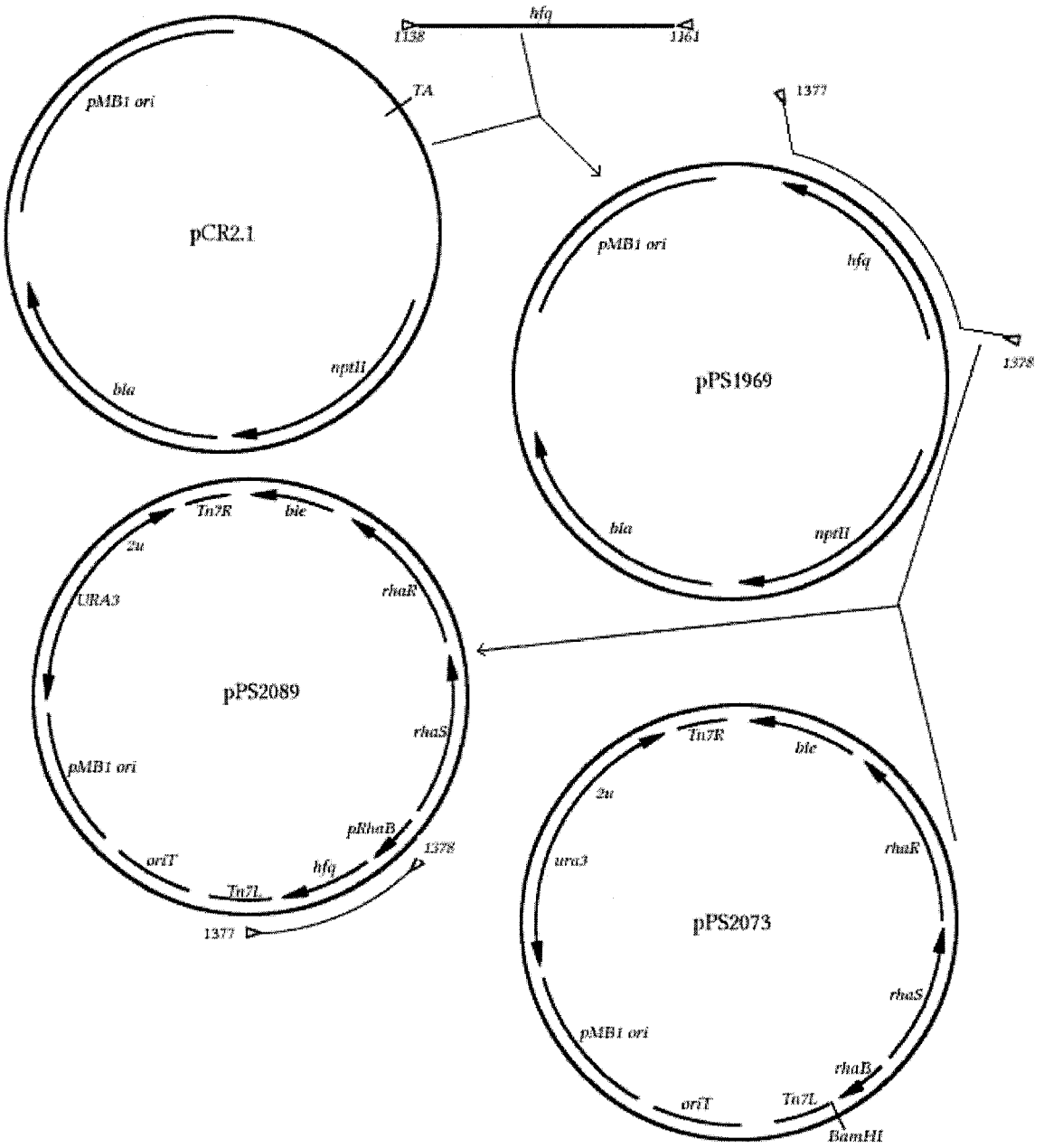


Figure 2.4 Cloning Strategy for *hfq* expression vector

Plasmid maps not drawn to scale. See text description under Materials and Methods section 2.7.3 Plasmid construction. Plasmid pPS1969 was used to verify if *hfq* driven from the P*lac* would be toxic in *E. coli*. The final plasmid pPS2073 was used to test *hfq* from a single copy integrated into the chromosome of *B. thailandensis* by way of the mini-Tn7 system.

growth curve comparison between the wild-type strain carrying the plasmid and the same strain carrying the empty vector. However, expression from a mini-Tn*7* insertion in single copy failed to show any growth defect in *B. thailandensis.* It is possible that the toxicity observed in *E. coli* was due to a very high overexpression of Hfq to levels that were not easily achievable in *B. thailandensis.* It is also possible that the inhibition of growth seen could be compensated by unknown factors after many hours of growth (overnight), meaning that the cells were growing slowly but were not being killed. Other more recent studies have shown that Hfq , although present in other bacteria, may not act the same way as it does in *E. coli* (Bohn et al. 2007). There is not enough data in the literature to explain why the high levels of Hfq were toxic in *E. coli.* Besides, no further experiments have been reported to try to clarify the issue, most articles in the literature deal with *hfq* mutants but do not mention anything about overexpressors.

2.5 *eradE*

Era (*E. coli* Ras-like protein) is a multifunctional GTPase with RNA-binding ability and homologues present in most bacteria. Reported functions include roles in ribosome biogenesis, cell division, cell growth and energy metabolism (Sharma et al. 2005). A mutant allele of *era* called *eradE* (deletion of Effector region) was shown to cause a growth defect in cells expressing it (in a dominant negative manner) and in the presence of acetate in the medium (Inoue et al. 2002). The fact that addition of acetate to the medium would cause a marked growth defect looked like an attractive feature for a CS marker, as it

meant that the toxicity of the marker could be more tightly regulated, than by using, for example, overexpression of Hfq. The hypothesis was that an *eradE* mutant would cause a growth defect in *B. thailandensis* grown in the presence of acetate, and this characteristic could be exploited to generate a gene replacement system. Only one strong homologue of the *E. coli era* was found in *B. thailandensis* and in *B. pseudomallei.*

EradE was made by deleting the effector region of the *B. thailandensis* Era by Soeing-PCR. The mutant was subcloned with its own ribosome binding site and expression driven from a P_{rhaB} promoter. See **Figure 2.5**. Experiments aimed at isolation of an efflux pump mutant, using a replacement cassette for the *bpeE* gene, failed. It was not possible to get the mutant because the merodiploid would not resolve after growth in the medium containing the inducer IPTG and acetate. The precise reasons why *eradE* caused a negative dominant growth defect in *E. coli* was the subject of speculation by the authors who described the initial findings (Inoue et al. 2002) . It was noted that *eradE* expressing strains had an altered gene expression profile that included genes in diverse metabolic pathways. Also, the *eradE* overexpressors were found to accumulate serine in their cytoplasm, however the known toxicity of serine reported in the literature can be overcome by addition of isoleucine (Isenberg and Newman 1974), and this was not the case here. *Burkholderia* have multiple accessory metabolic pathways that allow them to thrive in diverse environments, it is possible that the *eradE* mutant is not dominant negative in them because its effect can somehow be bypassed.

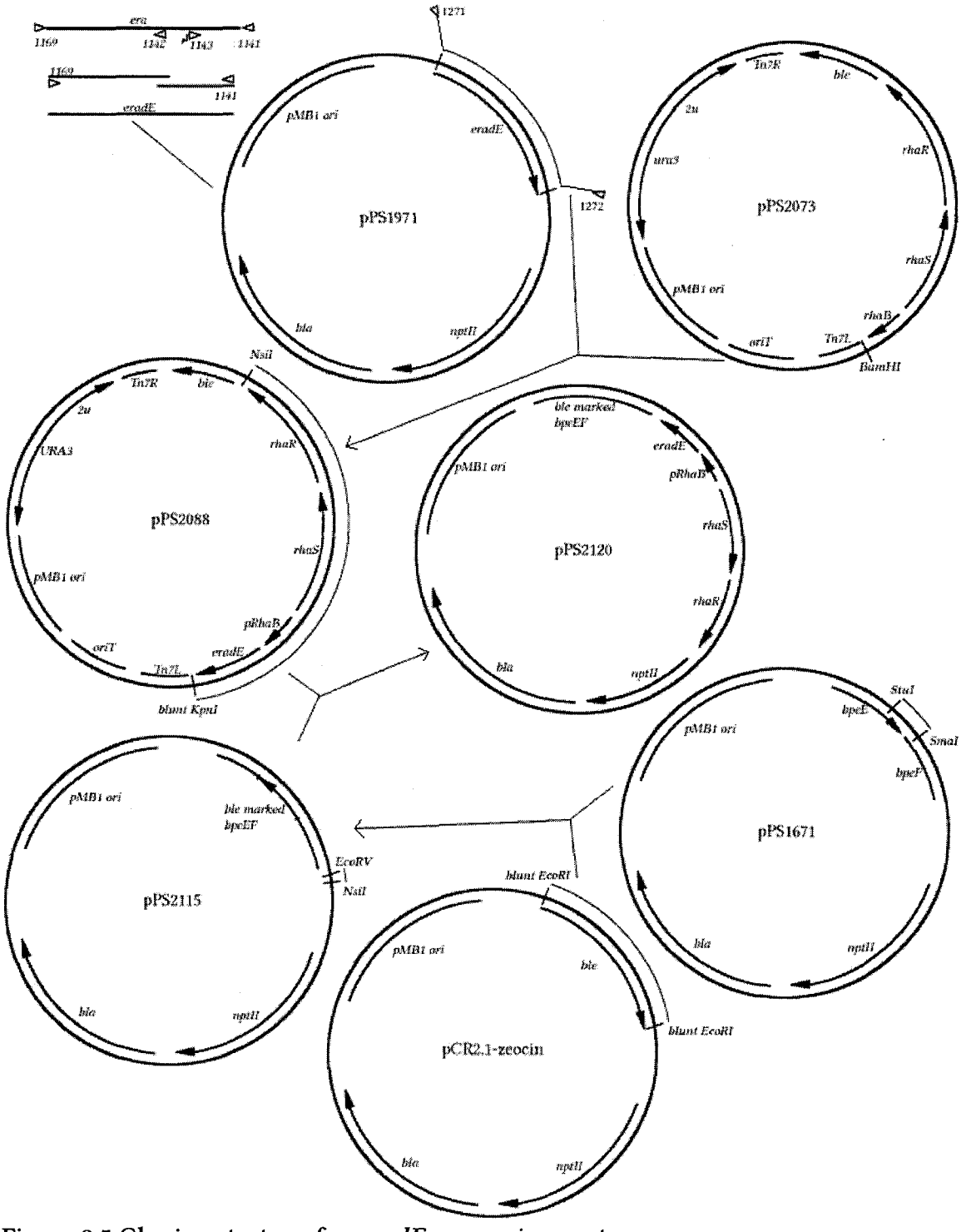


Figure 2.5 Cloning strategy for *eradE* expression vector

Plasmid maps not drawn to scale. See text description under Materials and Methods section 2.7.3 Plasmid construction. Final plasmid pPS2120 was used to try to obtain a *bpeEF* replacement in *B. thailandensis* using *eradE* as a CS marker.

2.6 Temperature Sensitive Replicon

The availability of a temperature-sensitive (TS) replicon (Choi et al. 2008) for *Burkholderia* species, the TS pRO1600 broad-host-range replicon, inspired the idea that it might be possible to use a replicon as a CS marker. In a paper dated back to 1989 other investigators explored the same idea and showed it could work (Hamilton et al. 1989), at least in *E. coli* and with the specific TS replicon they used.

The idea behind the technique is that an active replicon, integrated into the chromosome of a cell, should not be tolerated by the cell as it would disrupt the chromosomal replication mechanism. Therefore, a TS replicon placed in a gene replacement vector could allow for selection of plasmid cointegrates. At the non-permissive temperature (42°C), and using the antibiotic marker in the vector for selection, only cells that integrate the plasmid by homologous recombination would grow. Next, for isolation of strains that resolved the cointegrant, growth at the permissive temperature (30°C) should kill the cells that fail to resolve the cointegrant. The surviving cells would contain the plasmid replicating again in the cytoplasm at the permissive temperature, but some of them should have the mutant gene now replacing the wild-type in the chromosome, while the native gene would have been transferred to the plasmid. Curing the plasmids should be easy by propagating the cells again at the nonpermissive temperature, but this time without using antibiotic selection. After this step, one should be able to screen for mutants by making sure the resistance marker has been lost, and then for a phenotypic characteristic of the mutant, or by PCR. Alternatively, one could use a marker within the

mutant to facilitate the screening process (obtaining a marked mutant). An advantage of this method would be possibly that a selection for transformants, carrying the vector in the cytoplasm at the permissive temperature, before selecting for cointegrants at the non-permissive temperature, could allow for accumulation of cointegrates that underwent a recombination event, and make it possible to obtain mutants even in places in the chromosome where recombination events are very rare (Manna et al. 2004).

To test the hypothesis that the TS replicon can be used for counter-selection, replacement vectors were constructed containing the replacement cassettes for either the *sacB*::Tmp^R gene or the *bpeE*::Tmp^R gene (*bpeE* is an efflux pump gene) the TS pRO1600 replicon and the gene for kanamycin resistance as the marker in the plasmid backbone. See **Figure 2.6**. After selecting for transformants, cells were propagated at the non-permissive temperature and colonies were isolated that were trimethoprim resistant and kanamycin resistant. Switching cells to the permissive temperature and then again to the nonpermisiisve temperature, in the absence of antibiotic selection for kanamycin, failed to produce kanamycin sensitive cells. All isolates checked could still grow at 42°C in kanamycin containing media, showing that the cointegrates had not resolved when the merodiploids where grown at 30°C, and the integrated replicon must have not been detrimental to the cells.

Since not all replicons are regulated in the same way, and the knowledge in the field is still incipient, it is difficult to explain why a replicon in the chromosome of *B. thailandensis* would or would not alter the normal chromosomal replicon homeostasis. In

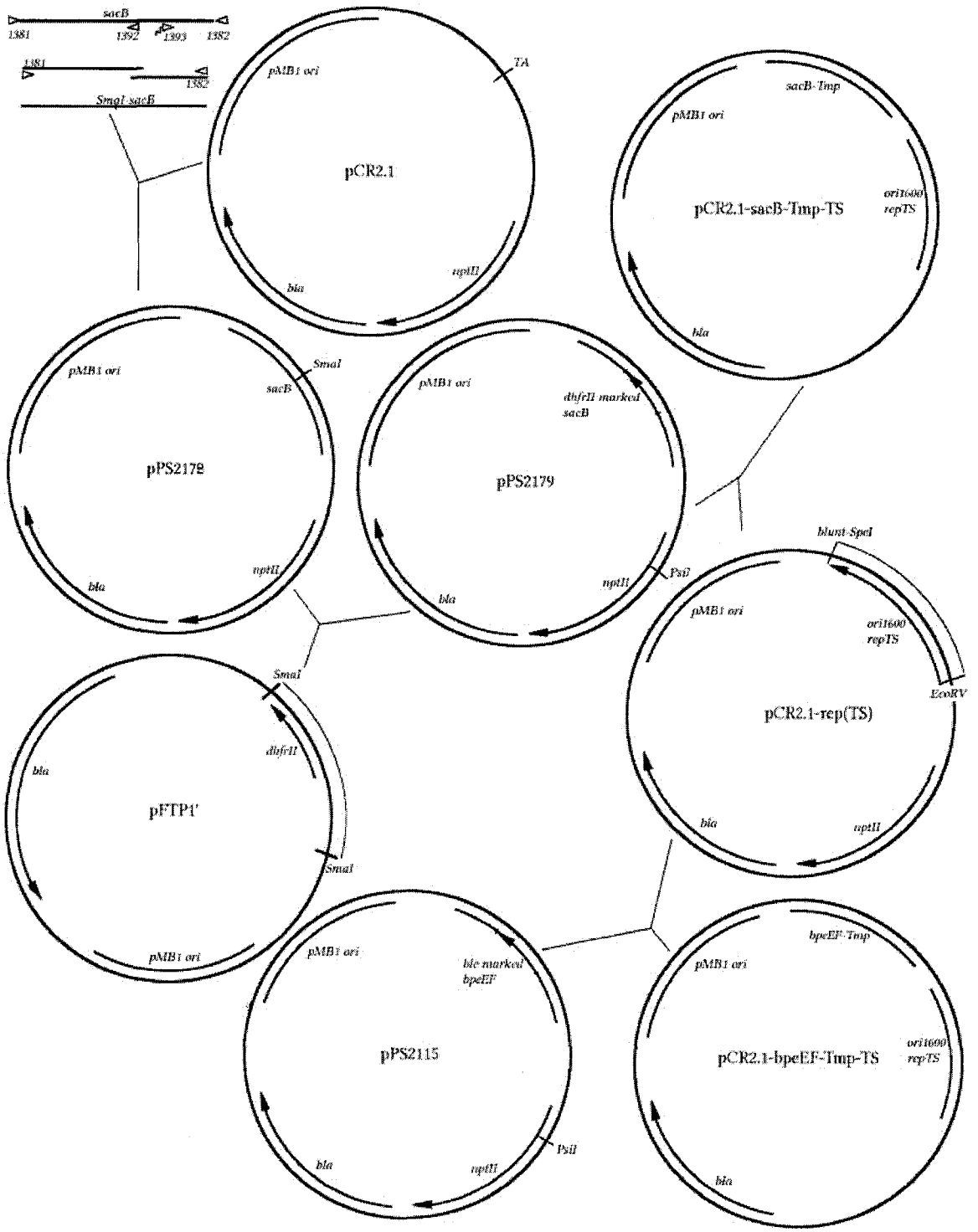


Figure 2.6 Cloning strategy for vectors to test TS replicon as a CS marker

Plasmid maps not drawn to scale. See text description under Materials and Methods section 2.7.3 Plasmid construction. Final plasmids pCR2.1sacB-Tmp-TS and pCR2.1-bpeEF-Tmp-TS were used to try to generate the *sacB* and *bpeEF* gene replacements in *B. thailandensis* using the TS replicon instead of a CS marker.

B. pseudomallei, for example, each replicon in each chromosome may be regulated by a separate control system as seen by the sequences and genes found at each origin site (Lessie et al. 1996). Even so, both chromosomes are replicated effectively in a timely fashion to produce daughter cells that carry both chromosomes.

2.7 Materials and Methods

2.7.1 Bacterial strains and growth conditions

The bacterial strains, plasmids and primers used in the above experiments are listed in **Table 2.1**. *E. coli*, *B. thailandensis* and *P. aeruginosa* strains were routinely grown at 37°C in LB medium (Lennox agar or Lennox broth, MoBio). IPTG 1 mM was added to the medium at a concentration of 1mM for induction of P_{lac} or P_{tac} promoter. Potassium acetate 50 mM was added to the medium in experiments for *eradE* CS. For plasmid maintenance in *E. coli*, the medium was supplemented with 100 μg/mL ampicillin, 15 μg/mL gentamycin or 35 μg/mL kanamycin. For selection of *B. thailandensis* exconjugants and counter selection against *E. coli* strains, LB agar plates were supplemented with 200 μg/mL rifampycin and 1oo μg/mL trimethoprim. For selection of *P. aeruginosa* the medium was supplemented with gentamycin 30 μg/mL or trimethoprim 100 μg/mL. Since *E. coli* cannot use citrate as its sole carbon source but *P. aeruginosa* can, VBMM medium (0.1 M citric acid, 0.57 M K_2HPO_4, 0.1 M Sodium citrate, 0.16 M Na NH_4PO_4, x 4 H_2O, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$) was used to counterselect *E. coli* in conjugations with *P. aeruginosa*.

TABLE 2.1 Strains, plasmids and primers used in this study

	Relevant characteristics	Reference/source
Strains		
Escherichia coli		
DH5α	F- φ80d*lacZ*M15 (*lacZ*YA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r k-, m k+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ-	Laboratory stock
HB101/pRK2013	Helper strain for conjugations	(Figurski et al 1979)
DB3.1	Strain with mutant gyrase that confers *ccdB* resistance	Invitrogen
DH5α λ pir	Phage λ pir lysogen of DH5α	C. López Lab collection
B. thailandensis		
P879	Rifampicin resistant strain derived from wild-type E264 (Holden et al. 2004)	K.White Lab collection
P. aeruginosa		
PAO1	Type-strain.	Laboratory collection
S. cerevisiae		
INVSc1	*MATa his3D1 leu2 trp1-289 ura3-52MAT his3D1 leu2 trp1-289 ura3-52*	Invitrogen
Plasmids		
pCR2.1	Ap^R Km^R, vector provided with TA cloning Kit	Invitrogen TM
pGATA	Ap^R, source of *GST-GATA* with MCS	(Truder et al. 1996)
pPS1735	Ap^R, source of *GST-GATA* without MCS	This study
pWSK29`	Ap^R, source of pSC101 origin	This study
pPS1849	Ap^R, pWSK29`-GSTGATA from pPS1735	This study
pCR2.1-lacIQptac	Ap^R Km^R, source of *lacI*^{q} and P_{tac} promoter	Lab collection
pPS1877	Ap^R; pSC101 origin with *lacI*^{q} and P_{tac} -GST-GATA	This study
pPS3122	Gen^R, pSC101 mini-Tn7 Gm	C. López Lab collection
pPS1878	Gen^R, pSC101 mini-Tn7 *lacI*^{q} and P_{tac} -GST-GATA Gm	This study
pFTP1`	Tmp^R, source of *dhfrII* gene for trimethoprim resistance	C. López Lab collection
pPS1964	Tmp^R; pSC101 mini-Tn7 *lacI*^{q} and P_{tac} -GST-GATA Tmp	This study
pDONR22	Ap^R, source of *ccdB*	Invitrogen

pPS1887	Ap^R Km^R; pCR2.1-ccdB	This study
pPS1850	Gen^R; pSC101 mini-Tn7 *lacI*^{q} and P_{tac}	C. López Lab collection
pPS1896	Gen^R; pSC101 mini-Tn7 *lacI*^{q} and P_{tac} *ccdB*	This study
pPS1969	Ap^R Km^R; pCR2.- hfq	This study
pPS2073	Zeo^R; pUC18 mini-Tn7 P*rhaB* Yeast-*E. Coli* shuttle vector	C. López Lab collection
pPS2089	Zeo^R; pUC18 mini-Tn7 zeocin P*rhaB hfq*	This study
pPS1971	Ap^R Km^R; pCR2.1-eradE	This study
pPS2088	Zeo^R; pUC18 mini-Tn7 zeocin P*rhaB* eradE	This study
pCR2.1-zeocin	Ap^R, Km^R, Zeo^R; source of *ble* gene	K.H. Choi Lab stock
pPS1671	Ap^R, Km^R, source of *bpeEF* fragment	A. Kumar Lab stock
pPS2115	Ap^R, Km^R, Zeo^R; pCR2.1 bpeEF::zeocin	This study
pPS2120	Ap^R, Km^R, Zeo^R; pPS2115 plus P*rhaB eradE*	This study
pPS2178	Ap^R, Km^R, pCR2.1 *sacB* (from E264)	This study
pPS2179	Ap^R, Km^R, Tmp^R; pCR2.1 *sacB::Tmp*	This study
pCR2.1-rep(TS)	Ap^R, Km^R, source of ori*1600* rep TS	K.H. Choi Lab stock
pCR2.1sacBTS	Ap^R, Km^R, Tmp^R; pCR2.1 *sacB::Tmp with ori1600 repTS*	This study
pTNS2	Ap^R, expression vector for *tnsABCD*	(Choi et al. 2006)

Primers

#930	5'-CTG GCG GCC GCG TAC
#931	5'-GCG GCC GCC AGA GCT
#1104	5'-CTC TTT TGC TGC AGA GAA CAG G
#954	5'-GCG AGG CGG ATC CTC AGT CAC
#992	5'-GGA ATT GTG AGC TCA TAA CAA TTT C
#1105	5'-AAG GCC ATT CCG CCG TAA GC
#470	5'-GCA CAT CGG CGA CGT GCT CTC
#478	5'-CAC AGC ATA ACT GGA CTG ATT TC
#479	5'-ATT AGC TTA CGA CGC TAC ACC C
#618	5'-GTT CGT CGT CCA CTG GGA TCA
#619	5'-AGATCGGATGGAATTCGTGGAG
#1138	5'-AAC GCA TAA CTC GAG ATT GAC GCC

#1161	5'-GAG TCG GCT TAT TGA CCG TC
#1377	5'-TCG TAA TGA AAT TCA GCA CCA TCA CAT ATG CCA TGG TCG TGC CAA TCT AGA TCA ACC TGC
#1378	5'-CCG GGC CCA AGC TTC TCG AGG AAT TCC TGC AGC CCG GGG TTA GGA CGC TGC TTC CGC ATC
#1169	5'-GGG TCA TTG ACA TCG TTC C
#1141	5'-GTC ATC CCG GTA CCG GAC G
#1142	5'-TTG CGC GAC GTG ATG CTG ATC
#1143	5'-TCA GCA TCA CGT CGC GCA AGA TTC GCA CGC TCG ACG ACG C
#1271	5'-GTG GAA TTG TGA GCG GAT AAC AAT TTC ACA CAG GAA ACA GTA GCG CTG CAG CTT CCG AAC
#1272	5'-TGC AAG GCC TTC GCG AGG TAC CGG GCC CAA GCT TCT CGA GGG TCA TCC CGG TAC CGG ACG
#1381	5'-GCT GGT GGA ACT TAC TGA CTG ACG ACA CGC
#1382	5'-GCT GGT GGT GCT TGA ATG CAT GCG CGA CG
#1392	5'-AAT TGA TGC CCG GGA AGT TCT GC
#1393	5'-GGA CAT TGC GCA GAA CTT CCC GCT CAT CAA TTT ACT ACC TGT TCA CGA TCA GCC

Saccharomyces cerevisiae INVSc1 cells were grown on YPD medium (10 g/L Yeast Extract, 20 g/L Bacto Peptone, 20 g/L dextrose and 0.32 g/L tryptophan) at 30°C.

2.7.2 DNA manipulations

Routine procedures were used for DNA manipulations (Sambrook and Russell 2001). *B. thailandensis* chromosomal DNA fragments (20-30 kb) were isolated using the QIAamp DNA Mini Kit (Qiagen), the DNA was suspended in 200 µL of the supplied buffer AE (10 mM Tris-HCL, 0.5 M EDTA, pH 9). Plasmid DNAs were isolated using QIAprep Spin miniprep Kit (Qiagen). Minipreps from *S. cerevisiae* were obtained using the same QIAprep Spin miniprep Kit (Qiagen) with the following modifications of the manufacturer's protocol: After addition of P1 buffer, 50 µL of acid washed glass beads were added and the mixture was vortexed for 5 min. After a short pause to let the beads settle at the bottom, the supernatant was transferred to a new microfuge tube and the protocol was continued as usual with addition of buffer P2. Plasmid DNA fragments were purified from 1% agarose gels using the QIAQuick Gel Extraction Kit (Qiagen). For colony PCR a single, large colony (usually from a colony patch) or the pellet of an overnight 0.5 mL culture, was transferred to a microcentrifuge tube containing 50 µL dH_2O, and was placed in a boiling water bath for 10 min. After a 2 min spin in a microcentrifuge (16000 x g), 5 µL of the supernatant were used for a 50 µL PCR reaction. Routine PCRs for checking constructs or strains were performed using standard *Taq* polymerase (NEB) as recommended by the vendor. PCRs for obtaining fragments for subsequent cloning procedures were done using Platinum *Taq* HiFi polymerase (Invitrogen) following the vendors instructions. Restriction

enzymes were purchased from NEB, and reactions were carried out using supplied buffers at the appropriate temperature for 1h in 20 to 30 µL volume reactions. Blunt ending of DNA fragments was performed using T4 DNA polymerase (NEB) right after restriction enzyme denaturation of 20 µL volume reactions (usually 65°C for 20 min), by addition of 1 µL of 10 mM dNTPs and 1 µL of enzyme and incubation at 12°C for 15 min. Ligation reactions were set up using DNA ligase (Invitrogen). Custom oligonucleotides were synthesized by Integrated DNA technologies (Coralville, IA).

2.7.3 Plasmid construction

For linker tailing of pGATA (Truder et al. 1996) to generate pPS1735, each primer 930 and 931 was resuspended in sterile water to a final concentration of 2 ug/µL. 12.5 µL of each pimer solution plus 25 µL of water were boiled for 1 min in a water bath, and then left to cool down slowly to room temperature in the water bath overnight to allow for linker hybridization. The next day, a ligation was set up using *Kpn* I/*Sac* I digested pGATA (~1 µg) and the linkers (~1 µgof hybridized linkers). After ~ 16 h at 14°C, the ligation mixture was heated to 70°C for 10 min and then it was ran in a 0.8% agarose gel electrophoresis. After purification of the single smiling band on the gel, the 50 µL eluate was mixed with 50 µL of Hybridization Buffer (20 µL of 5 M NaCL, 10 µL of 1 M Tris- HCl at pH 7.5 and 2 µL of 0.5 M EDTA at pH 8 in 968 µL of sterile water), and the 100 µL solution was heated at 80°C for 5 min in a heat block. The heat block was shut down and let cool down overnight before using the ligation for transformation of *E. coli*.

GATA was PCR amplified using primers #992 and #954 (Table 2.1) from pPS1735 and the

resulting 1036 bp fragment was ligated into the *EcoR*V site of pWSK29`. pWSK29' is a low copy number plasmid containing the *pSC101* origin of replication and it was chosen to prevent possible toxic effects of the *GATA* gene in *E. coli.* Next, the P_{tac} $lacI^q$ expression cassette was excised form pCR2.1-lacIQptac on a 1596 bp *Hind*III-*Xho*I fragment, which was then ligated between the same sites of pPS1849 to form pPS1877. Finally, the $lacI^q$ P_{tac} *GATA* fragment from pPS1877 was inserted into the mini-Tn7 delivery vector pPS3122 for chromosomal integration in pPS1878, see figure 2.2. To be able to select for the mini-Tn7 integration into the chromosome of *B. thailandensis*, the gentamycin resistance marker *aacC1,* from pPS1878, was replaced with a trimethoprim resistance marker, *dhfrII,* from pFTP1' resulting in pPS1964.

ccdB was amplified with primers #1104 and #1105 (Table 2.1) from the pDONR22 plasmid and the resulting 382 bp fragment was ligated into pCR2.1 to obtain pPS1887. Next, the 365 bp PstI-HindIII fragment containing ccdB was cloned between the same sites of pPS1850 to create pPS1896, a mini-Tn7 delivery vector containing the pSC101 origin of replication and the gentamycin resistance marker, *aacC1,* for integration into *P. aeruginosa.*

For yeast gap repair *Saccaromyces cerevisiae* INVSc1 cells were used as the host to transform linearized fragments and PCR products (1-2 μg of DNA total) seen in the figures for *hfq* and *eradE* cloning strategies. Yeast competent cells were prepared in the lab following the Lithium Acetate protocol and kept at -80°C. A 50 μL aliquot from the frozen stock was placed on ice, 5 μL of salmon sperm DNA were added to the cells. Then the DNA fragments for transformation were added. Finally 300 μL LiAc/PEG (100 mM

Lithium acetate, 10 mM Tris-HCl pH 7, 1 mM EDTA pH 8 and 40% PEG3350) were added before incubating the cells at 30°C for 30 min. Heat shock was performed at 42°C for 20 min. Cells were plated onto YPD media for selection of trasnformants carrying the URA3 gene.

hfq was amplified from chromosomal DNA of *B. thailandensis* E264 using primers #1138 and #1161 (Table 2.1) the 677 bp product was ligated into pCR2.1 to obtain pPs1969. Primers #1377 and #1378 (Table 2.1) were used to amplify an *hfq* containing fragment (737 bp) from pPS1969 used together with *BamHI* digested pPS2073 to perform yeast gap repair and obtain pPS2089, see Figure 2.4.

The *era* gene was amplified form the chromosome of *B. thailandensis* E264 using primers #1169 and #1141 and the 1003 bp fragment was used as a template in a second set of PCR reactions to obtain fragments #1169-#1142 (204 bp) and #1143-#1141 (788 bp), which were further used as templates, in a Soeing PCR reaction with primers #1169 and #1141, to obtain *eradE* (972 bp). See Figure 2.5. The *eradE* PCR product was ligated into pCR2.1 to obtain pPS1971. Using pPS1971 as a template, a 1052 bp PCR product of amplification with primers #1271 and #1272 was used together with *BamHI* digested pPS2073, in yeast gap repair, to obtain pPS2088. On a second sequence of plasmid design paths, pPS1671, containing bpeE and bpeF sequences from *B. thailandensis* E264, was digested with *Stu*I and *Sma*I, to insert a blunt ended *EcoR*I zeocin resistance cassette from pCR2.1-zeocin (546 bp) into it, resulting in pPS2115, a plasmid carrying a zeocin marked *bpeEF* mutant cassette. Finally, pPS2115 was digested with *EcoR*V and *Nsi*I, and a blunt ended-*Kpn*I-*Nsi*I

fragment from pPS2088, containing *eradE* driven by the P_{RhaB} promoter (2068 bp) was inserted into it, to obtain pPS2120.

The *sacB* gene from *B. thailandensis* E264 was amplified from the chromosome using primers #1381 and #1382 (Table 2.1). The 1727 bp PCR product was then used as a template in a set of two PCR reactions using primer sets #1381 and #1392, and #1393 and #1382 to obtain fragments of 325 bp and 577 bp respectively. Both these PCR products were used again as templates in a Soeing PCR reaction using primers #1381 and #1382 to obtain a mutant *sacB* (882 bp) which was ligated into pCR2.1 to obtain pPS2178. PPS2178 was digested with *Sma*I and a trimethorpim resistance cassette (*dhfrII*), from pFTP1' digested with the same enzyme (862 bp), was ligated into it, to obtain pPS2179. Next, pPS2179 was digested with *Psi*I and the blunt-ended-*Spe*I-*EcoR*V fragment, of 1303 bp, containing the ori_{1600} *rep*TS from pCR2.1-rep(TS) was inserted into it, resulting in pCR2.1-sacB-Tmp-TS. On a different path of plasmid constructions, pPS2115 was digested with *Psi*I and the 1303 bp blunt-ended-*Spe*I-*EcoR*V fragment from pCR2.1-rep(TS) was ligated into it resulting in pCR2.1-bpeEF-Tmp-TS.

2.7.4 Introduction of plasmids inside the cells

For integration of mini-Tn7 plasmids into *P. aeruginosa* or *B. thailandensis* 100 µL of overnight cultures, at 37°C, of *E. coli* helper strain HB101/pRK2013 (kanamycin resistant) (Figurski and Helinski 1979) , *E. coli* carrying the respective mini-Tn7 vector and *E. coli* carrying the helper pTNS2, and the intended host (*P. aeruginosa* PAO1 or *B. thailandensis*

P898) were washed in 1 mL 10mM $MgSO_4$. The pellets of the four bacterial strains were all combined into a microfuge tube, resuspended in 1 mL 10 mM $MgSO_4$ and spun down for 1 min at maximum speed in a microfuge. The supernatant was discarded and the pellet was placed on top of a nitrocellulose conjugation filter on an LB agar plate. The filter was incubated at 37°C overnight. Next day the filter was taken from the plate and placed against the inside wall of a microfuge tube with 1 mL 0.9% (w/vol) NaCl solution. The cells were resuspended in the saline solution by pipetting up and down with a pipette, and 50 μL aliquots were plated onto either a VBMM plate plus the necessary antibiotic for selection in the case of PAO1, or an LB plate with Rifampicin 200 μg/mL plus the necessary antibiotic for selection of *B. thailandensis*. The remainder of the cells were pelleted in a microcentrifuge at maximum speed for 30 s, suspended in 50 μL of 0.9% NaCl solution and the suspension plated onto a second selective plate. Typically after 24 h at 37°C tiny colonies appeared on the plates. Usually a background of tiny colonies appeared on the plates in the case of *B. thailandensis*. Mini-Tn7 integrations into the chromosome of *P. aeruginosa* were checked by PCR using primers #470 and #478, which result in a 272 bp PCR product. Integrations into *B. thailandensis*, which could happen in two different regions in the chromosome 9downstream of glmS1 or glmS2, were checked with primers #479 and #618 which result in a 328 bp product, or with primers #479 and #619, which result in a 394 bp product (Choi et al. 2006) .

For electroporations of other non-replicative plasmids into *P. aeruginosa* or *B. thailandensis* reported methods were used (Choi et al. 2006).

CHAPTER THREE

Exploring *pheS* as a Counter-Selection Marker for *B. pseudomallei*

3.1 Introduction

In recent years *Burkholderia pseudomallei*, has received increased attention from the scientific community since it has been classified as a Select Agent by the United States Government. The availability of various completed genome sequences of different strains brings a great opportunity for all those interested in studying different aspects of the molecular biology of this microorganism. The ability to create a targeted mutation in the chromosome is an important tool allowing scientists to evaluate hypotheses concerning the function of those regions.

A useful and reliable technique employed to generate targeted mutagenesis using circular plasmids as delivery vectors in bacteria, requires a counter-selectable marker in the backbone, for resolution of inevitable merodiploids resulting from plasmid integration into the chromosome. The widely used *sacB* and *rpsL* counter-selection markers are of very limited value in wild-type *B. pseudomallei* because of endogenous *sacB* genes and

intrinsic streptomycin resistance. This chapter describes the evaluation of a *pheS* gene allele as a counter-selection marker in *Burkholderia* species and its use in development of a versatile gene replacement system.

PheS is the alpha subunit of phenylalanine tRNA synthase, the enzyme catalyzing acylation of phenylalanine to its cognate tRNA. While tRNA synthases are known for their substrate specificity, it was shown that a substitution of the amino acid alanine for glycine at position 294, in the *E. coli* enzyme, resulted in a protein with relaxed substrate specificity (Kast and Hennecke 1991). When this mutant enzyme is expressed, it catalyzes incorporation of halogenated phenylalanine analogs, like *para*-chloro-phenylalanine, into proteins resulting in non-functional proteins and eventual cell death. This finding has led to use of the $pheS^{A294G}$ gene as a counter-selectable marker in different bacteria (Kast 1994; Kristich et al. 2007; Frirdich, Vinogradov, and Whitfield 2004). The working hypothesis for this chapter is, therefore, that a change in the corresponding amino acid of the alpha subunit of the *B. pseudomallei* phenylalanine tRNA synthetase would result in relaxed specificity of the *B. pseudomallei* enzyme, and lead to cell toxicity and cell death in the presence of *p*-chloro-phenylalanine, regardless of strain background. The new gene replacement system was used to isolate unmarked *amrAB-oprA* operon and *purM* gene mutants (see **Figure 3.1**) in different strains.

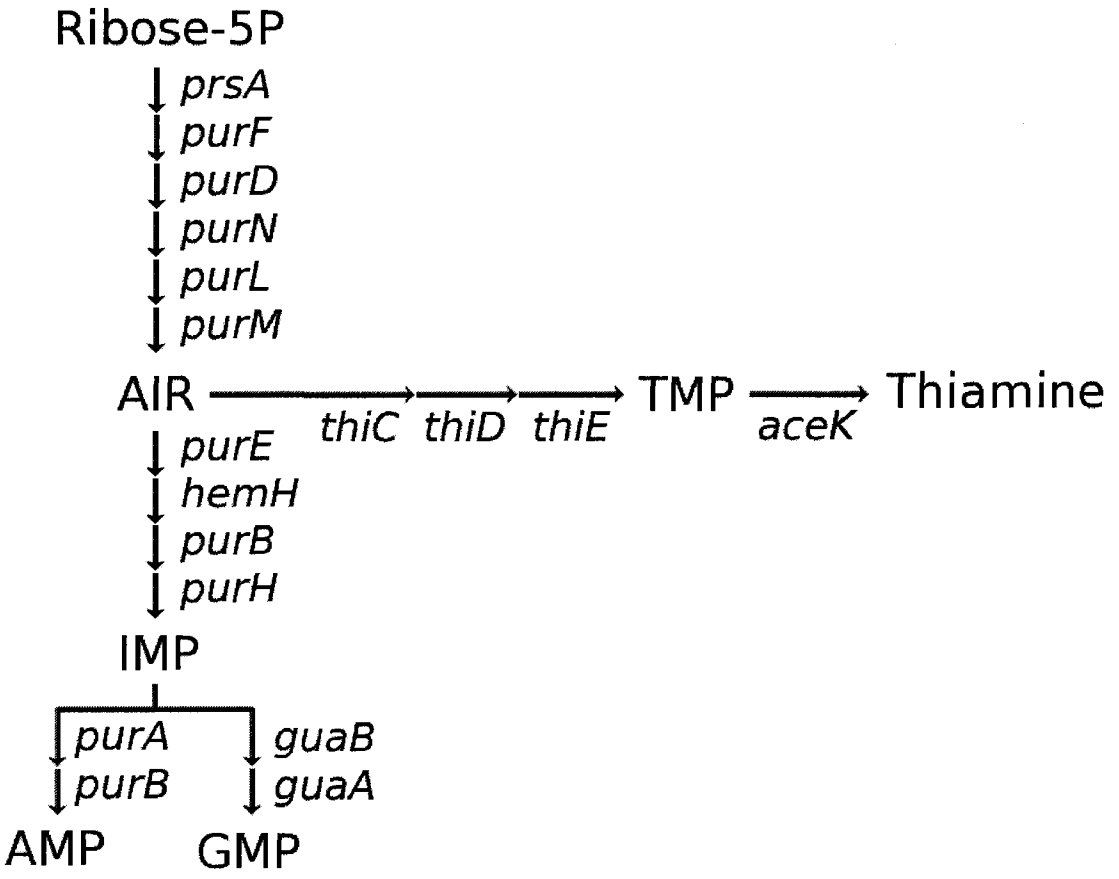


Figure 3.1 Purine biosynthesis pathway

Predicted Purine biosynthesis pathway in *B. pseudomallei* showing the role of *purM* in adenine and thiamine synthesis.

The *purM* gene encodes for aminoimidazole ribonucleotide synthetase (AIR synthase) (Li et al. 1999). *purM* mutants have been shown to be avirulent in different bacteria including *B. pseudomallei* (Frodyma, Rubio, and D. M. Downs 2000; Levine and Maurer 1958), probably due to the inability to obtain sufficient free purines in host tissues for growth and maintenance. The *purM* mutants are adenine and thiamine auxotrophs (Ebbole and Zalkin 1987; Zilles et al. 2001).

3.2 Materials and methods

3.2.1 Bacterial strains and growth conditions

The bacterial strains and plasmids used in the following experiments are listed in **Table 3.1**. *E. coli*, *B. pseudomallei* and *B. mallei* strains were routinely grown at 37°C in LB medium (Lennox agar or Lennox broth, MoBio). YEG-Cl with *p*-Cl-phenylalanine (5 g/L yeast extract, 5 g/L NaCl, bacto agar 15 g/L, 10 mM glucose, 10 mM *p*-Cl-phenylalanine) or M9 medium with *p*-Cl-phenylalanine (15 g/L KH_2PO_4, 2.5 g/L NaCl, 30 g/L Na_2HPO_4, 5 g/NH_4Cl L, 15 g/L bacto agar, 0.25 mM CaCl, 1.25 mM M $MgSO_4$, 1 µM thiamine, 10 mM glucose, 10 mM *p*-Cl-phenylalanine) was used for counter-selection against merodiploids. Whenever necessary medium was supplemented with 0.06 mM adenine. For plasmid maintenance in *E. coli*, the medium was supplemented with 100 µg/mL ampicillin, 15 µg/mL gentamycin or 35 µg/mL kanamycin. For selection of *B. pseudomallei* exconjugants and counter selection against *E. coli* strains, LB agar plates were supplemented with 1000 µg/mL kanamycin and 100 µg/mL polymyxin *B*. For verifying *purM* mutants phenotypically M9 glucose agar plates with and without 0.06 mM adenine were used. For verifying *B. pseudomallei* AmrAB-OprA efflux pump mutants LB agar plates with 250 µg/mL gentamycin were used.

TABLE 3.1 Strains, plasmids and primers used in this study

	Relevant characteristics	Reference/source
Strains		
Escherichia coli		
DH5α	F⁻ φ80d*lacZ*M15 (*lacZ*YA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_K^-, m_K^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ⁻	Laboratory stock
HB101/pRK2013	Helper strain for conjugations	(Figurski et al 1979)
B. pseudomallei		
K96243	Wild-type strain, clinical isolate	(Holden et al. 2004)
1026b	Wild-type strain, clinical isolate	(DeShazer et al. 1997)
2188a-derivative		L. Trunk, Lab stock
708a-derivative		L. Trunk, Lab stock
B. mallei		
ATCC 23344	Type-strain; clinical isolate from 1944	(Nierman et al. 2004)
Plasmids		
pCR2.1	Ap^R Km^R, vector provided with TA cloning Kit	Invitrogen ™
pEX100T	Ap^R; backbone with *oriT* and *Sma*I site in *lacZα*	(Schweizer et al 1995)
pFKm2	Ap^R Km^R; pFRT3 with *nptII*	(Choi et al. 2008)
pBADSceTSZ	Zeo^R; source of P_{BAD}	This study, Chapter 3
pJRC117	Km^R;, expresses $pheS^{A304G}$ *B. thailandensis*	J.Chandler
pBBRMCS-2	Km^R, backbone of pJRC117, empty vector	J. Chandler
pACΩGm	Gen^R; source of *aacC1* gentamycin resistance marker	Lab stock
E4102	Ap^R; source of *amrRAB-oprA*::Kan cassette	T. Mima Lab stock
pPS2336	Ap^R Zeo^R; source of Δ*purM* cassette	K.H. Choi Lab stock
pPS2165	Ap^R Km^R Zeo^R; source of ori_{i600}-*rep*(TS_{Bt}) and *ble*	K.H. Choi Lab stock
pCR2.1-pheS	Ap^R Km^R, pCR2.1 with promoterless *pheS*	This study
PCR2.1-pheS*	Ap^R Km^R, pCR2.1 with $pheS^{A304G}$	This study
pCR2.1-UPpheS	Ap^R Km^R, pCR2.1 with *pheS* (and its promoter)	This study
pEXKan	Km^R; pEX100T backbone with *nptII* from pFKm2	This study
pEXKanpheS	Km^R; pEXKan with *pheS* from pCR2.1-UPpheS	This study
pEXKanpheS*	Km^R; $pheS^{A304G}$ obtained by mutagenesis with QCpheS	This study

pBADpheS*	Ap^R ; P_{BAD} from *p*BADSceTSZ driving *pheS** from pCR2.1-pheS*	This study
pEXpheS*	Ap^R ; pBADpheS* with *oriT* from pEX100T	This study
pEXpheS*Gen	Gen^R; peXpheS* with *aacC1* from pACΩGm	This study
pGEMS12	Ap^R; pGEM-T-easy backbone from E4102 contiaining *S12* promoter from primers S12-UP & S12-DW	This study
pS12pheS*	Ap^R; *S12* promoter from pGEMS12 driving $pheS^{A304G}$ from pCR2.1pheS*S	This study
pS12pheS*Gen	Gen^R; pS12pheS* with *bla* replaced with *aacC1* from pACΩGm	This study
pPS2345	Gen^R; pEXpheS*Gen with Δ(*amrRAB-oprA)*::Kan from E4102	This study
pPS2354	Gen^R; pS12pheS*Gen with Δ(*amrRAB-oprA)*::Kan from E4102	This study
pS12control	Gen^R; pPS2354 with *pheS* mutagenized with pimers set QCpheS & pEXSce	This study
pPS2402	Km^R; pEXKanpheS* with *ΔpurM* from pPS2336	This study
Primers		
pheS-UP	5'-TTT CGC TCG CAG AGC ACA AC	
pheS-DW	5'-TTA CGC GAA CTG CCG CAG G	
5u*p*-pheS-UP	5'-GAT CGT GAA GCA GGT GAA GG	
WTpheS	5-'Phosphorylated/CTA CAT CGG CTT CGC GTT CGG CAG CGG C	
QCpheS*	5'-/Phosphorylated/CTA CAT CGG CTT CGG GTT CGG CAG CGG C	
pEXSce	5'-/Phosphorylated/CTG TTA TCC CTA GAG CTT GGC GTA ATC ATG G	
#536	5'-TCC GCT GCA TAA CCC TGC TTC	
#537	5'-CAG CCT CGC AGA GCA GGA TTC	
#1546	5'-TAC ATG GCG ATA GCT AGA CTG G	
#597	5'-CGA ATT GGG GAT CTT GAA GTT CCT	
#1505	5'-GAT CTT CCA TAC CTG CTC GC	
#1508	5'-GAA TCC TCC GAA ATC CGC TC	

3.2.2 DNA manipulations

Routine procedures were used for DNA manipulations (Sambrook and Russell 2001). *B. pseudomallei* chromosomal DNA fragments (20-30 kb) were isolated using the QIAamp DNA Mini Kit (Qiagen), the DNA was suspended in 200 µL of the supplied buffer AE (10 mM Tris-HCL, 0.5 M EDTA, pH 9). Plasmid DNAs were isolated using Mini-spin Kit (Fermentas). Plasmid DNA fragments were purified from 1% agarose gels using the Sigma Gel Extraction Kit (Sigma) or the DNA Gel Extraction Kit (Fermentas) for more concentrated samples. For colony PCR a single, large colony (usually from a colony patch) or the pellet of an overnight 0.5 mL culture, was transferred to a microcentrifuge tube containing 50 µL dH_2O, and was placed in a heat block at 100°C for 12 min. After a 2 min spin in a microcentrifuge (16000 x g), 5 µL of the supernatant were used for a 50 µL PCR reaction. Routine PCRs for checking constructs or strains were performed using standard *Taq* polymerase (NEB) as recommended by the vendor. PCRs for obtaining fragments for subsequent cloning procedures were done using Platinum *Taq* HiFi polymerase (Invitrogen) following the vendors instructions. Restriction enzymes were purchased from NEB, and reactions were carried out using supplied buffers at the appropriate temperature for 1h in 20 to 30 µL volume reactions. Blunt ending of DNA fragments was performed using T4 DNA polymerase (NEB) right after restriction enzyme denaturation of 20 µL volume reactions (usually 65°C for 20 min), by addition of 1 µL of 10 mM dNTPs and 1 µL of enzyme and incubation at 12°C for 15 min. Ligation reactions were set up using DNA ligase (Invitrogen). Site-directed mutagenesis of plasmids *in vitro* were performed using

the QuickChange multi Kit (Stratagene) with 5' phosphorylated primers listed in **Table 3.1** (see above). Custom oligonucleotides were synthesized by Integrated DNA technologies (Coralville, IA).

3.2.3 BSL3 experiments

All experiments involving live *B. pseudomallei* or *B. mallei* strains were conducted under BSL3 conditions. Chromosomal DNA samples or samples for colony PCR to be taken out of the biocontainment laboratory were inspected routinely to verify that no living bacteria were present, by plating 5 µL of each sample onto an LB plate and verifying that there was no growth after 3 days at the appropriate temperature.

3.2.4 Plasmid construction

The *pheS* gene was amplified from *B. pseudomallei* strain 1026b genomic DNA by using either primers pheS-UP and pheS-DW, which amplify a region including a tentative ribosome binding site (RBS); or primers 5u*p*-pheS-UP and pheS-DW, which include also the 5' intergenic region and the tentative promoter. The 1057 bp and 1199 bp PCR products were used in different ligation reactions with pCR2.1 linearized plasmid (TA cloning Kit, Invitrogen) to obtain pCR2.1-pheS and pCR2.1-UPpheS respectively. pCR2.1-pheS was mutagenized using the Quick Change Kit and primer QCpheS to obtain pCR2.1-pheS*.

To obtain pEXKan, pEX100T was digested with *Dra*I, the fragment containing *oriT* was then used in a ligation with the *nptII*-containing *Sma*I fragment derived from digestion of pFKm2. pEXKanpheS* was built by cleaving pEXKan with *Hinc*II and

inserting a *Pvu*II digested *pheS*-containing fragment derived from pCR2.1-UPpheS. Next, the *pheS* in pEXKanpheS was mutagenized following instructions in the QuickChange Mutagenesis Kit (Stratagene), using 5' phosphorylated primer QCpheS.

Plasmid pBADSceTSZ was digested with *Sna*BI and *Nhe*I and the resulting P_{BAD}-containing fragment was ligated to blunt-ended-*Spe*I/*Xba*I digested $pheS^{A304G}$ from pCR2.1-pheS*, to obtain pBADpheS*. Plasmid pBADpheS* was then digested with *Sma*I and *Bpm*I and, the resulting P_{BAD}-containing fragment, was used in a ligation with an *oriT*-containing fragment resulting from *Psi*I/*Sma*I digestion of pEX100T, to obtain pEXpheS*. Finally, a *Sca*I/*Bpm*I fragment, containing the P_{BAD} from pEXpheS*, was ligated to a *Psi*I/*Hin*cII fragment containing the *aacC1* from pACΩGm, to obtain pEXpheS*Gen.

Plasmid pGEMS12 was constructed by linker tailingusing an *Apa*I/*Nsi*I pGEMT-easy fragment from E4102 as the backbone, and the primers S12-UP and S12-DW annealed to each other as the insert. To obtain the insert, 24 µg of each primer (the excess of DNA ensures each digested plasmid end has a linker bound to it), in a total volume of 50 µL of dH_2O, were boiled together for 1 minute inside a 1.5 µL microfuge tube in a water bath. The tubes were then left in the water bath cooling down slowly overnight. To obtain the plasmid pS12pheS*, pGEMS12 was then digested with *Dra*III (blunt-ended) and *Apa*I, and the resulting fragment containing the *S12* promoter was used in a ligation with a $pheS^{A304G}$ containing fragment obtained by digestion of pCR2.1-pheS* with *Spe*I (blunt-

ended) and *Apa*I. Finally,plasmid pS12pheS*Gen was build from a blunt ended *Sca*I/*Bpm*I fragment carrying the P_{S12} derived from pS12pheS*, and an *aacC1* containing fragment derived from pACΩGm digestion with *Psi*I and *Hinc*II.

The *amrRAB-oprA*::Km cassette for mutating the efflux pump was obtained from Takehiko Mima' s E4102 (unpublished). This plasmid contains the *nptII* gene flanked on one end by a fragment from the 5' end of *amrR* and a fragment from the 3' end of *oprA* on the other end. The cassette (3091 bp) was obtained by digestion with *Eco*RI and blunt ending, to be inserted in the *Sma*I site of pEXpheS*Gen or pS12pheS*Gen, obtaining pPS2345 or pPS2354 respectively. Plasmid pS12control was obtained by mutagenizing the $pheS^{A304G}$ in pPS2354 back to *pheS* using primers WTpheS and pEXSce and the Quick Change Mutagenesis Kit (Stratagene).

purM gene deletion cassette was obtained from pPS2336 (KH Choi unpublished). The plasmid contains the 5' and 3' ends of *purM*, from *B. pseudomallei* 1026b, flanking a zeocin marker. pPS2336 was first digested with *Nru*I to delete the zeocin marker and generate an unmarked deletion of *purM*. The mutant gene was then excised with *Eco*RV and *Spe*I (blunt ended) and it was inserted into the *Sma*I site of pEXKanpheS* and obtain pPS2402.

3.2.5 Conditional growth inhibition experiments.

Replicative plasmids pJRC117 and pBBRMCS-2 were introduced independently into *B. pseudomallei* 1026b and *B. mallei* ATCC 23344 by electroporation. The *Burkholderia* cells were made electrocompetent using a microcentrifuge-based procedure modified

slightly from that previously published for *P. aeruginosa* (Choi et al. 2006). Briefly, one milliliter of an overnight culture grown in LB broth at 37˚C, was centrifuged at room temperature for 30 s at 16000 x g. The pellet was then washed twice with 1 mL of 300 mM sucrose solution and the final pellet was resuspended in a total of 100 µL using the same sucrose solution. For electroporation, ~100 ng of plasmid DNA (1 µL in EB from Qiagen) were mixed with 100 µL of electrocompetent cells and the mixture was transferred to a 2 mm gap width electroporation cuvette. The electroporator apparatus (Bio-Rad Gene Pulser Xcell TM) was set at 25 µF, 200Ω and 2.5 kV, immediately after applying a pulse inside the biosafety cabinet, 900 µL of LB broth were added to the cuvette and the suspension was transferred to a culture tube. The 1 mL culture was shaken at 37˚C for 1 h before 10 µL of the culture plus 90 µL of LB broth were plated onto an LB + 1000 µg/mL kanamycin plate for *B. pseudomallei* and 200 µg/mL kanamycin for *B. mallei.* The plates were incubated overnight at 37˚C until colonies appeared the next day.

For the conditional growth inhibition assays, a single colony from transformants of 1026b or ATCC 23344 with each of the replicative plasmids was grown overnight in 3 mL of LB broth at 37˚C in a shaker. The next morning, 20 µL aliquots were subcultured into 2 mL LB broth. When an optical density of 1 (OD_{600} nm) was reached after approximately 8h, both cultures were standardized so that each one had an approximate equal number of cells in 1 mL of 0.9% (w/vol) NaCl solution. 10-fold serial dilutions were prepared in an 96-well plate out to 10^{11}fold dilution. Using a multi-channel pipette 10 µL from each well were spotted onto a YEG-Cl plate containing 1000 µg/mL kanamycin and either 10 mM *p*-

Cl-phenylalanine or no *p*-Cl-phenylalanine. Plates were incubated at 37˚C overnight. Pictures of the plates were taken after 24 h for *B. pseudomallei* 1026b, and after 48 h for *B. mallei* ATCC23344.

3.2.6 Gene replacement vector delivery

Tri-parental matings were performed to deliver the non-replicative plasmids into *B. pseudomallei* or *B. mallei* strains. To do this, 100 µL of overnight cultures, at 37°C of *E. coli* helper strain HB101/pRK2013 (kanamycin resistant) (Figurski and Helinski 1979) and *E. coli* carrying the respective replacement vector were washed in 1 mL 10mM $MgSO_4$. Pellets were placed in a microcentrifuge tube to be taken inside the BSL3 laboratory. In the same way, 100 µL of the respective *B. pseudomallei* or *B. mallei* strain overnight culture (grown at 37°C in the BSL3) were washed in 1 mL 10 mM $MgSO_4$. The pellets of the three bacterial strains were all combined into a microfuge tube, resuspended in 1 mL 10 mM $MgSO_4$ and spun down for 1 min at maximum speed in a microfuge. The supernatant was discarded and the pellet was placed on top of a nitrocellulose conjugation filter on an LB agar plate. The filter was incubated at 37°C overnight. Next day the filter was taken from the plate and placed against the inside wall of a microfuge tube with 1 mL 0.9% (w/vol) NaCl solution. The cells were resuspended in the saline solution by pipetting up and down with a pipette, and 50 µL aliquots were plated onto an LB plate with 1000 µg/mL kanamycin, 100 µg/mL polymyxin B for selection of *B. pseudomallei* exconjugants, and onto an LB plate with 200 µg/mL kanamycin plus 200 µg/mL carbenicillin for selection of

B. mallei exconjugants. The remainder of the cells were pelleted in a microcentrifuge at maximum speed for 30 s, suspended in 50 µL of 0.9% NaCl solution and the suspension plated onto a second selective plate. Typically after 24 h at 37°C tiny colonies appeared on the plates (approximately 15 to 50 colonies appeared each time) which were left in the incubator until the next day. After 48 h incubation the bigger colonies were approximately 3 mm in diameter (smaller in the case of *B. mallei*), but usually a background of tiny colonies appeared on the plates in the case of *B. pseudomallei* (*B. mallei* plates did not have a background). Some of the bigger colonies were picked for isolation of single colonies and streaked onto a new plate containing the same antibiotics used for selection of exconjugants. Merodiploids were verified by PCR to see sequences present only in the plasmid introduced into them. Primers for *oriT* (#536 & #537), kanamycin (#1546 & #597) and *purM* (#1505 & #1508) were used. The PCR for *oriT* was carried out under the following cycle conditions: one cycle of 95°C for 5 min, 30 cycles of 95°C for45 s, 60°C for 30 s and 72°C for 20 s; and a final extension at 72°C for 10 min. The PCR for *nptII* followed these cycle conditions: one cycle of 95°C for 5 min, 30 cycles of 95°C for45 s, 62°C for 30 s and 72°C for 1 min 10 s; and a final extension at 72°C for 10 min. For *purM* the cycle conditions were the following: one cycle of 95°C for 5 min, 30 cycles of 95°C for45 s, 55°C for 30 s and 72°C for 2 min; and a final extension at 72°C for 10 min.

3.2.7 Counter-selection with *para*-Chloro-phenylalanine

Two purified exconjugant colonies were picked and streaked onto a YEG-Cl plate containing 10 mM *p*-Cl-phenylalanine. The next day bigger colonies were obvious upon a

background of tiny ones. Six of the big colonies were streaked again for purification onto a new plate. The next day, pure colonies from the counter-selection plates were used to screen for the presence of mutants.

3.2.8 Phenotypic s creening of putative mutant isolates

Purified colonies from *p*-Cl-phenylalanine counter-selection plates were used to screen for mutants. In the case of efflux pump mutants, ~40 colonies were picked and replica patched onto an LB plate containing 1000u g/mL kanamycin and onto a plate containing 250 µg/L gentamycin. For the *amrAB-oprA* mutant in the 2188a-derivative an MIC (Minimal Inhibitory concentration) was used to compare the sensitivities to four antibiotics to the known sensitivities in 1026*B*. MIC assays were carried out in 96-well microtiter plates by a standard broth microdilution method (NCCLS, 2006). 5 mL of Muller-Hinton broth (MHB, Beckton Dickinson) were inoculated with 50 µL of an overnight culture, and the mixture was incubated for approximately 5 h at 37°C in a shaker, until the OD 600 reached ~0.7. After adjustment of the culture with MHB to a 2 McFarland nephelometer standard, the culture was further diluted 50-fold before inoculation of 20 µL into 180 µL of MH*B*. Bacterial growth was determined 18 h after incubation at 37°C. MICs of carbenicillin, gentamycin, erythromycin and streptomycin were determined.

For genetic confirmation, PCR was performed, using primers that amplify the *oriT* (#536 and #537) in the vector backbone and the kanamycin marker (#1546 & #597) in the replacement cassette.

In the case of *purM* mutants, approximately 40 single colonies were patched onto an LB plate containing kanamycin (1000 μg/mL for *B. pseudomallei* and 200 μg/mL for *B. mallei*), onto an M9 plate and onto an M9 plus adenine and thiamine plate. For confirmation at the genetic level, colony PCRs were done using primer sets #1505 & #1508 and #536 & #537.

3.3 Results

3.3.1 Non-replicative delivery vectors for mutagenesis

Three different replacement vectors were designed in this study. They all contain the $pheS^{A304G}$, s missense mutation in *B. pseudomallei pheS*, which corresponds to the reported *E. coli* $pheS^{A294G}$ allele. All of the vectors also contain the ColE1 replicon and do not replicate outside of the *Enterobacteriaceae*; they are, therefore, effective non-replicative plasmids in *Burkholderia* species. The *oriT* for transfer by conjugation is also present in each of the vectors, so that they can be delivered into *B. pseudomallei* or *B. mallei* by tri-parental matings, as electroporation is not efficient enough to obtain plasmid integration (a few trials yielded no transformants). Only gentamycin or kanamycin resistance determinants approved for work in Select Agents were included as selection markers. A unique *Sma*I site embedded within the *lacZα* gene allows for blue/white screening of recombinants. Plasmid pEXKanpheS* contains $pheS^{A304G}$ driven by its own promoter and the *nptII* gene encoding kanamycin resistance, in the backbone. In plasmid

pEXpheS*Gen, the P_{BAD} promoter drives the expression of *pheS*^{A304G} and the *aacC1* gene, encoding gentamycin resistance, is present in the backbone. Finally, plasmid pS12pheS* has *pheS*^{A304G} driven by the *S12* promoter, and the plasmid also contains the *aacC1* gene in the backbone. See **Figure 3.2**. Diverse versions of these vectors containing different replacement mutation cassettes were created to perform proof of concept experiments where a corresponding mutant was isolated after counter-selection with *p*-Cl-phenylalanine. The three main types of vectors were tested in *B. pseudomallei* 1026b and allow for selection of mutants without significant differences amongst them. Only pEXKanpheS* was tried in *B. mallei* and allowed for resolution of merodiploids after counter-selection.

3.3.2 Conditional growth inhibition mediated by *pheS*^{A304G}

The replicative pJRC117 and pBBRMCS-2 plasmids provided by Josephine Chandler (University of Washington) were each introduced into *B. pseudomallei* 1026b and *B. mallei* ATCC 23344. Transformants were recovered in all cases with an efficiency higher than 107 per microgram of plasmid DNA. Transformants were used in an assay to determine if *pheS*^{A304G} would inhibit growth of the strains harboring the plasmid, when plated on media containing *p*-Cl-phenylalanine. The results showed that *pheS*^{A304G} strongly inhibited the growth of both strains in the presence of *p*-Cl-phenylalanine, but cells containing no plasmid or empty vector grew normally. See **Figure 3.3**.

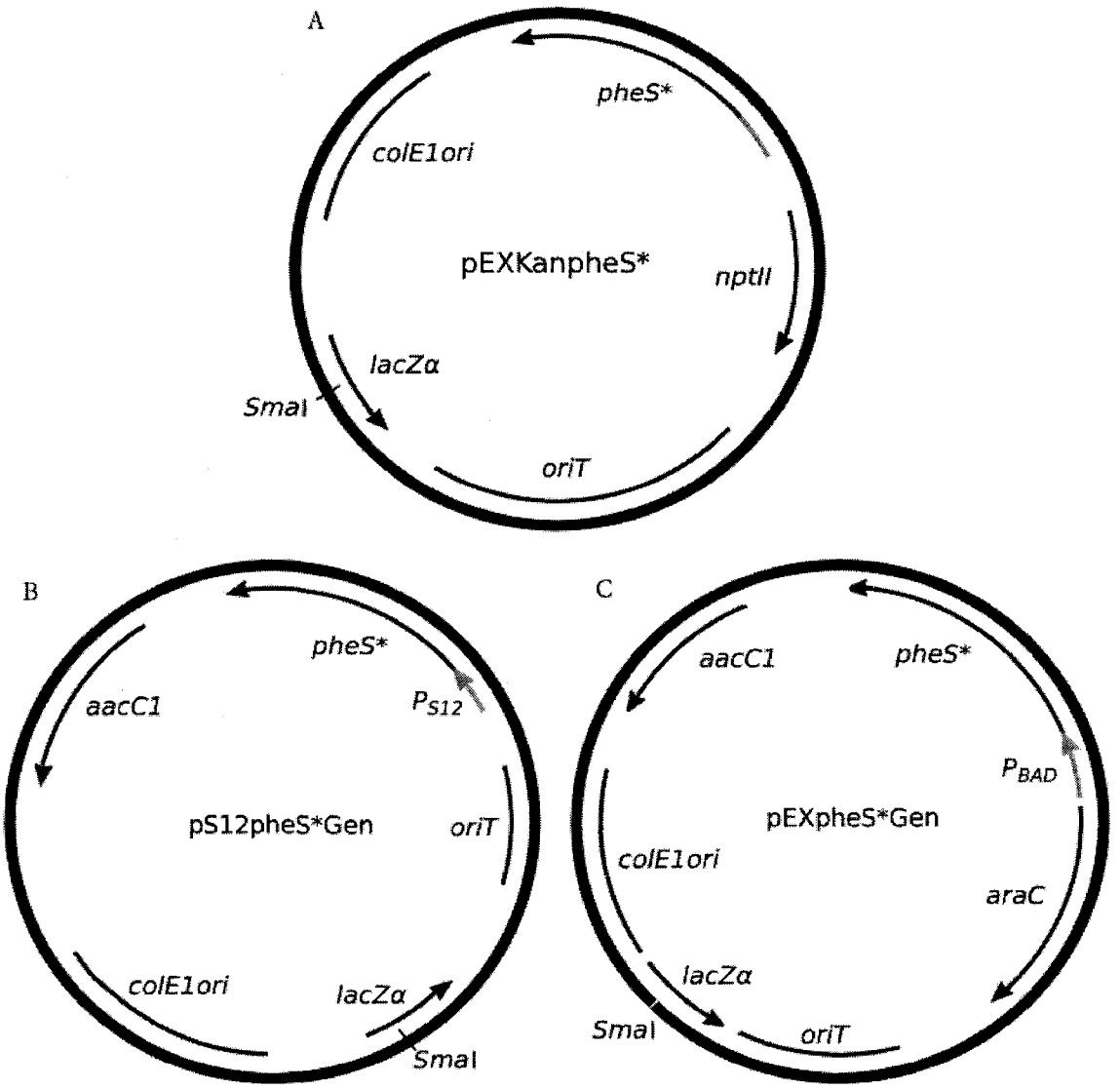


Figure 3.2 Plasmid maps of suicide vectors

Suicide plasmids used for targeted mutagenesis (not drawn to scale).
Features in all vectors include a ColE1 replicon , an origin of transfer (*oriT*), the $pheS^{A304G}$ (*pheS**), and a *Sma*I site within the *lacZα* gene for blue/white screening in *E. coli*, of inserts containing the replacement cassettes to be delivered (thin line dissecting the plasmid circle), also all plasmids contain I-*Sce*I sites flanking the *Sma*I site.

A. Plasmid pEXKanpheS* has its own promoter driving expression of $pheS^{A304G}$ and the *nptII* kanamycin resistance marker.

B. Plasmid pS12pheS*Gen has the P_{S12} promoter driving $pheS^{A304G}$ and the gentamycin marker *aacC1*.

C. Plasmid pEXpheS*Gen has the P_{BAD} promoter driving $pheS^{A304G}$ and the *aacC1*.

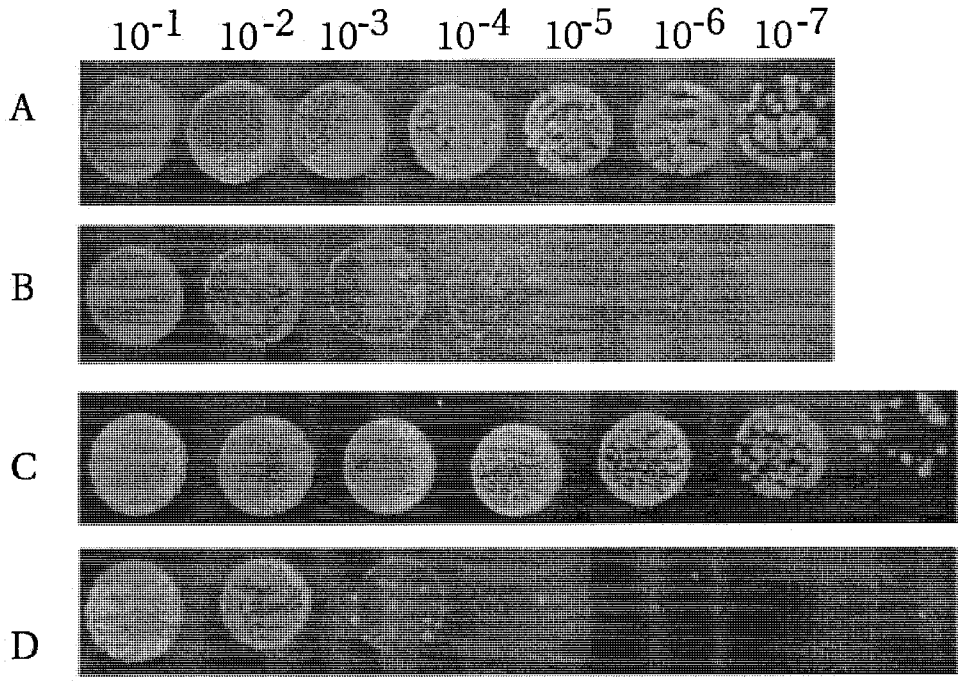


Figure 3.3 Conditional inhibition of *Burkholderia* strains

Pictures showing conditional inhibition of *Burkholderia* strains carrying a replicative plasmid that either expresses $pheS^{A304G}$ (pJRC117) or not (pBBRMCS2) when plated on media containing *p*-Cloro-phenylalanine.

A. Serial dilutions of *B.pseudomallei* 1026b/pBBRMCS2 on YEG-Cl plus *p*-Cl-phenylalanine media.
B. Serial dilutions of *B.pseudomallei* 1026b/pJRC117 on YEG-Cl plus *p*-Cl-phenylalanine media.
C. Serial dilutions of *B. mallei* ATCC 23344/pBBRMCS2 on YEG-Cl plus *p*-Cl-phenylalanine media.
D. Serial dilutions of *B. mallei* ATCC 23344/pJRC117 on YEG-Cl plus *p*-Cl-phenylalanine media.

3.3.3 Isolation of a marked AmrAB-OprA mutant in *B. pseudomallei* 1026b

Initial experiments used the pPS2345, pPS2354 and pS12control (control containing wild-type *pheS* gene) non-replicative vectors in 1026b to demonstrate that $pheS^{A304G}$ functions as a CS marker in *B. pseudomallei.* To provide evidence that the isolation of a second crossover event is due to the presence of $pheS^{A304G}$, and does not occur by chance at noticeable rates, care was taken to first isolate merodiploids. After counter-selection on YEG-Cl with *p*-Cl-phenylalanine, marked mutants were obtained only when pPS2345 and pPS2354 were used. Plasmid pS12control (carrying the wild-type *pheS*) did not inhibit the growth of merodiploids, when grown in the presence of *p*-Cl-phenylalanine, and all the single colonies isolated from the plate were still gentamycin resistant. See **Figure 3.4.** Merodiploids that have integrated the delivery plasmid allow for amplification of both *oriT* and *nptII* sequences. On the other hand, mutants that have replaced the efflux pump operon with the mutant cassette, by a second crossover, only allow for amplification of the *nptII* sequence. These mutants became gentamycin susceptible.

3.3.4 Isolation of marked mutants in different strains of *B. pseudomallei*

To ensure the $pheS^{A304G}$ counter-selectable marker works equally well in different strains, the pPS2354 vector was used to generate AmrAB-OprA mutants in strains K96243, a 2188a-derivative and a 708a-derivative. Marked mutants were obtained in all cases after counter-selection on M9 with *p*-Cl-phenylalanine or YEG-Cl with *p*-Cl-phenylalanine. See **Figure 3.5.** All mutants became gentamycin susceptible, confirming inactivation of the

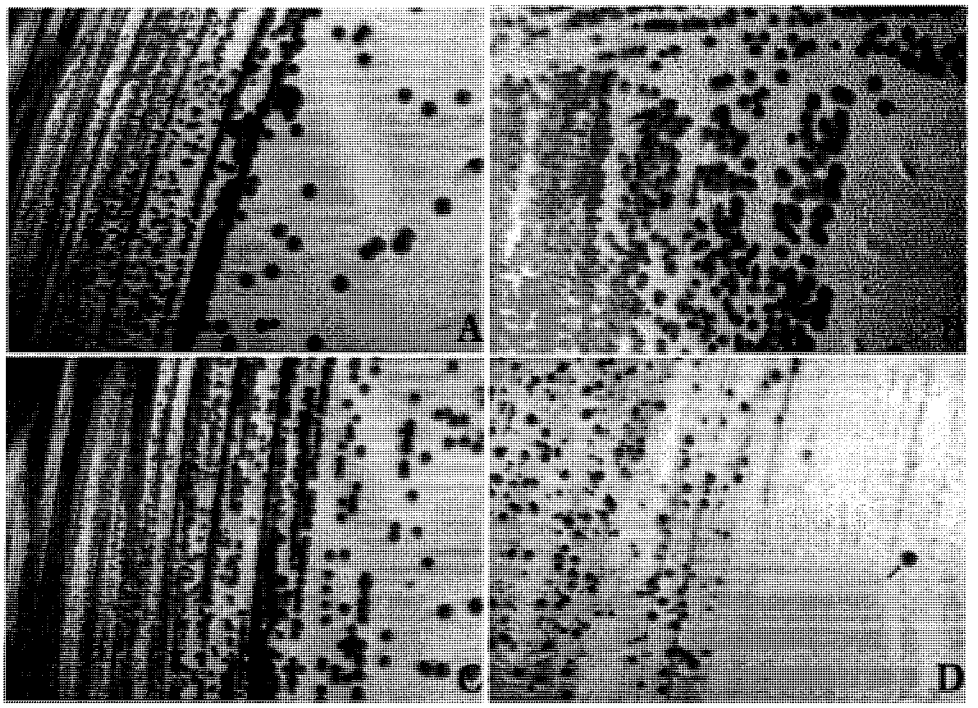


Figure 3.4 Streakings of merodiploids of *B. pseudomallei*

Pictures of plates containing streakings of two different merodiploids of *B. pseudomallei* 1026b on media with or without *p*-Cl-phenylalanine.

The rightmost area of each picture contains well isolated colonies, however, only in D, it is possible to see a big colony close to many tiny ones (arrow). The cells in this large colony have undergone a second crossover event, losing integrated plasmid backbone and the $pheS^{A304G}$. Tiny colonies are still merodiploids expressing $pheS^{304AG}$

A.1026bΔ *amrRAB-oprA*::Kan-*pheS* grown on YEG-Cl.

B. 1026bΔ *amrRAB-oprA*::Kan-$pheS^{A304G}$ grown on YEG-Cl.

C. 1026bΔ*amrRAB-oprA*::Kan-*pheS* grown on YEG-Cl plus *p*-Cl-phe.

D. 1026bΔ *amrRAB-oprA*::Kan-$pheS^{A304G}$ grown on YEG-Cl plus *p*-Cl-phe.

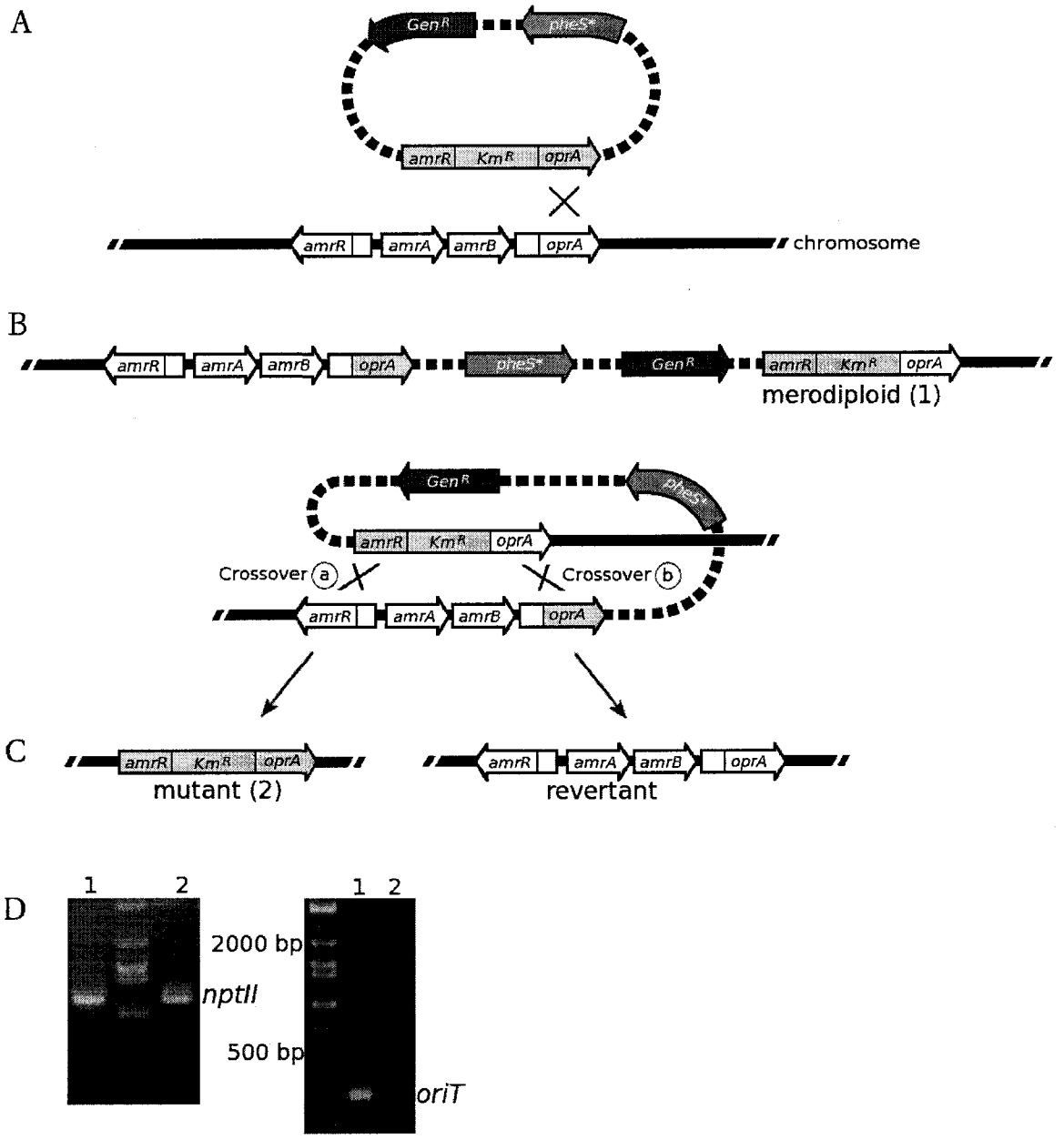


Figure 3.5. Isolation of marked efflux pump mutants in *B. pseudomallei*

A. Plasmid pPS2354 was ligated to an insert containing a kanamycin marker flanked by regions homologous to the 3' end of *amrR* and the 3' end of *oprA*. **B.** After introduction into recipient cells, selection for kanamycin resulted in the isolation of merodiploids. **C.** Counter-selection in the presence of *p*-Cl-phenylalanine resulted in either efflux pump mutants or revertants towild-type. **D.** Gel pictures showing *nptII* (kanamycin resistance) and *oriT* amplicons in a *B. pseudomallei* 2188a merodiploid(1) and a mutant (2).

AmrAB-OprA efflux pump. MIC determinations showed that the mutant in the 2188a-derivative had the expected antibiotic resistance profile. See **Table 3.2**. It became susceptible to gentamycin, streptomycin and erythromycin, while it remained resistant to carbenicillin.

3.3.5 Isolation of unmarked *B. pseudomallei* K96243 *purM* mutant

To provide evidence that the $pheS^{A304G}$ based counter-selection system can be used to generate unmarked, non-polar mutations in the *B. pseudomallei* chromosome, plasmid pEXKanpurM was used to isolate merodiploids after tri-parental mating. Merodiploids were streaked onto YEG-Cl *p*-Cl-phenylalanine plates and big colonies were purified. Of these approximately 20 out of 40 tested lost the kanamycin resistance marker, three of these colonies were adenine auxotrophs. See **Figure 3.6.**

3.3.6 Utilization of $pheS^{A304G}$ for merodiploid resolution in *B. mallei*

The same peXKanpurM non-replicative vector used in *B. pseudomallei* K96243 was used to generate merodiploids in *B. mallei* ATCC 23344. After tri-parental matings exconjugants were streaked onto a YEG-Cl plate containing *p*-Cl-phenylalanine. Approximately one third of the surviving cells screened for loss of the kanamycin marker by plating onto LB kanamycin 200 μg/mL had lost the marker. PCR for the kanamycin resistance marker confirmed these findings. All of the isolates that lost the marker grew on M9 without adenine, which means they were all revertants to wild-type. See **Figure 3.7.**

Strain	MIC (ug/mL)			
	carbenicillin	gentamycin	streptomycin	erythromycin
1026b	256	256	>1024	256
2188a merodiploid	256	>1024	>1024	128
2188a-mutant	512	4	16	16

Table 3.2 Antibiotic susceptibilities of *B. pseudomallei* wild-type and mutant isolates

MICs of two aminoglycosides (gentamycin and streptomycin) and one macrolide (erythromycin)for strain 2188a-derivative merodiploid and efflux pump mutant (strain 2188a-derivative was generated from clinical isolate 2188a by serial passage on increasing concentrations of gentamycin). 1026b is a wild-type laboratory strain. Carbenicillin was included as a control, to show the efflux pump mutant does not affect the MIC of antibiotics which are not substrates for the AmrAB-OprA efflux pump.

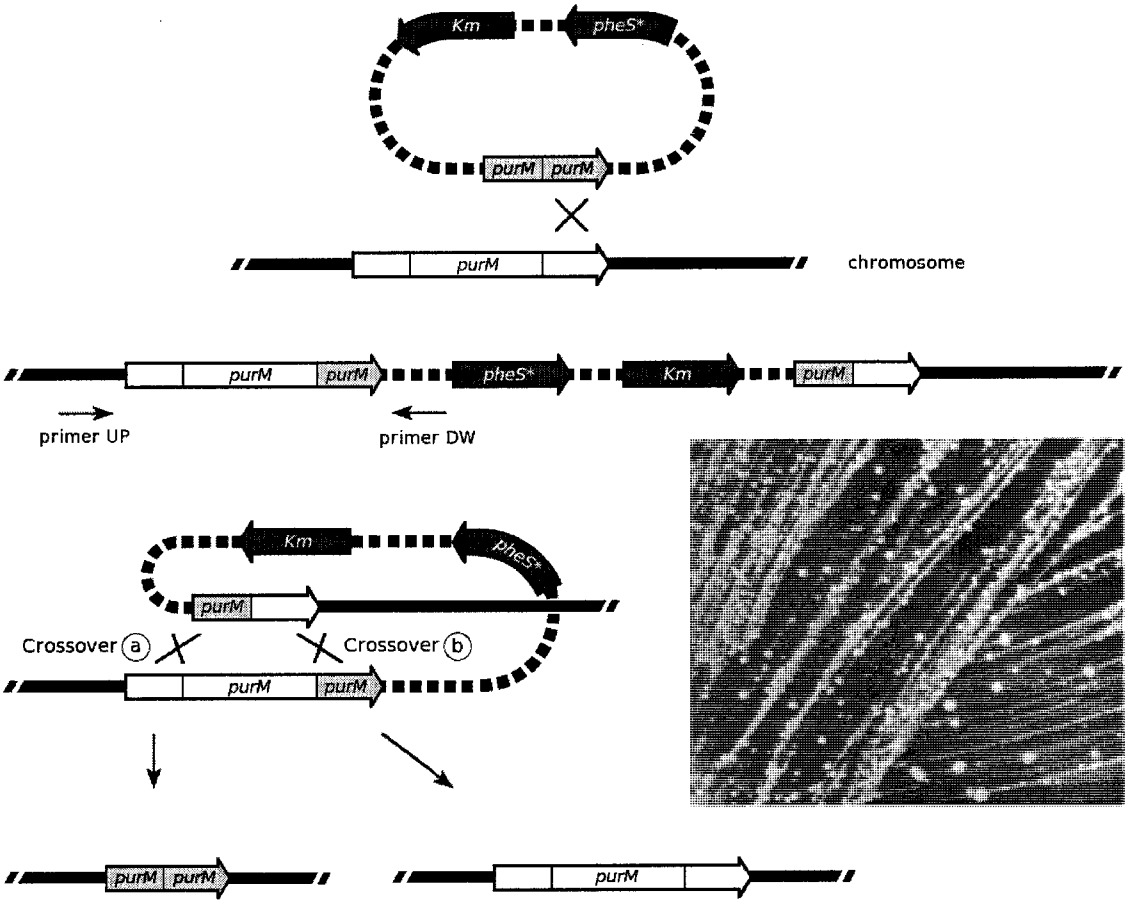


Figure 3.6 Schematic of strategy to obtain *purM* mutants

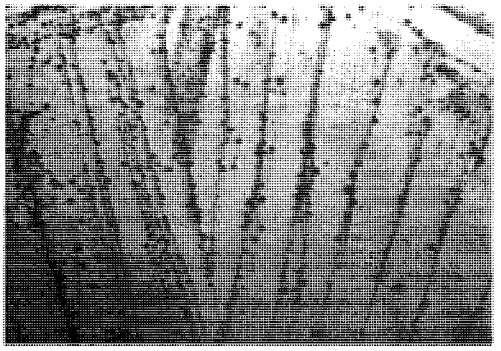
A. pEXKanpheS* was ligated to an insert containing segments of the 5' and 3' ends of *purM* from strain 1026*B*.

B. Merodiploids were obtained after selection of exconjugants on kanamycin plates.

C. After counterselection on *p*-Cl-phenylalanine, big colonies were either mutants or revertants.

D. Picture showing the streaking of merodiploids on media containing *p*-Cl-phenylalanine to show colonies that undergo a second crossover and a background of tiny merodiploid colonies for which the phenylalanine analog is toxic.

A



B

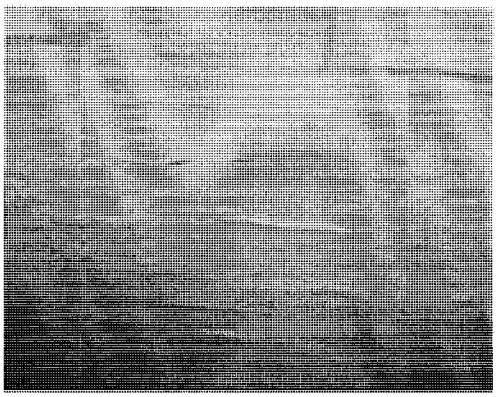


Figure 3.7 Use of the *pheS*^{A304G} marker in *B. mallei*

A. *purM* merodiploids of B *mallei* ATCC23344 streaked onto YEG-Cl *p*-Cl-phenylalanine plate. As in the case of *B. pseudomallei* it is possible to distinguish colonies from cells that have resolved the merodiploid (larger colonies) from those that are still merodiploids (tiny colonies).

B. Single colonies from the counter-selection plate were struck onto a plate containing 200 ug/mL kanamycin. *B. mallei* cells that have resolved the merodiploid have lost the *nptII* and, therefore, do not grow on this plate.

3.4 Discussion

The usual method employed to create targeted mutagenesis relies on the host recombination machinery and a non-replicative delivery vector that carries the mutation destined for exchange with the wild-type sequence. In some strains of *B. pseudomallei* it is possible to use linear DNA fragments to introduce the desired mutation, but in many other cases a circular plasmid needs to be used. The circular plasmid is designed so that the replacement region is flanked by two fragments homologous to the cognate chromosomal target sequences, and selection and counter-selectable markers are included in the plasmid backbone.

This chapter describes the implementation and use of a *pheS*-based system for markerless gene replacements in *B. pseudomallei*. It was shown that expression of a $pheS^{A304G}$ (PheS*) in *B. pseudomallei* and *B. mallei* from either single or multiple copy constructs led to cell death in the presence of *p*-Cl-phenylalanine. The utility of PheS* based counter-selection was demonstrated by employing the newly engineered gene replacement vectors for construction of marked *amrAB-oprA* and unmarked *purM* mutants in various *B. pseudomallei* strains, as demonstrated with strains K96243, a 708a-derivative and a 2188a-derivative which are all refractory to linear PCR fragment mutagenesis. Strains 708a and 2188a are rare gentamycin susceptible isolates from Thailand (Simpson et al. 1999) and the Schweizer Lab is currently determining the molecular mechanism(s) underlying this susceptibility. The newly developed gene replacement system will greatly facilitate these efforts.

The isolation of unmarked *purM* mutants in *B. pseudomallei* attests to the fact that the PheS* system can be utilized to readily isolate unmarked mutants. Unmarked mutations exhibit fewer polar effects on downstream genes. PheS* based counter-selection will also allow transfer of point mutations to the chromosome. Markerless gene replacement is a big advantage in view of the strict regulations governing use of antibiotic selection markers in Select Agent bacteria.

Recombination at the chromosomal *pheS* copy is possible in theory, since the the *pheS* and the $pheS^{A304G}$ are identical with the exception of a single nucleotide. However, in the course of these studies this homology did not cause problems. It is possible that this integration at the chromosomal *pheS* locus does occur frequently because it causes deleterious polar effects to the cell, but this was not studied in any detail. Nevertheless, this possible drawback has been eliminated by other investigators who engineered a synthetic $pheS^{A304G}$ gene that contains a very different nucleotide sequence from that of the wild-type gene (Barrett et al. 2008). However, the synthetic gene was only tested in *B. thailandensis* and other *Burkholderia* species, but not yet in *B. pseudomallei.*

CHAPTER FOUR

A homing endonuclease based system for the construction of targeted mutations in *B. pseudomallei*

4.1 Introduction

This chapter describes a different approach for targeted mutagenesis that does not rely on a counter-selection marker. In this approach, the gene encoding I-*Sce*I, a homing endonuclease, and its target sequence are used in lieu of a counter-selectable marker.

The homing endonucleases are enzymes present in introns or inteins of eukaryotes, that are able to create double stranded breaks in DNA molecules *in vivo*, whenever they encounter a recognition sequence. The recognition sequences are usually fairly long and asymmetric and are only rarely found in genomic sequences.

I-*Sce*I is a homing endonuclease present in the mitochondria of *Saccharomyces cerevisiae*. The enzyme is encoded in an intron of the large rRNA gene and its recognition sequence is present at the intron homing site (Beylot and Spassky 2001). The recognition site is asymmetric and 18 bp long and, if present randomly, would be found only once

every 70 billion base pairs (it should be noted that the human genome is roughly 3.2 billion base pairs).

Experiments conducted in several different organisms show that the enzyme is able to generate double stranded breaks *in vivo* in the genome if recognition sites are present. One of the proposed mechanisms of homologous recombination initiation is the creation of a double stranded break in a molecule of DNA (the other mechanism of initiation is the presence of a gap in one of the DNA strands). Double stranded breaks in cells appear constantly because of free radical and ionizing radiation damage, and it is necessary for the cell to repair these breaks to be able to survive. Researchers have found that I-*Sce*I generated double stranded breaks are repaired by homologous recombination of nearby sequences flanking each side of the break (Jasin 1996). This concept has been used to generate systems where a recognition site is integrated into a chromosome, between homologous sequences. Upon subsequent expression of I-*Sce*I, only those cells survive which have successfully repaired the break by homologous recombination (Posfai et al. 1999; Wong and Mekalanos 2000), a result which is the same as that obtained after counter-selection with any of the known traditional CS markers.

The genome of *B. pseudomallei* K96243 was searched for I-*Sce*I recognition sites and no sites were found. The hypothesis , therefore, was that expression of the endonuclease I-*Sce*I from a replicative vector, in a merodiploid containing chromosomally integrated recognition sites, would allow for recovery of isolates that had resolved the merodiploid.

The experimental system involves an I-*Sce*I expression vector with a temperature sensitive replicon for easy curing of the plasmid, and a non-replicative gene replacement

vector containing I-*Sce*I recognition sites. To assess the functionality of the system in *B. pseudomallei*, *purM* mutants similar to those obtained by $pheS^{A304G}$ counter-selection were created in *B. pseudomallei* strains K96243 and 1026*B*.

Also, to show that the system can be used to introduce single nucleotide changes into the chromosome, the creation of a temperature-sensitive *fabD* mutant was attempted. The gene *fabD* encodes an essential fatty acid biosynthesis enzyme, malonyl-CoA:ACP acyltransferase. This enzyme catalyzes the transfer of the malonyl group of malonyl-CoA to the sulfhydryl group of the acyl carrier protein (ACP) (See **Figure 4.1**). Malonyl-ACP is the key building block of de novo fatty acid biosynthesis in bacteria studied to date. Fatty acids are essential for cellular function as they provide precursors for synthesis of membranes, lipopolysaccharides, virulence factors and quorum sensing molecules. The enzymes of bacterial fatty acid biosynthesis, provide good targets for antibacterial drug design because the separation of the machinery in different proteins (type II fatty acid biosynthesis) as opposed to the large multifunctional proteins characteristic of the eukaryotes (type I fatty acid biosynthesis) provides distinct targets that differ from the eukaryotic counterparts. Temperature-sensitive *fabD* mutations of *E. coli* specifically block fatty acid synthesis at nonpermissive temperatures (Verwoert et al. 1994). Since FabD enzymes are fairly conserved it seemed reasonable to attempt isolation of a *fabD*(TS) mutant by changing the key amino acid tryptophan at position 255 for a glutamine, in a way analogous to the W254Q that confers temperature sensitivity to the *P. aeruginosa* *fabD*, reported in the literature (Kutchma et al. 1999). Isolation of a temperature sensitive

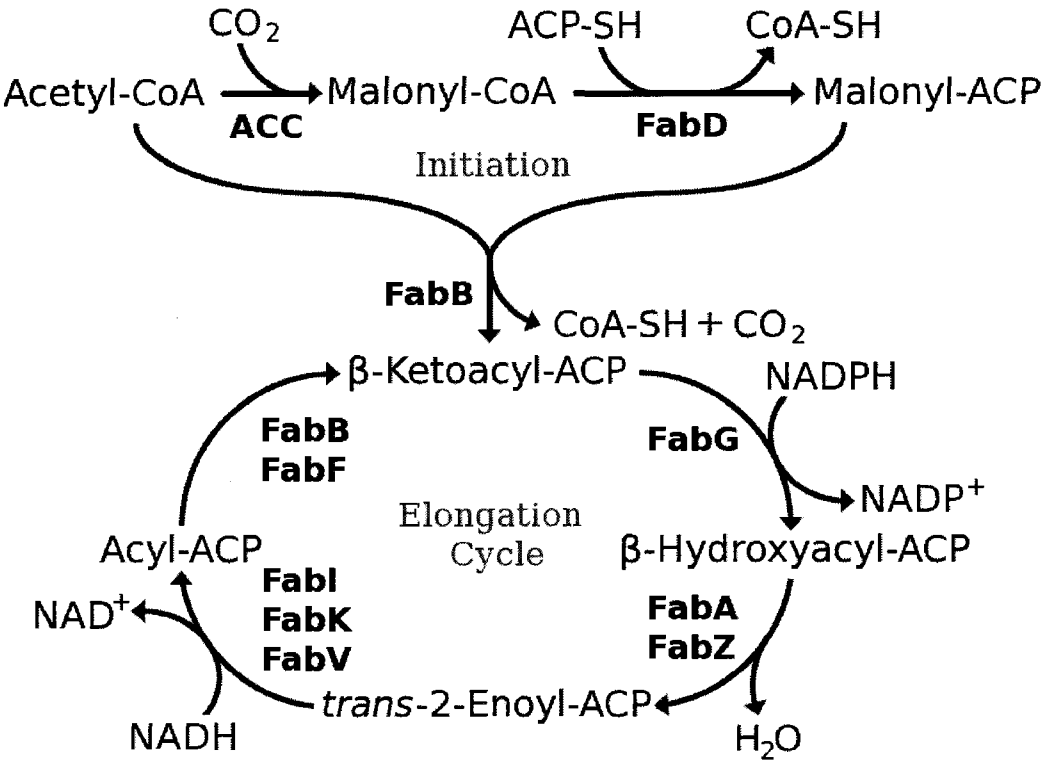


Figure 4.1 The bacterial fatty acid biosynthetic pathway

Acetyl-CoA is carboxylated by the acetyl-CoA carboxylase complex (ACC) to form malonyl-CoA, which is then transferred to holoacyl-carrier protein (ACP-SH) by malonyl-CoA:ACP transacylase (FabD). Fatty acid synthesis is then performed in a repeated cycle of condensation (FabB or FabF), reduction (FabG), dehydration (FabA or FabZ) and reduction (FabI, FabK or FabV). Other abbreviations:CoA-SH, reduced coenzyme A; ACP, acyl carrier protein.

The fatty acid biosynthesis pathway provides essential substrates for cellular reactions requiring acyl-ACP donors, like those leading to byosyntheis of phospholipids for membrane biogenesis, homoserine lactone for quorum sensing molecules, lipopoly-saccharide and others (e.g., protein modifications, etc.).

FabD in *B. pseudomallei* would provide evidence that the protein is essential for growth and it would make an effective target for drug design.

4.2 Materials and methods

4.2.1 Bacterial strains and growth conditions

The strains and plasmids used in these experiments are listed in **Table 4.1**. All bacteria were routinely grown at 30°C or 37°C in Luria-Bertani (LB)medium. M9 minimal glucose medium was prepared as described in Chapter 3. Antibiotics for *E. coli* were used at the following concentrations: ampicillin 100 μg/mL, kanamycin 35 μg/mL, gentamycin 15 μg/mL and zeocin 25 μg/mL. For *B. pseudomallei*, antibiotics were added to the media at the following concentrations: kanamycin 1000 μg/mL and zeocin 2000 μg/mL. Zeocin plates were kept in the dark, at 4°C and used within 3 weeks of preparation. For induction of I-*Sce*I expression, L-arabinose was added to the media at a concentration of 0.5% (w/vol). Polymyxin B (100 μg/mL) was used to counter select against *E. coli* in tri-parental matings.

4.2.2 DNA manipulations

Routine procedures were used for DNA manipulations (Sambrook and Russell 2001). *B. pseudomallei* chromosomal DNA fragments (20-30 kb) were isolated using the QIAamp DNA Mini Kit (Qiagen) and a modified protocol that makes use of lysozyme. The DNA was suspended in 200 μL of the supplied buffer AE (10 mM Tris-HCL, 0.5 M EDTA, pH 9). Plasmid DNAs were isolated using Mini-spin Kit (Fermentas). Plasmid DNA fragments

TABLE 4.1 Strains, plasmids and primers used in this study

	Relevant characteristics	Reference/source
Strains		
Escherichia coli		
DH5α	F⁻ φ80d*lac*ZM15 (*lacZ*YA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ⁻	Laboratory stock
HB101/pRK2013	Helper strain for conjugations	(Figurski et al 1979)
B. pseudomallei		
K96243	Wild-type strain, clinical isolate	(Holden et al. 2004)
1026b	Wild-type strain, clinical isolate	(DeShazer et al. 1997)
B. mallei		
ATCC 23344	Type-strain; clinical isolate from 1944	(Nierman et al. 2004)
Plasmids		
pBAD-*I-sceI*	Ap^R source of I-*Sce*I and P_{BAD}	(Tischer et al. 2006)
pCR2.1	Ap^R Km^R, vector provided with TA cloning Kit	Invitrogen ™
pPS2165	Ap^R Km^R Zeo^R; source of ori_{1600}-*rep*(TS_{Bt}) and *ble*	K.H. Choi Lab stock
pBADSceTSZ	Ap^R,pBAD-I-*sce*I carrying ori_{1600}-*rep*(TS_{Bt}) and *ble* from pPS2165	This study
pBADSce	Zeo^R; pBAD-I-*sce*I with *bla* gene deletion	This study
pEXKan	Km^R; pEX100T backbone with *nptII* from pFKM2	This study, Chapter 3
pCR2.1-fabD	Ap^R Km^R, pCR2.1 *B. pseudomallei fabD* fragment	This study
pCR2.1-fabD(TS)	*Ap^R Km^R*, pCR2.1-fabD mutagenized with QCfabD	This study
pEXKanfabd(TS)	Km^R; *fab*D(TS) from pCR2.1-fabD(TS) into pEXKan	This study
pPS2336	Ap^R Zeo^R; source of Δ*purM* cassette	K.H. Choi Lab stock
pEXKanpurM	Km^R; Δ*purM* cassette from pPS2336 in pEXKan	This study
Primers		
fabD-UP	5'-GCT CGA ATC GAT TCA ATT GGG	
fabD-DW	5'-TCG GTT GAA TCG GCA AGC TCG	
TSfabD	5'/Phosphorylated/CCC CGT CGC CCA GGT CGA GTG CGT GCA GCA CAT C	
#536	5'-TCC GCT GCA TAA CCC TGC TTC	

#537	5'-CAG CCT CGC AGA GCA GGA TTC
#1546	5'-TAC ATG GCG ATA GCT AGA CTG G
#597	5'-CGA ATT GGG GAT CTT GAA GTT CCT
#939	5'-CGA CGC GTA ACT CAC GTT AA
#940	5'-ATC TCT GAT GTT ACA TTG CAC A

were purified from 1% agarose gels using the Sigma Gel Extraction Kit (Sigma) or the DNA Gel Extraction Kit (Fermentas) for more concentrated samples. For colony PCR a single, large colony (usually from a colony patch) or the pellet of an overnight 500 µL culture, was transferred to a microcentrifuge tube containing 50 µL dH_2O, and the cell suspension was placed in a heat block at 100°C for 12 min. After a 2 min spin in microcentrifuge (16000 x g), 5 µL aliquots of the supernatant were used for a 50 µL PCR reaction. Routine PCRs for checking constructs or strains were performed using standard *Taq* polymerase (NEB) as recommended by the vendor. PCRs for obtaining fragments for subsequent cloning procedures were done using Platinum *Taq* HiFi polymerase (Invitrogen) following the vendor's instructions. Restriction enzymes were purchased from NEB, and reactions were carried out using supplied buffers at the appropriate temperature for 1h in 20 to 30 µL volume reactions. Blunt ending of DNA fragments was performed using T4 DNA polymerase (NEB) immediately after restriction enzyme denaturation of 20 µL volume reactions (usually 65°C for 20 min), by addition of 1 µL of 10 mM dNTPs and 1 µL of enzyme and incubation at 12°C for 15 min. Ligation reactions were set up using T4 DNA ligase (Invitrogen) at room temperature for 1 h for sticky ends and overnight at 14°C for blunt ends. Site-directed mutagenesis of plasmids *in vitro* were performed using the QuickChange multi Kit (Stratagene).

4.2.3 Biosafety Level 3 experiments

All experiments involving live *B. pseudomallei* strains were conducted under BSL3 conditions. Chromosomal DNA samples or samples for colony PCR to be taken out of the

bio-containment laboratory were inspected routinely to verify that no living bacteria were present, by plating 5 μL of each sample onto an LB plate and verifying that there was no growth after 3 days at the appropriate temperature.

4.2.4 Plasmid constructions

To generate pBADSceTSZ, plasmid pBAD-I-sceI was restricted with *Afe*I and *Bpm*I, and the insert containing the ori$_{1600}$-rep(TS$_{Bt}$), was isolated from pPS2165 by digesting it with *Bpm*I and *Fsp*I.

Plasmid pBADSce was obtained by deleting the *bla* gene present in plasmid pBADSceTSZ by restriction with *Bpm*I (blunt ended) and *Sca*I and self-ligation of the plasmid backbone.

Plasmid pEXKan was generated as described in chapter 3.

The *fabD* gene was amplified from genomic DNA of *B. pseudomallei* 1026b by using primers fabD-UP and fabD-DW which amplify a fragment spanning the 5' upstream intergenic region of the gene and its 3' end. The PCR was run under the following cycle conditions: one step of 95°C for 5 min; 30 cycles of 95°C for 30 s, 55°C for 30 s and 68°C for 1 min; and a final extension step at 68°C for 10 min. The expected 994 bp amplified fragment was excised from a 1% agarose gel, purified and used as the insert in a ligation with the TA cloning vector pCR2.1 (Invitrogen) to obtain pCR2.1-fabD. Plasmid pCR2.1-fabD was then mutated *in vitro* using the QuickChange Mutagenesis Kit (Stratagene), and a 5'phosphorylated primer, QCfabD, to obtain *fabD*^{W255Q} in pCR2.1-fabD(TS).

pCR2.1-fabD(TS) was digested with *Eco*RV, to release an insert containing most of the gene (including the change introduced by the QCfabD primer). This insert was ligated into the *Sma*I site of pEXKan, to generate pEXKanfabD(TS).

To obtain plasmid pEXKanpurM, pPS2336 was first digested with *Nru*I to delete the zeocin resistance marker and generate an unmarked deletion of *purM*. The mutant Δ*purM* gene was then released by digestion with *Eco*RV and *Spe*I (blunt ended) before ligating it into the *Sma*I site of pEXKan.

4.2.5 Gene replacement vector delivery

Non-repliocative plasmids carrying the appropriate mutated allele were introduced into *B. pseudomallei* by tri-parental mating. Plasmid pEXKanfabD(TS) was used to generate *fabD*(TS) merodiploids, and plasmid pEXKanpurM to generate *purM* merodiploids. For tri-parental matings, 100 µL of overnight cultures of *E. coli* helper strain HB101/pRK2013 (kanamycin resistant) (Figurski and Helinski 1979) and *E. coli* carrying the respective replacement vector were washed in 1 mL 10 mM MgSO4. In the BSL3 lab, the same was done for the respective *B. pseudomallei* recipient strains. The three strain pellets were then pooled into a microfuge tube and the combined pellets were placed on top of a conjugation filter on an LB plate. The filter was incubated at 37°C until the next day. The filter was then taken from the plate and immersed into 1 mL 0.9% NaCl solution in a microfuge tube. After resuspending the cells using a pipette, 50 µL were spread onto an LB plate with 1000 µg/mL kanamycin and 100 µg/mL polymyxin B for counter-selection against *E. coli* and selection of merodiploids. The remaining cells were centrifuged, the

supernatant was discarded and the rest of the cells suspended in 50 µL NaCl before plating onto the same selection plates. The plates were incubated at 37°C until some colonies appeared. After approximately 48 h the bigger colonies on the plate (~3mm in diameter) were picked and transferred to a new plate to obtain pure single colonies. These colonies were then screened by PCR to make sure they contained the integrated plasmid using primer sets (#536 & #537 and #1546 & #597) detailed in **Table 4.1** (see above). The PCR cycle reaction conditions were those described in Chapter Three.

4.2.6 Introduction of the I-*Sce*I expression plasmid into the merodiploids

The I-*Sce*I expression plasmid, pBADSce was introduced into the cells by electroporation. Electrocompetent cells were made by a slightly modified method to that previously published (Choi et al, 2006). Briefy, *B. pseudomallei* strains were grown overnight at 37°C in LB broth. 1 mL of overnight culture was washed twice in 300mM sucrose solution and then the pellet was resuspended in 100 µL of the same sucrose solution. 1 µL (~ 100ng) of the expression plasmid was added to the electrocompetent cells and the whole mixture was pulsed inside the biosafety cabinet, in a 2 mm gap width cuvette using an electroporation apparatus (Bio-Rad Gene Pulser Xcell TM) using the following settings: 25 µF, 200Ω and 2.5 kV. Immediately after the pulse 900 µL of LB were added and the mixture was transferred to a culture tube before incubation at 30°C for 1 h. After the 1 h recovery time, 10 µL of culture plus 90 µL of LB broth were spread onto an LB plate containing 2000 µg/mL zeocin , and the plates were incubated at 30°C until colonies appeared. Multiple single colonies usually appeared the next day,and some were

patched onto a fresh selection. Colony PCR for the zeocin resistance marker (primer set #939 & #940) was used to verify the presence of the replicative plasmid in the transformants. The PCR was carried out under the following cycle conditions: one cycle of 95°C for 5 min, 30 cycles of 95°C for 45 s, 59°C for 30 s and 72°C for 35 s; and a final extension at 72°C for 10 min.

4.2.7 Induction of I-*Sce*I expression and isolation of mutants

Induction of I-*Sce*I was done by spreading the pBADSce containing merodiploids onto an LB plate containing 2000 μg/mL zeocin and 0.5% arabinose for single colonies, and incubating the plates at 30°C.

For isolation of *purM* mutants, single colonies were patched the next day (after more than 24 h) onto kanamycin plates, to screen for those that had lost the marker, and onto M9 glucose plates to identify adenine auxotrophs.

In the case of the *fabD*(TS) mutant, single colonies were screened for the loss of the kanamycin resistance marker and for temperature sensitivity by plating on LB agar and incubating the plates at 42°C (at the same time a replica plate with patches was incubated at 30°C) . In some cases, a second purification procedure by isolating single colonies on the same LB zeocin-arabinose plates was necessary to verify complete lack of growth at 42°C. Alternatively, a transformant was used to inoculate 2 mL LB broth supplemented with 2000 μg/mL zeocin and 0.5% arabinose. After overnight incubation at 30°C, 10 μL of the broth culture were spread onto an LB zeocin-arabinose plate, for isolation of single colonies and phenotypic screens. Once temperature-sensitive colonies were identified, the

corresponding colonies in the replica patch plate grown at 30°C were picked and used to inoculate 3 mL LB broth. These cultures were grown overnight at 30°C. After more than 24 h, 20 µL aliquots were spread for single colonies on an LB plate. The plates were incubated at 30°C until colonies appeared on them (48 h later). Purified colonies were patched onto an LB 2000 µg/mL LB plate and onto an LB plate to chose colonies that had lost the zeocin resistance. After 24 h in the 30°C incubator 15 colonies out of 40 were zeocin susceptible. Some of these colonies were used to make frozen stocks. Briefly, 2 mL LB broth were inoculated with a toothpick dipped in the corresponding patch culture and incubated in at 30°C for ~36 h. The next day, 1.5 mL of the overnight culture were mixed with 0.5 mL of a sterile 80% glycerol solution and the cell suspensions were kept at -80°C in a cryovial. To verify the nucleotide changes intended to cause a TS phenotype were indeed present in the chromosome of the mutants, a PCR was done using genomic DNA isolated from the isolates in frozen stocks. Primers fabD-UP and fabD-DW were used and the PCR was performed by following the same cycle conditions described above. The amplicons were purified from a 1% agarose gel and sent for sequencing.

Once the *purM* mutant was isolated, growth at 42°C resulted in elimination of the replicative I-*Sce*I expression plasmid from the cells.

For curing of the TS plasmid from the *fabD*(TS) mutants, the cells were grown in LB broth without antibiotic selection at 30°C overnight, before streaking for single colonies on LB plates without antibiotic selection.

4.3 Results

4.3.1 Construction of delivery vector and I-*Sce*I expression vectors

A non-replicative plasmid containing the kanamycin resistance determinant for selection in *B. pseudomallei*, an *oriT* for conjugational transfer and a unique *Sma*I site in a *lacZα* peptide sequence flanked by two I-*Sce*I recognition sites. This plasmid allows for cloning of replacement cassettes by blue-white screening and delivery of the cassette to the *B. pseudomallei* chromosome by conjugation.

A replicative temperature sensitive plasmid for I-*Sce*I expression was also constructed. This plasmid contains the P_{BAD} promoter for regulated expression of the I-*Sce*I endonuclease, contains a zeocin marker for selection (*ble* gene), and can be cured easily from its host by growing it at the non-permissive temperature (See **Figure 4.2**).

4.3.2 Isolation of *purM* mutants by I-*Sce*I endonuclease induction

In a proof of concept experiment, the *purM* gene from *B. pseudomallei* strains K96243 and 1026b was replaced with a markerless mutant allele (See **Figure 4.3**). A merodiploid strain was first created by introducing the pEXKanpurM into the cells by conjugation This merodiploid contained two I-*Sce*I recognition sites flanking the vector backbone sequences in the genome. Next, the I-*Sce*I endonuclease expression vector pBADSce was introduced into the merodiploid cells by electroporation. After arabinose induction of the expression of endonuclease, mutant colonies were isolated, ~30 out of 40 colonies had lost the

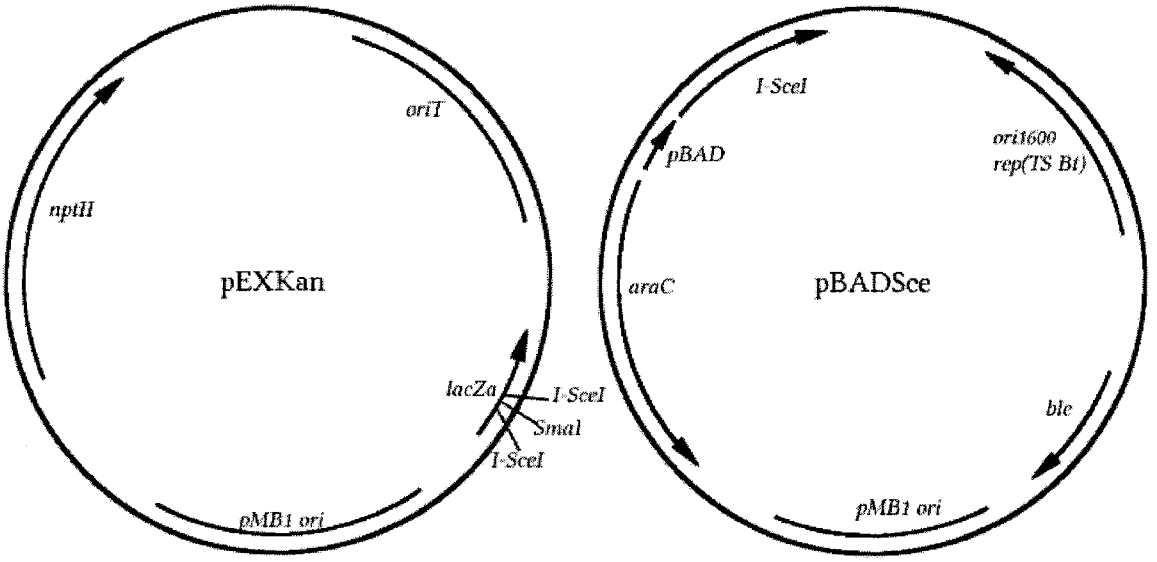


Figure 4.2 Maps of the mutagenesis and I-*Sce*I expression vectors

The plasmid maps are not drawn to scale.

A. The suicide plasmid pEXKan is used to create the merodiploids in *Burkholderia* species. A unique *Sma*I site within the *lacZa* peptide coding sequence is used for cloning of replacement cassettes. Two I-*Sce*I sites flank the *Sma*I site to allow I-SceI catalyzed double stranded breaks in the chromosome in the integrated plasmid backbone. The plasmid also contains a kanamycin resistance marker for merodiploid selection.

B. pBADSce is a plasmid with a replicon that is temperature sensitive in *Burkholderia* species. It is used to express the endonuclease I-*Sce*I under the control of the P_{BAD} promoter. After electroporation into merodilploid cells, zeocin resistant transformants are selected (*ble* is a bleomycin resistance gene that confers zeocin resistance). Growth in the presence of L-arabinose induces I-*Sce*I expression.

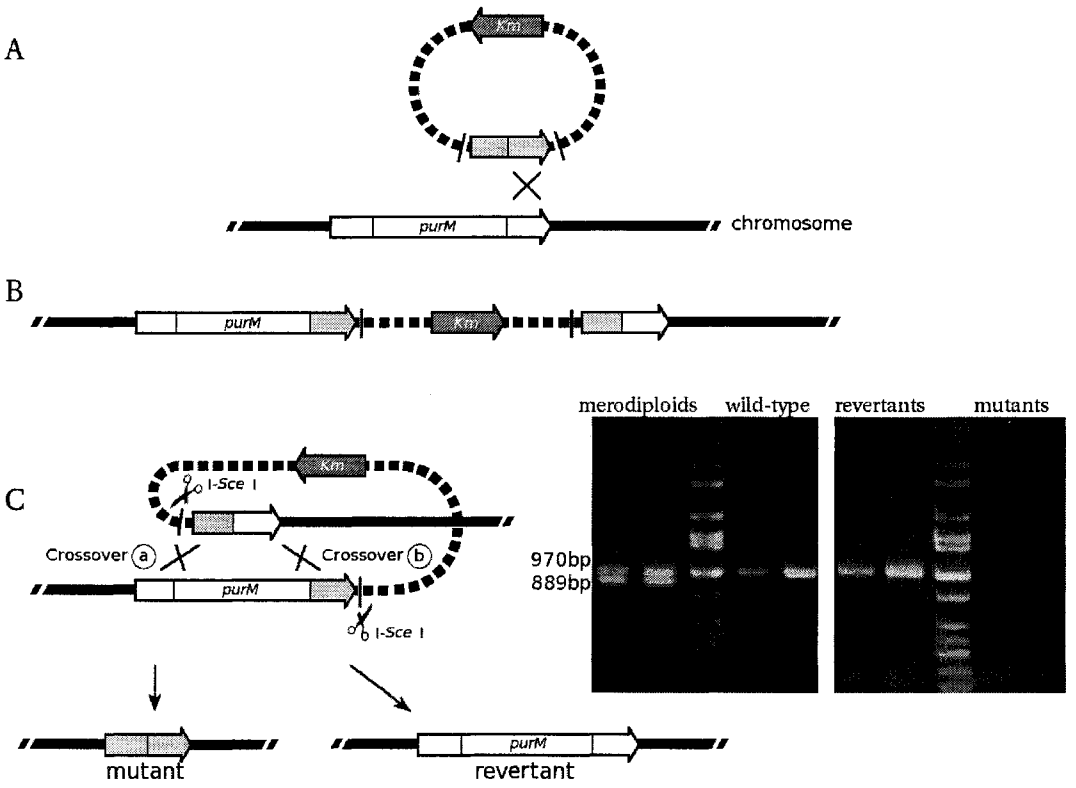


Figure 4.3 Isolation of *B. pseudomallei purM* mutants using the I-*Sce*I system.

A. Plasmid pEXKanpurM, containing the *purM* 5' and 3'end sequences is introduced into the cells by conjugation.

B. Selection for kanamycin resistance results in merodiploid formation via a single crossover event

C. Introduction of pBADSce into the merodiploid cells and induction with arabinose results in expression of I-*Sce*I (indicated by scissors).I-*Sce*I creates double stranded breaks in the chromosome at the integrated I-*Sce*I recognition sites (lines dissecting the plasmid in the top panel). After induction isolates are either *purM* mutants or revertants to wild-type.

D. Agarose gels showing amplicons of PCR using *purM* specific primers. The mutant *purM* is 889 bp, while the wild-type gene is 970 bp.

kanamycin resistance marker, and 12 of these colonies were adenine auxotrophs in K96243 and 8 in 1026*B*. See **Figure 4.4**.

4.3.3 Attempt of isolation of *fabD*(TS) mutants

To verify that I-*Sce*I can be used to incorporate single nucleotide changes into the chromosome, creation of a *fabD*(TS) mutant was attempted. After isolation of merodiploids of *fabD* in *B. pseudomallei* K96243 and 1026b and expression of I-*Sce*I endonuclease from the replicative plasmid pBADSce, colonies were isolated that did not grow 42°C but grew at 30°C. From 40 purified colonies after I-*Sce*I expression, 18 had lost the kanamycin resistance marker in the case of K96243 and 21 in 1026b. Of these, only one K96243 colony and two 1026b colonies were temperature sensitive. The sequencing results showed that the expected amino acid change was not present in any of the isolates, the whole sequence of the gene matched that of the wild-type *fabD*. After checking again the same isolates for temperature sensitivity, it was obvious that they were growing at 42°C albeit very slowly and with a different colony morphology than that seen when the same isolates were grown at 30°Cs (See **Figure 4.5**).

4.4 Discussion

In this chapter, a system for obtaining markerless mutations in the chromosome of *B. pseudomallei* was evaluated. The system is based on the ability of the I-*Sce*I endonuclease to create double stranded breaks in the DNA when it binds to its recognition sequence. By incorporating I-*Sce*I recognition sites, instead of a counter-selectable marker, in the

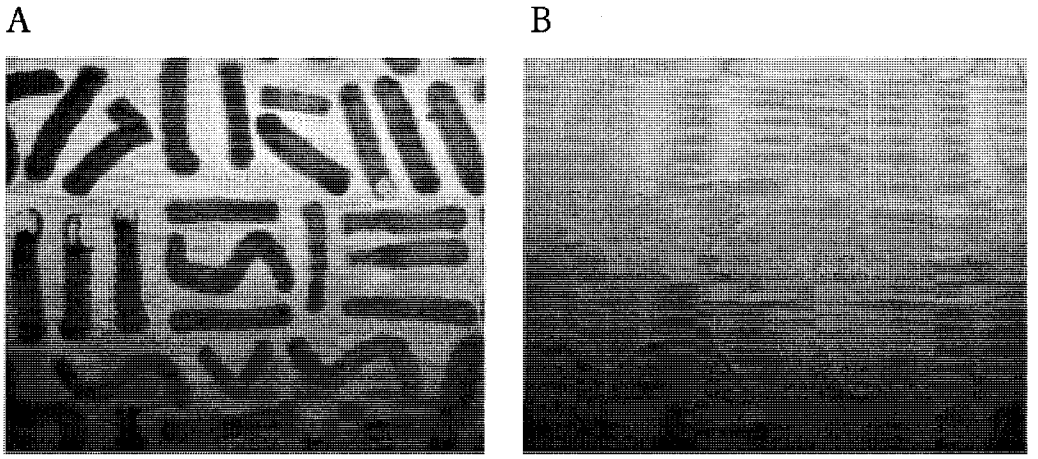


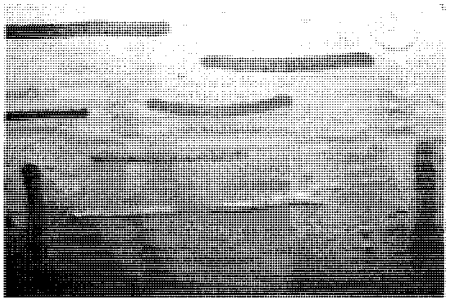
Figure 4.4 Adenine and thiamine auxotrophs of *B. pseudomallei* K96243

Purified colonies of *purM* mutants after I-*Sce*I expression and grown at 42°C to cure the pBADSce plasmid were replicated onto M9 glucose plates with and without added adenine and thiamine and incubated at 37°C.

A. Patches of mutants grown on media containing adenine and thiamine.

B. Patches of mutants displaying auxotrophy for adenine and thiamine.

A



B

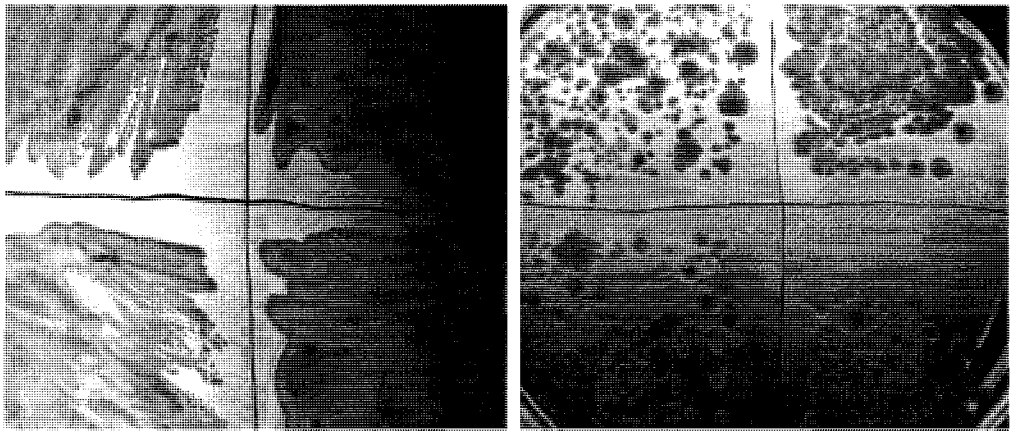


Figure 4.5 Loss of temperature sensitivity of isolated mutants of *B. pseudomallei*

A. After induced expression of I-*Sce*I in *fabD* merodiploids, single colonies were picked, and replica-patched onto an LB plate grown at 30°C or at 42°C. The picture shows patches on the plate incubated at 42°C, two patches in the middle did not grow at this temperature.

B. The TS colonies were rescued from the plates grown at 30°C. A PCR using the *fabD* primer set was done and the amplicons were sequenced, the result showed that the changes necessary to maintain the temperature sensitivity were not present in any of the isolates. When the cells from the frozen stocks were tested again for temperature sensitivity, by streaking them on LB plates, the plate incubated at 30°C showed normal growth (first panel), but the plate incubated at 42°C showed small, slowly growing colonies (second panel) indicating loss of the temperature sensitivity.

backbone of a non-replicative plasmid designed to deliver mutations to the chromosome of *B. pseudomallei* by homologous recombination, it was possible to select for mutants that had undergone double recombination events between homologous sequences and had replaced the wild-type sequence with a mutant sequence.

The isolation of unmarked *purM* deletion mutants proved that the technique can be used to create unmarked scarless mutations with minimal polar effects on downstream sequences.

The failure to isolate, or more specifically to maintain, a *fabD*(TS) mutant, probably has something to do with the instability of such mutations in *B. pseudomallei*, but it would be strange if it had something to do with the method employed in creating the mutant, the finding that merodiploids could be resolved and that the amount of isolated temperature sensitive mutants was so little agrees with the explanation that the fitness of the mutants was low.

All the components of the system described here are compliant with Select Agent rules. The selectable markers used can all be recycled and used again for repeated mutagenesis, as they do not persist in the resultant strains. The targeted mutagenesis is achieved in three steps: First a mutant cassette is cloned at the correct place in the delivery vector, flanked by I-*Sce*I recognition sites, exploiting the unique *Sma*I site and the blue/white screening capabilities. Then a merodiploid is created by homologous recombination of the delivery plasmid into the chromosome. Finally, I-*Sce*I expression results in a large proportion of mutants which have undergone a second recombination event.

This system requires no special media preparations, apart from the addition of arabinose to induce the promoter driving expression of I-*Sce*I. This can be an advantage because colonies can be grown in rich media allowing them to grow fast. Also, experimenting with diverse compositions of media possibly leads to isolation of specific mutants.

The experiments described here, proved indirectly that expression of I-*Sce*I is possible using the P_{BAD} promoter. The P_{BAD} promoter allows for a very tight regulation of expression in *E. coli* and other bacteria (Lefebre and Valvano 2002; Guzman et al. 1995). The promoter is present in the L-arabinose operon of *E. coli* where it regulates the transcription of the gene products of *araB* (ribulokinase), *araA* (isomerase) and *araD* (epimerase). The promoter is regulated by the gene product of *araC*, whose transcription is repressed by AraC itself. In the absence of L-arabinose, AraC forms a homodimer that binds to an initiator region and an operator region, forming a DNA loop, which resembles a termination loop and which represses the transcription from the promoter. When L-arabinose is present and is bound by AraC its conformation changes and the DNA loop formation is abolished. Furthermore, AraC as a homodimer with L-arabinose bound acts as an activator by binding to initiator regions upstream of the promoter, allowing both binding of the activating complex formed by cyclic AMP (cAMP) and cAMP receptor protein, as well as allowing the binding of RNA polymerase, thus effectively initiating transcription from the promoter (Reeder and Schleif 1991). When these experiments were initiated there was no evidence that expression of I-*Sce*I could not be carried just as well

using a constitutive promoter, but since there was no evidence either to suggest that the P_{BAD} promoter would not work well in *B. pseudomallei*, the promoter was tried. A recent paper published while this dissertation was being written describes a very similar system based on I-*Sce*I for gene replacement in other *Burkholderia* species, employing a strong constitutive promoter (Flannagan et al. 2008). Also, in theory, the use of a regulated promoter would allow for integration of the I-*Sce*I gene into the same plasmid used for delivery of the mutation, potentially reducing the steps required for isolation of the desired mutant.

Another important feature of this system is the use of the temperature sensitive replicon pRO1600 (Choi et al. 2008) which allows rapid curing of the I-*Sce*I expression vector.

The ability of I-*Sce*I to create double stranded breaks has shown to promote homologous recombination in other bacteria (Posfai et al. 1999). If this is true in the case of *B. pseudomallei*, then this system should be more efficient than any system based on a counter-selectable marker, because I-*Sce*I would not only allow for selection of double crossover recombinants, but it also would promote the second recombination event, that otherwise would only occur spontaneously. As discussed in Chapter One, the frequency with which one can isolate mutants as opposed to isolating just revertants (strains that loose the integrated plasmid and the mutated sequence) depends on factors such as the distance in base pairs between homologous regions. It has been shown that the linear PCR fragment mutagenesis procedure can be used to isolate deletions in *B. pseudomallei* 1026b

more than 25 kb in length (T. Mima's unpublished results). In systems using circular plasmids, the deletion of such long fragments can be difficult to obtain, because of the long distance between the homologous regions required to undergo a crossover event. The frequency of recombination between direct repeats depends on the distance between them, therefore, the resolution of the cointegrate would probably yield a wild-type allele in most cases. With an I-*Sce*I system, in which double stranded breaks in the chromosome would promote recombination between homologous regions, it should be possible to isolate very long deletions much more frequently than with a counter-selectable marker. Furthermore, it should be possible to generate very large deletions by integrating two different non-replicative plasmids in the chromosome, each carrying an I-*Sce*I recognition site close to each end of the desired deletion.

It is of note that the I-*Sce*I system seemed to be more efficient for generating markerless *purM* mutants than the $pheS^{A304G}$ based system. On average 75% ofthe colonies lost the kanamycin marker after I-*Sce*I expression compared to the 50% that lost the marker after counter-selection with $pheS^{A304G}$. Nevertheless these observations should not be taken as prove that I-*Sce*I indeed promotes intra molecular recombination in *B. pseudomallei*, as opposed to just eliminating the isolates that do not undergo a second crossover. Detailed analyses to address this point were not performed.

Another advantage of the system involving I-*Sce*I as opposed to $pheS^{A304G}$ is the fact that integration of the non-replicative plasmid can only occur at the site where the mutated sequence is homologous to the chromosomal sequence, as opposed to the case of

pheS^{A304G} where the homologous recombination integrating the plasmid can theoretically also occur at the wild-type *pheS* site in the chromosome.

A possible downside of the system is that there is no selective pressure for the presence of intact I-*Sce*I sites in the chromosome, as is the case with the counter-selectable markers sequences, and cumulative mutations could change the sites so that I-*Sce*I would not recognize them anymore. However, this did not seem to be a problem in these studies.

The fact that not all the secondary colonies, after the induction of I-*Sce*I expression, had lost the kanamycin resistance marker, is probably the result of a combination of factors that lead to resolution of the cointegrates or stability of merodiploids. Sufficient levels of endonuclease activity will ensure that cleavage will occur at most I-*Sce*I sites resulting in higher frequencies of merodiploid resolution. Conversely, insufficient levels of endonuclease activity may not result in cleavage of all I-*Sce*I sites, thus resulting in a higher frequency of unresolved merodiploids. Adjustments to I-*Sce*I expression could possibly be made using different concentrations of arabinose in the media.

CHAPTER FIVE

Summary and Conclusions

Despite the availability of numerous *Burkholderia* genome sequences, including several *B. pseudomallei* strains, most aspects of the biology and pathogenesis of these bacteria are still unknown. These bacteria contain a wealth of genetic information that allows them to thrive in vastly different environments (soil, plants, mammals) and under hostile conditions (dryness, nutrient deficiency, acidic niches). While the biological fitness and capabilities of many *Burkholderia* species can be harnessed for beneficial biotechnological purposes (for example bioremediation of noxious organic pollutants) many members of this genus can also be deadly opportunistic pathogens (for example *B. cepacia*, *B. pseudomallei* and *B. mallei*).

The Centers for Disease Control and Prevention, listed *B. pseudomallei* and *B. mallei* as Category B Select Agents that have the potential to pose a severe threat to public health and safety, i.e. they both are considered to be amongst those pathogens that could be used as biological weapons. Research with Select Agents provides privileged sources of funding but is also governed by strict rules and regulations.

The arsenal of genetic tools available for the study of *B. pseudomallei* and *B. mallei* has been steadily improving in recent years. However, many basic procedures are still

cumbersome and slow the pace of discovery. Until very recently, one of the main obstacles hampering genetic analyses in these bacteria was lack of reliable and Select Agent compliant allelic replacement procedures. Earlier this year, two groups published a method using marked linear DNA fragments that are delivered into cells by and natural transformation. However, this method does not work in all *B. pseudomallei* strains. Because it requires a selectable marker the method also does not allow for routine transfer of unmarked single nucleotide alleles into the *Burkholderia* chromosome. Such procedures require merodiploid formation and subsequent resolution into a mixture of wild-type and mutant cells. Resolution of merodiploids requires a CS marker. Unfortunately, the two CS markers most commonly used in Gram-negative bacteria, *sacB* and *rpsL*, are not routinely applicable in wild-type strains of *Burkholderia* species because most of them possess endogenous *sacB* genes and are naturally streptomycin resistant. Amongst several possible CS markers examined in this study, methods using the *pheS CS* marker and the endonuclease I-*Sce*I were shown to be most effective in *B. pseudomallei.*

The methods described in this dissertation are shown to work in different strains of *B. pseudomallei* and at least in one *B. mallei* strain. All their components are Select Agent compliant and allow for isolation of markerless chromosomal mutations, including deletion and single nucleotide changes as evidenced by the fact that introduced changes need not be designed with inclusion of a selection marker.

Even though both of these methods work well in *B. pseudomallei*, differences in the procedures can provide advantages or disadvantages for use under different circumstances.

For example, use of *pheS*^{A304G} can be used without the need to introduce a second plasmid into the cells and works well as a CS marker for use with different applications. The I-*Sce*I system does not require any special media and is probably more efficient than CS marker based systems as it seems to promote second crossovers. Similar to the PCR fragment mutagenesis procedure the I-*Sce*I system could probably be used to generate large chromosomal deletions if two plasmids are integrated into the chromosome prior to I-*Sce*I expression.

The availability of these two proven methods should allow scientists to introduce mutations at will into the chromosomes of *B. pseudomallei* and *B. mallei* and accelerate research into the biology and pathogenesis of these fascinating yet understudied bacteria. The results of this study also show that procedures developed for other bacteria can be adapted for use in more rarely studied bacteria.

REFERENCES

Achouak, W, R Christen, M Barakat, M H Martel, and T Heulin. 1999. *Burkholderia* caribensis sp. nov., an exopolysaccharide-producing bacterium isolated from vertisol microaggregates in Martinique. International Journal of Systematic Bacteriology 49 Pt 2 (April): 787-94. doi:10319504.

Aguirre-Ramírez, Marisela, Jesús Ramírez-Santos, Laurence Van Melderen, and M Carmen Gómez-Eichelmann. 2006. Expression of the F plasmid *ccd* toxin-antitoxin system in *Escherichia coli* cells under nutritional stress. Canadian Journal of Microbiology 52, no. 1 (January): 24-30. doi:w05-107.

Alice, Alejandro F, Claudia S López, Carolyn A Lowe, Maria A Ledesma, and Jorge H Crosa. 2006. Genetic and transcriptional analysis of the siderophore malleobactin biosynthesis and transport genes in the human pathogen *Burkholderia pseudomallei* K96243. Journal of Bacteriology 188, no. 4 (February): 1551-66. doi:188/4/1551.

Amemiya, Kei, Jennifer L Meyers, David Deshazer, et al. 2007. Detection of the host immune response to *Burkholderia mallei* heat-shock proteins GroEL and DnaK in a glanders patient and infected mice. Diagnostic Microbiology and Infectious Disease 59, no. 2 (October): 137-47. doi:S0732-8893(07)00185-X.

Aronoff, S C. 1988. Outer membrane permeability in *Pseudomonas cepacia*: diminished porin content in a beta-lactam-resistant mutant and in resistant cystic fibrosis isolates. Antimicrobial Agents and Chemotherapy 32, no. 11 (November): 1636-9. doi:2855296.

Ashdown, L R. 1979. An improved screening technique for isolation of *Pseudomonas pseudomallei* from clinical specimens. Pathology 11, no. 2 (April): 293-7. doi:460953.

Ashdown, L R, and J M Koehler. 1990. Production of hemolysin and other extracellular enzymes by clinical isolates of *Pseudomonas pseudomallei.* Journal of Clinical Microbiology 28, no. 10 (October): 2331-4. doi:2229359.

Baldwin, Adam, Pamela A Sokol, Julian Parkhill, and Eshwar Mahenthiralingam. 2004. The *Burkholderia cepacia* epidemic strain marker is part of a novel genomic island encoding both virulence and metabolism-associated genes in *Burkholderia* cenocepacia. Infection and Immunity 72, no. 3 (March): 1537-47. doi:14977960.

Ballard, R W, N J Palleroni, M Doudoroff, R Y Stanier, and M Mandel. 1970. Taxonomy of the aerobic pseudomonads: *Pseudomonas cepacia, P. marginata, P. alliicola and P. caryophylli.* Journal of General Microbiology 60, no. 2 (February): 199-214. doi:5488054.

Baltz, R H, and T J Hosted. 1996. Molecular genetic methods for improving secondary-metabolite production in actinomycetes. Trends in Biotechnology 14, no. 7 (July): 245-50. doi:8771797.

Barrett, Ashley R, Yun Kang, Ken S Inamasu, et al. 2008. Genetic Tools for Allelic-Replacement in *Burkholderia* Species. Applied and Environmental Microbiology (May 23). doi:AEM.00531-08.

Baxter, I A, and P A Lambert. 1994. Isolation and partial purification of a carbapenem-hydrolysing metallo-beta-lactamase from *Pseudomonas cepacia.* FEMS Microbiology Letters 122, no. 3 (October 1): 251-6. doi:7527361.

Bentley, Ronald. 2008. A fresh look at natural tropolonoids. Natural Product Reports 25, no. 1 (February): 118-38. doi:10.1039/b711474e.

Beylot, B, and A Spassky. 2001. Chemical probing shows that the intron-encoded endonuclease I-*Sce*I distorts DNA through binding in monomeric form to its homing site. The Journal of Biological Chemistry 276, no. 27 (July 6): 25243-53. doi:11279183.

Blomfield, I C, V Vaughn, R F Rest, and B I Eisenstein. 1991. Allelic exchange in *Escherichia coli* using the *Bacillus subtilis sacB* gene and a temperature-sensitive pSC101 replicon. Molecular Microbiology 5, no. 6 (June): 1447-57. doi:1686293.

Bochner, B R, H C Huang, G L Schieven, and B N Ames. 1980. Positive selection for loss of tetracycline resistance. Journal of Bacteriology 143, no. 2 (August): 926-33. doi:6259126.

Boddey, Justin A, Cameron P Flegg, Chris J Day, Ifor R Beacham, and Ian R Peak. 2006. Temperature-regulated microcolony formation by *Burkholderia pseudomallei* requires *pilA* and enhances association with cultured human cells. Infection and Immunity 74, no. 9 (September): 5374-81. doi:74/9/5374.

Bohn, Chantal, Candice Rigoulay, and Philippe Bouloc. 2007. No detectable effect of RNA-binding protein Hfq absence in *Staphylococcus aureus.* BMC Microbiology 7. doi: 10.1186/1471-2180-7-10.

Boonbumrung, Khaemaporn, Vanaporn Wuthiekanun, Sirirat Rengpipat, Nicholas P J Day, and Sharon J Peacock. 2006. *In vitro* motility of a population of clinical *Burkholderia pseudomallei* isolates. Journal of the Medical Association of Thailand = Chotmaihet thangphaet 89, no. 9 (September): 1506-10. doi:17100392.

van Borm, Steven, Alfred Buschinger, Jacobus J Boomsma, and Johan Billen. 2002. Tetraponera ants have gut symbionts related to nitrogen-fixing root-nodule bacteria. Proceedings. Biological Sciences / The Royal Society 269, no. 1504 (October 7): 2023-7. doi: 12396501.

Brämer, C O, P Vandamme, L F da Silva, J G Gomez, and A Steinbüchel. 2001. Polyhydroxyalkanoate-accumulating bacterium isolated from soil of a sugar-cane plantation in Brazil. International Journal of Systematic and Evolutionary Microbiology 51, no. Pt 5 (September): 1709-13. doi:11594600.

Brans, Alain, Patrice Filee, Andy Chevigne, Aurore Claessens, and Bernard Joris. 2004. New Integrative Method To Generate *Bacillus subtilis* Recombinant Strains Free of Selection Markers. Appl. Environ. Microbiol. 70, no. 12 (December 1): 7241-7250. doi: 10.1128/AEM.70.12.7241-7250.2004.

Brenner, Don J., George M. Garrity, Noel R. Krieg, James T. Staley, and David R. Boone. 2005. Bergey's Manual of Systematic Bacteriology: Part C the Alpha-, Beta-, Delta. Springer.

Brett, P J, D DeShazer, and D E Woods. 1998. *Burkholderia thailandensis* sp. nov., a *Burkholderia pseudomallei*-like species. International Journal of Systematic Bacteriology 48 Pt 1 (January): 317-20. doi:9542103.

Brett, P. J., D. DeShazer, and D. E. Woods. 1998. *Burkholderia thailandensis* sp. nov., a *Burkholderia pseudomallei*-like species. International Journal of Systematic and Evolutionary Microbiology 48, no. 1: 317-320.

Brook, M D, B Currie, and P M Desmarchelier. 1997. Isolation and identification of *Burkholderia pseudomallei* from soil using selective culture techniques and the polymerase chain reaction. Journal of Applied Microbiology 82, no. 5 (May): 589-96. doi: 9172400.

Bros, X. 1893. The use of tuberculin and mallein for the diagnosis of tuberculosis and glanders in animals. Science (New York, N.Y.) 22, no. 560 (October 27): 226. doi:ns-22/560/226.

Brown, Nat F., Ala E. Lew, and Ifor R. Beacham. 2000. Identification of New Transposable Genetic Elements in *Burkholderia pseudomallei* Using Subtractive Hybridisation. FEMS Microbiology Letters 183, no. 1 (February): 73-79. doi:10.1111/j.1574-6968.2000.tb08936.x.

Burkholder WH. Sour skin: a bacterial rot of onion bulbs. Phytopathology 1950;40.

Chain, Patrick S. G., Vincent J. Denef, Konstantinos T. Konstantinidis, et al. 2006. Inaugural Article: *Burkholderia xenovorans* LB400 harbors a multi-replicon, 9.73-Mbp genome shaped for versatility. Proceedings of the National Academy of Sciences 103, no. 42 (October 17): 15280-15287. doi:10.1073/pnas.0606924103.

Chan, Y Y, T M C Tan, Y M Ong, and K L Chua. 2004. BpeAB-OprB, a multidrug efflux pump in *Burkholderia pseudomallei.* Antimicrobial Agents and Chemotherapy 48, no. 4 (April): 1128-35. doi:15047512.

Chan, Ying Ying, Hao Sheng Bian, Theresa May Chin Tan, et al. 2007. Control of quorum sensing by a *Burkholderia pseudomallei* multidrug efflux pump. Journal of Bacteriology 189, no. 11 (June): 4320-4. doi:JB.00003-07.

Chan, Ying Ying, and Kim Lee Chua. 2005. The *Burkholderia pseudomallei* BpeAB-OprB efflux pump: expression and impact on quorum sensing and virulence. Journal of Bacteriology 187, no. 14 (July): 4707-19. doi:187/14/4707.

Chantratita, Narisara, Vanaporn Wuthiekanun, Khaemaporn Boonbumrung, et al. 2007. Biological Relevance of Colony Morphology and Phenotypic Switching by *Burkholderia pseudomallei.* J. Bacteriol. 189, no. 3 (February 1): 807-817. doi:10.1128/JB.01258-06.

Cheng, Allen C., and Bart J. Currie. 2005. Melioidosis: Epidemiology, Pathophysiology, and Management. Clin. Microbiol. Rev. 18, no. 2 (April 1): 383-416. doi:10.1128/CMR.18.2.383-416.2005.

Cheung, Terence K M, P L Ho, Patrick C Y Woo, K Y Yuen, and P Y Chau. 2002. Cloning and expression of class A beta-lactamase gene *blaA* (BPS) in *Burkholderia pseudomallei.* Antimicrobial Agents and Chemotherapy 46, no. 4 (April): 1132-5. doi:11897607.

Choi, Kyoung-Hee, Jared B Gaynor, Kimberly G White, et al. 2005. A Tn7-based broad-range bacterial cloning and expression system. Nature methods 2, no. 6 (June): 443-8. doi:nmeth765.

Choi, Kyoung-Hee, Ayush Kumar, and Herbert P Schweizer. 2006. A 10-min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: application for DNA fragment transfer between chromosomes and plasmid transformation. Journal of Microbiological Methods 64, no. 3 (March): 391-7. doi:S0167-7012(05)00158-2.

Choi, Kyoung-Hee, Takehiko Mima, Yveth Casart, et al. 2008. Genetic tools for select-agent-compliant manipulation of *Burkholderia pseudomallei*. Applied and Environmental Microbiology 74, no. 4 (February): 1064-75. doi:AEM.02430-07.

Coenye, T, B Holmes, K Kersters, J R Govan, and P Vandamme. 1999. *Burkholderia cocovenenans* (van Damme et al. 1960) Gillis et al. 1995 and *Burkholderia vandii* Urakami et al. 1994 are junior synonyms of *Burkholderia gladioli* (Severini 1913) Yabuuchi et al. 1993 and *Burkholderia plantarii* (Azegami et al. 1987) Urakami et al. 1994, respectively. International Journal of Systematic Bacteriology 49 Pt 1 (January): 37-42. doi:10028245.

Coenye, T, P Vandamme, J R Govan, and J J LiPuma. 2001. Taxonomy and identification of the *Burkholderia cepacia* complex. Journal of Clinical Microbiology 39, no. 10 (October): 3427-36. doi:11574551.

Conway, Barbara-Ann, and E P Greenberg. 2002. Quorum-sensing signals and quorum-sensing genes in *Burkholderia vietnamiensis*. Journal of Bacteriology 184, no. 4 (February): 1187-91. doi:11807080.

Court, Donald L, James A Sawitzke, and Lynn C Thomason. 2002. Genetic engineering using homologous recombination. Annual Review of Genetics 36: 361-88. doi:12429697.

Cox, A D, and S G Wilkinson. 1991. Ionizing groups in lipopolysaccharides of *Pseudomonas cepacia* in relation to antibiotic resistance. Molecular Microbiology 5, no. 3 (March): 641-6. doi:2046549.

Cuccui, J, A Easton, K K Chu, et al. 2007. Development of signature-tagged mutagenesis in *Burkholderia pseudomallei* to identify genes important in survival and pathogenesis. Infection and Immunity 75, no. 3 (March): 1186-95. doi:IAI.01240-06.

Currie, B J, D A Fisher, D M Howard, and J N Burrow. 2000. Neurological melioidosis. Acta tropica 74, no. 2-3 (February 5): 145-51. doi:10674643.

Currie, B J, M Mayo, N M Anstey, et al. 2001. A cluster of melioidosis cases from an endemic region is clonal and is linked to the water supply using molecular typing of *Burkholderia pseudomallei* isolates. The American Journal of Tropical Medicine and Hygiene 65, no. 3 (September): 177-9. doi:11561699.

Dance, D A. 1991. Melioidosis: the tip of the iceberg? Clin. Microbiol. Rev. 4, no. 1 (January 1): 52-60.

Dance, 2000. Ecology of *Burkholderia pseudomallei* and the interactions between environmental *Burkholderia* spp. and human-animal hosts. Acta Tropica 74, no. 2-3 (February 5): 159-68.

Dao-Thi, Minh-Hoa, Laurence Van Melderen, Erwin De Genst, et al. 2005. Molecular basis of gyrase poisoning by the addiction toxin Ccd*B*. Journal of Molecular Biology 348, no. 5 (May 20): 1091-102. doi:S0022-2836(05)00329-3.

Darling, P, M Chan, A D Cox, and P A Sokol. 1998. Siderophore production by cystic fibrosis isolates of *Burkholderia cepacia*. Infection and Immunity 66, no. 2 (February): 874-7. doi:9453660.

DeShazer, D, P J Brett, R Carlyon, and D E Woods. 1997. Mutagenesis of *Burkholderia pseudomallei* with Tn5-OT182: isolation of motility mutants and molecular characterization of the flagellin structural gene. Journal of Bacteriology 179, no. 7 (April): 2116-25. doi:9079894.

DeShazer, D, P J Brett, and D E Woods. 1998. The type II O-antigenic polysaccharide moiety of *Burkholderia pseudomallei* lipopolysaccharide is required for serum resistance and virulence. Molecular Microbiology 30, no. 5 (December): 1081-100. doi:9988483.

Donlan, Rodney M, and J William Costerton. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. Clinical Microbiology Reviews 15, no. 2 (April): 167-93. doi:11932229.

Druar, Chris, Fei Yu, Jodie L Barnes, et al. 2008. Evaluating *Burkholderia pseudomallei* Bip proteins as vaccines and Bip antibodies as detection agents. FEMS Immunology and Medical Microbiology 52, no. 1 (January): 78-87. doi:FIM345.

Duerkop, Breck A, Jake P Herman, Ricky L Ulrich, Mair E A Churchill, and E Peter Greenberg. 2008. The *Burkholderia mallei* BmaR3-BmaI3 Quorum-Sensing System Produces and Responds to N-3-Hydroxy-Octanoyl Homoserine Lactone. Journal of Bacteriology (May 16). doi:J*B*.00246-08.

Eberl, Leo. 2006. Quorum sensing in the genus *Burkholderia*. International journal of medical microbiology : IJMM 296, no. 2-3 (April): 103-10. doi:S1438-4221(06)00036-1.

Ebbole, D J, and H Zalkin. 1987. Cloning and characterization of a 12-gene cluster from *Bacillus subtilis* encoding nine enzymes for de novo purine nucleotide synthesis. The Journal of Biological Chemistry 262, no. 17 (June 15): 8274-87. doi:3036807.

Essex-Lopresti, Angela E, Justin A Boddey, Richard Thomas, et al. 2005. A type IV pilin, PilA, Contributes To Adherence of *Burkholderia pseudomallei* and virulence *in vivo*. Infection and Immunity 73, no. 2 (February): 1260-4. doi:73/2/1260.

Faa, Antony G, and Peter J Holt. 2002. Melioidosis in the Torres Strait islands of far North Queensland. Communicable Diseases Intelligence 26, no. 2: 279-83. doi:12206383.

Fabret, Céline, S Dusko Ehrlich, and Philippe Noirot. 2002. A new mutation delivery system for genome-scale approaches in *Bacillus subtilis*. Molecular Microbiology 46, no. 1 (October): 25-36. doi:12366828.

Figurski, D H, and D R Helinski. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. Proceedings of the National Academy of Sciences of the United States of America 76, no. 4 (April): 1648-52. doi:377280.

Flannagan, Ronald S, Thomas Linn, and Miguel A Valvano. 2008. A system for the construction of targeted unmarked gene deletions in the genus *Burkholderia*. Environmental Microbiology (March 13).

Frirdich, Emilisa, Evgeny Vinogradov, and Chris Whitfield. 2004. Biosynthesis of a novel 3-deoxy-D-manno-oct-2-ulosonic acid-containing outer core oligosaccharide in the lipopolysaccharide of *Klebsiella pneumoniae*. The Journal of Biological Chemistry 279, no. 27 (July 2): 27928-40. doi:15090547.

Frodyma, Michael, Aileen Rubio, and D. M. Downs. 2000. Reduced Flux through the Purine Biosynthetic Pathway Results in an Increased Requirement for Coenzyme A in Thiamine Synthesis in *Salmonella enterica* Serovar *Typhimurium*. Journal of Bacteriology 182, no. 1 (January 1): 236-240. doi:10.1128/JB.182.1.236-240.2000.

Fujii, Shingo, Asako Isogawa, and Robert P Fuchs. 2006. RecFOR proteins are essential for Pol V-mediated translesion synthesis and mutagenesis. The EMBO Journal 25, no. 24 (December 13). doi:10.1038/sj.emboj.7601474.

Fushan, Alexey, Galina Monastyrskaya, Igor Abaev, et al. 2005. Genome-wide identification and mapping of variable sequences in the genomes of *Burkholderia mallei* and *Burkholderia pseudomallei*. Research in Microbiology 156, no. 2 (March): 278-88. doi:S0923-2508(04)00259-1.

Gabant, P, T Van Reeth, P L Drèze, et al. 2000. New positive selection system based on the parD (kis/kid) system of the R1 plasmid. BioTechniques 28, no. 4 (April): 784-8. doi: 10769758.

Garcia, R A, J H Hotchkiss, and K H Steinkraus. 1999. The effect of lipids on bongkrekic (Bongkrek) acid toxin production by *Burkholderia cocovenenans* in coconut media. Food Additives and Contaminants 16, no. 2 (February): 63-9. doi:10435074.

Gay, P, D Le Coq, M Steinmetz, E Ferrari, and J A Hoch. 1983. Cloning structural gene *sacB*, which codes for exoenzyme levansucrase of *Bacillus subtilis*: expression of the gene in *Escherichia coli*. Journal of Bacteriology 153, no. 3 (March): 1424-31. doi:6402497.

Gee, Jay E, Mindy B Glass, Ryan T Novak, et al. 2008. Recovery of a *Burkholderia thailandensis*-like isolate from an Australian water source. BMC Microbiology 8: 54. doi: 1471-2180-8-54.

Glass, Mindy *B.*, Jay E. Gee, Arnold G. Steigerwalt, et al. 2006. Pneumonia and Septicemia Caused by *Burkholderia thailandensis* in the United States. J. Clin. Microbiol. 44, no. 12 (December 1): 4601-4604. doi:10.1128/JCM.01585-06.

Godoy, Daniel, Gaynor Randle, Andrew J. Simpson, et al. 2003. Multilocus Sequence Typing and Evolutionary Relationships among the Causative Agents of Melioidosis and Glanders, *Burkholderia pseudomallei* and *Burkholderia mallei.* J. Clin. Microbiol. 41, no. 5 (May 1): 2068-2079. doi:10.1128/JCM.41.5.2068-2079.2003.

Goris, Johan, Winnie Dejonghe, Enevold Falsen, et al. 2002. Diversity of transconjugants that acquired plasmid pJP4 or pEMT1 after inoculation of a donor strain in the A- and B-horizon of an agricultural soil and description of *Burkholderia hospita* sp. nov. and *Burkholderia terricola* sp. nov. Systematic and Applied Microbiology 25, no. 3 (October): 340-52. doi:12421072.

Graves, M, T Robin, A M Chipman, et al. 1997. Four additional cases of *Burkholderia gladioli* infection with microbiological correlates and review. Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America 25, no. 4 (October): 838-42. doi:9356798.

Gronow, Sabine, Christian Noah, Antje Blumenthal, Buko Lindner, and Helmut Brade. 2003. Construction of a deep-rough mutant of *Burkholderia cepacia* ATCC 25416 and characterization of its chemical and biological properties. The Journal of Biological Chemistry 278, no. 3 (January 17): 1647-55. doi:12427755.

Guglierame, Paola, Maria Rosalia Pasca, Edda De Rossi, et al. 2006. Efflux pump genes of the resistance-nodulation-division family in *Burkholderia* ceno*cepacia* genome. BMC microbiology 6: 66. doi:1471-2180-6-66.

Gutterson, N I, and D E Koshland. 1983. Replacement and amplification of bacterial genes with sequences altered *in vitro.* Proceedings of the National Academy of Sciences of the United States of America 80, no. 16 (August): 4894-8. doi:6308658.

Guzman, L M, D Belin, M J Carson, and J Beckwith. 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. Journal of Bacteriology 177, no. 14 (July): 4121-30. doi:7608087.

Hamill, R J, E D Houston, P R Georghiou, et al. 1995. An outbreak of *Burkholderia* (formerly *Pseudomonas*) *cepacia* respiratory tract colonization and infection associated with nebulized albuterol therapy. Annals of Internal Medicine 122, no. 10 (May 15): 762-6. doi:7536401.

Hamilton, C M, M Aldea, B K Washburn, P Babitzke, and S R Kushner. 1989. New method for generating deletions and gene replacements in *Escherichia coli.* Journal of Bacteriology 171, no. 9 (September): 4617-22. doi:2548993.

Harley, V S, D A Dance, B S Drasar, and G Tovey. 1998. Effects of *Burkholderia pseudomallei* and other *Burkholderia* species on eukaryotic cells in tissue culture. Microbios 96, no. 384: 71-93. doi:10093229.

Harms, Klaus, Johann de Vries, and Wilfried Wackernagel. 2007. A double kill gene cassette for the positive selection of transforming non-selective DNA segments in *Acinetobacter baylyi* BD413. Journal of Microbiological Methods 69, no. 1 (April): 107-115. doi: 10.1016/j.mimet.2006.12.006.

Heine, Henry S., Marilyn J. England, David M. Waag, and W. Russell Byrne. 2001. *In Vitro* Antibiotic Susceptibilities of *Burkholderia mallei* (Causative Agent of Glanders) Determined by Broth Microdilution and E-Test. Antimicrob. Agents Chemother. 45, no. 7 (July 1): 2119-2121. doi:10.1128/AAC.45.7.2119-2121.2001.

Henderson, P J, and H A Lardy. 1970. Bongkrekic acid. An inhibitor of the adenine nucleotide translocase of mitochondria. The Journal of Biological Chemistry 245, no. 6 (March 25): 1319-26. doi:4245638.

Hoang, T T, R R Karkhoff-Schweizer, A J Kutchma, and H P Schweizer. 1998. A broad-host-range Fl*p*-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene 212, no. 1 (May 28): 77-86. doi:9661666.

Holden, M. T. & Parkhill, J.2003. The sequencing and analysis of the *Burkholderia cenocepacia* genome. Pediatric. Pulmonology. Suppl. 25, 286.

Holden, Matthew T. G., Richard W. Titball, Sharon J. Peacock, et al. 2004. From the Cover: Genomic plasticity of the causative agent of melioidosis, *Burkholderia pseudomallei.* Proceedings of the National Academy of Sciences 101, no. 39 (September 28): 14240-14245. doi:10.1073/pnas.0403302101.

Howe, C, A Sampath, and M Spotnitz. 1971. The *pseudomallei* group: a review. The Journal of Infectious Diseases 124, no. 6 (December): 598-606. doi:5001752.

Inglis, Timothy J. J., and Jose-Luis Sagripanti. 2006. Environmental Factors That Affect the Survival and Persistence of *Burkholderia pseudomallei.* Appl. Environ. Microbiol. 72, no. 11 (November 1): 6865-6875. doi:10.1128/AEM.01036-06.

Inoue, Koichi, Jingqiu Chen, Ikunoshin Kato, and Masayori Inouye. 2002. Specific growth inhibition by acetate of an *Escherichia coli* strain expressing Era-dE, a dominant negative Era mutant. Journal of Molecular Microbiology and Biotechnology 4, no. 4 (July): 379-88. doi:12125819.

Isenberg, S, and E B Newman. 1974. Studies on L-serine deaminase in *Escherichia coli* K-12. Journal of Bacteriology 118, no. 1 (April): 53-8. doi:4595204.

Jasin, Maria. 1996. Genetic manipulation of genomes with rare-cutting endonucleases. Trends in Genetics 12, no. 6 (June): 224-228. doi:10.1016/0168-9525(96)10019-6.

Jeddeloh, J A, D L Fritz, D M Waag, J M Hartings, and G P Andrews. 2003. Biodefense-driven murine model of pneumonic melioidosis. Infection and Immunity 71, no. 1 (January): 584-7. doi:12496217.

Jenney, A W, G Lum, D A Fisher, and B J Currie. 2001. Antibiotic susceptibility of *Burkholderia pseudomallei* from tropical northern Australia and implications for therapy of melioidosis. International Journal of Antimicrobial Agents 17, no. 2 (February): 109-13. doi:11165114.

Ji, Y, B Zhang, S F Van, et al. 2001. Identification of critical staphylococcal genes using conditional phenotypes generated by antisense RNA. Science (New York, N.Y.) 293, no. 5538 (September 21): 2266-9. doi:11567142.

Jiang, Chun-Yu, Xia-Fang Sheng, Meng Qian, and Qing-Ya Wang. 2008. Isolation and characterization of a heavy metal-resistant *Burkholderia* sp. from heavy metal-contaminated paddy field soil and its potential in promoting plant growth and heavy metal accumulation in metal-polluted soil. Chemosphere 72, no. 2 (May): 157-64. doi:S0045-6535(08)00162-8.

Johnston, D M, and J G Cannon. 1999. Construction of mutant strains of *Neisseria gonorrhoeae* lacking new antibiotic resistance markers using a two gene cassette with positive and negative selection. Gene 236, no. 1 (August 5): 179-84. doi:10433979.

Kast, P. 1994. pKSS--a second-generation general purpose cloning vector for efficient positive selection of recombinant clones. Gene 138, no. 1-2 (January 28): 109-14. doi: 8125286.

Kast, P, and H Hennecke. 1991. Amino acid substrate specificity of *Escherichia coli* phenylalanyl-tRNA synthetase altered by distinct mutations. Journal of Molecular Biology 222, no. 1 (November 5): 99-124. doi:1942071.

van Kessel, Julia C, and Graham F Hatfull. 2007. Recombineering in *Mycobacterium tuberculosis*. Nature Methods 4, no. 2 (February): 147-52.

Kim, H Stanley, Mark A Schell, Yan Yu, et al. 2005. Bacterial genome adaptation to niches: divergence of the potential virulence genes in three *Burkholderia* species of different survival strategies. BMC Genomics 6: 174. doi:1471-2164-6-174.

Komatsu, Harunobu, Yoshiyuki Imura, Akira Ohori, Yuji Nagata, and Masataka Tsuda. 2003. Distribution and Organization of Auxotrophic Genes on the Multichromosomal Genome of *Burkholderia* multivorans ATCC 17616. J. Bacteriol. 185, no. 11 (June 1): 3333-3343. doi:10.1128/*JB*.185.11.3333-3343.2003.

Kornder, J D. 2002. Streptomycin revisited: molecular action in the microbial cell. Medical Hypotheses 58, no. 1 (January): 34-46. doi:11863397.

Krauss, H., H. G. Schieffer, A. Weber, and Wenrich Slenczka. 2003. Zoonoses: Infectious Diseases Transmissible from Animals to Humans. ASM Press.

Kristich, Christopher J., Josephine R. Ch, ler, and Gary M. Dunny. 2007. Development of a host-genotype-independent counterselectable marker and a high-frequency conjugative delivery system and their use in genetic analysis of *Enterococcus faecalis*. Plasmid 57, no. 2 (March): 131-144. doi:10.1016/j.plasmid.2006.08.003.

Kumar, Ayush, Kim-Lee Chua, and Herbert P. Schweizer. 2006. Method for Regulated Expression of Single-Copy Efflux Pump Genes in a Surrogate *Pseudomonas aeruginosa* Strain: Identification of the BpeEF-OprC Chloramphenicol and Trimethoprim Efflux Pump of *Burkholderia pseudomallei* 1026*B*. Antimicrobial Agents and Chemotherapy 50, no. 10 (October). doi:10.1128/AAC.00440-06.

Lawes, M, and S Maloy. 1995. MudSacI, a transposon with strong selectable and counterselectable markers: use for rapid mapping of chromosomal mutations in *Salmonella typhimurium*. Journal of Bacteriology 177, no. 5 (March): 1383-7. doi:7868615.

Lederberg, J. 1951. Streptomycin resistance; a genetically recessive mutation. Journal of Bacteriology 61, no. 5 (May): 549-50. doi:14832197.

Lee, Taewoo, and Andrew L Feig. 2008. The RNA binding protein Hfq interacts specifically with tRNAs. RNA (New York, N.Y.) 14, no. 3 (March): 514-23. doi:rna.531408.

Lefebre, Matthew D, and Miguel A Valvano. 2002. Construction and evaluation of plasmid vectors optimized for constitutive and regulated gene expression in *Burkholderia cepacia* complex isolates. Applied and Environmental Microbiology 68, no. 12 (December): 5956-64. doi:12450816.

Lertpatanasuwan, N, K Sermsri, A Petkaseam, et al. 1999. Arabinose-positive *Burkholderia pseudomallei* infection in humans: case report. Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America 28, no. 4 (April): 927-8. doi: 10825075.

Lesic, Biliana, and Laurence G Rahme. 2008. Use of the lambda Red recombinase system to rapidly generate mutants in *Pseudomonas aeruginosa.* BMC molecular biology 9: 20. doi: 1471-2199-9-20.

Levine, H. *B.*, and R. L. Maurer. 1958. Immunization with an Induced Avirulent Auxotrophic Mutant of *Pseudomonas pseudomallei.* Journal of Immunology 81, no. 5 (November 1): 433-438.

Li, C, T J Kappock, J Stubbe, T M Weaver, and S E Ealick. 1999. X-ray crystal structure of aminoimidazole ribonucleotide synthetase (*purM*), from the *Escherichia coli* purine biosynthetic pathway at 2.5 A resolution. Structure (London, England: 1993) 7, no. 9 (September 15): 1155-66. doi:10508786.

Li, Zexuan, Yishu Huang, Jing Ge, et al. 2007. The Crystal Structure of MCAT from Mycobacterium tuberculosis Reveals Three New Catalytic Models. Journal of Molecular Biology 371, no. 4 (August 24): 1075-1083. doi:10.1016/j.jm*B*.2007.06.004.

Lim, Boon-San. 2007. Genome-wide prediction and annotation of *Burkholderia pseudomallei* AraC/XylS family transcription regulator.. In Silico Bio. 2007;7(4-5):389-97.

Lessie, T G, W Hendrickson, B D Manning, and R Devereux. 1996. Genomic complexity and plasticity of *Burkholderia cepacia.* FEMS microbiology letters 144, no. 2-3 (November 1): 117-28.

Li, H Q, I Matsuda, Y Fujise, and A Ichiyama. 1999. Short-chain acyl-CoA-dependent production of oxalate from oxaloacetate by *Burkholderia glumae*, a plant pathogen which causes grain rot and seedling rot of rice via the oxalate production. Journal of Biochemistry 126, no. 1 (July): 243-53. doi:10393345.

Ling, Jessmi M L, Richard A Moore, Michael G Surette, and Donald E Woods. 2006. The mviN homolog in *Burkholderia pseudomallei* is essential for viability and virulence. Canadian Journal of Microbiology 52, no. 9 (September): 831-42. doi:w06-042.

Link, A J, D Phillips, and G M Church. 1997. Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. Journal of Bacteriology 179, no. 20 (October): 6228-37. doi:9335267.

Loprasert, S, R Sallabhan, W Whangsuk, and S Mongkolsuk. 2000. Characterization and mutagenesis of fur gene from *Burkholderia pseudomallei*. Gene 254, no. 1-2 (August 22): 129-37. doi:10974543.

Lorenz, Michael G., and Johannes Sikorski. 2000. The potential for intraspecific horizontal gene exchange by natural genetic transformation: sexual isolation among genomovars of *Pseudomonas stutzeri*. Microbiology 146, no. 12 (December 1): 3081-3090.

Lumjiaktase, Putthapoom, Stephen P Diggle, Suvit Loprasert, et al. 2006. Quorum sensing regulates *dpsA* and the oxidative stress response in *Burkholderia pseudomallei*. Microbiology (Reading, England) 152, no. Pt 12 (December): 3651-9. doi:152/12/3651.

Lutter, E, S Lewenza, J J Dennis, M B Visser, and P A Sokol. 2001. Distribution of quorum-sensing genes in the *Burkholderia cepacia* complex. Infection and Immunity 69, no. 7 (July): 4661-6. doi:11402012.

Magnuson, R, and M B Yarmolinsky. 1998. Corepression of the P1 addiction operon by Phd and Doc. Journal of Bacteriology 180, no. 23 (December): 6342-51. doi:9829946.

Maloy, S R, and W D Nunn. 1981. Selection for loss of tetracycline resistance by *Escherichia coli*. Journal of Bacteriology 145, no. 2 (February): 1110-1. doi:7007341.

Manna, Dipankar, Adam M Breier, and N Patrick Higgins. 2004. Microarray analysis of transposition targets in *Escherichia coli*: the impact of transcription. Proceedings of the National Academy of Sciences of the United States of America 101, no. 26 (June 29): 9780-5. doi:15210965.

Mandon, K, N Michel-Reydellet, S Encarnación, et al. 1998. Poly-beta-hydroxybutyrate turnover in *Azorhizobium caulinodans* is required for growth and affects *nifA* expression. Journal of Bacteriology 180, no. 19 (October): 5070-6. doi:9748438.

Martinsohn, Jann T, Miroslav Radman, and Marie-Agnès Petit. 2008. The lambda red proteins promote efficient recombination between diverged sequences: implications for bacteriophage genome mosaicism. PLoS genetics 4, no. 5: e1000065. doi: 10.1371/journal.pgen.1000065.

Meng, Xiangdong, Robin M Smith, Astrid V Giesecke, J Keith Joung, and Scot A Wolfe. 2006. Counter-selectable marker for bacterial-based interaction trap systems. BioTechniques 40, no. 2 (February): 179-84. doi:16526407.

Merritt, J, P Tsang, L Zheng, W Shi, and F Qi. 2007. Construction of a counterselection-based in-frame deletion system for genetic studies of *Streptococcus mutans*. Oral Microbiology and Immunology 22, no. 2 (April): 95-102. doi:OMI329.

Moore, R A, D DeShazer, S Reckseidler, A Weissman, and D E Woods. 1999. Efflux-mediated aminoglycoside and macrolide resistance in *Burkholderia pseudomallei*. Antimicrobial Agents and Chemotherapy 43, no. 3 (March): 465-70. doi:10049252.

Moore, R A, and R E Hancock. 1986. Involvement of outer membrane of *Pseudomonas cepacia* in aminoglycoside and polymyxin resistance. Antimicrobial Agents and Chemotherapy 30, no. 6 (December): 923-6. doi:3028253.

Moore, Richard A., Shauna Reckseidler-Zenteno, Heenam Kim, et al. 2004. Contribution of Gene Loss to the Pathogenic Evolution of *Burkholderia pseudomallei* and *Burkholderia mallei*. Infect. Immun. 72, no. 7 (July 1): 4172-4187. doi:10.1128/IAI.72.7.4172-4187.2004.

Moreno, Edgardo. 1998. Genome Evolution Within the Alpha Proteobacteria: Why Do Some Bacteria Not Possess Plasmids and Others Exhibit More Than One Different Chromosome? FEMS Microbiology Reviews 22, no. 4 (October): 255-275. doi:10.1111/j.1574-6976.1998.tb00370.x.

Murphy, Kenan C. 1998. Use of Bacteriophage lambda Recombination Functions To Promote Gene Replacement in *Escherichia coli*. J. Bacteriol. 180, no. 8 (April 15): 2063-2071.

National Committee for Clinical Laboratory Standards. 2006. Methods for dilution.

Nair, Bindu M, K-John Cheung, Adam Griffith, and Jane L Burns. 2004. Salicylate induces an antibiotic efflux pump in *Burkholderia cepacia* complex genomovar III (*B. cenocepacia*). The Journal of clinical investigation 113, no. 3 (February): 464-73. doi:14755343.

Nefedov, M, R Williamson, and P A Ioannou. 2000. Insertion of disease-causing mutations in BACs by homologous recombination in *Escherichia coli*. Nucleic acids research 28, no. 17 (September 1): E79. doi:10954612.

Neubauer, H, L D Sprague, R Zacharia, et al. 2005. Serodiagnosis of *Burkholderia mallei* infections in horses: state-of-the-art and perspectives. Journal of Veterinary Medicine. Infectious Diseases and Veterinary Public Health 52, no. 5 (June): 201-5. doi:JVB855.

Nierman, William C, David DeShazer, H Stanley Kim, et al. 2004. Structural flexibility in the *Burkholderia mallei* genome. Proceedings of the National Academy of Sciences of the United States of America 101, no. 39 (September 28): 14246-51. doi:15377793.

Nierman, William C., David DeShazer, H. Stanley Kim, et al. 2004. From the Cover: Structural flexibility in the *Burkholderia mallei* genome. Proceedings of the National Academy of Sciences 101, no. 39 (September 28): 14246-14251. doi:10.1073/pnas.0403306101.

Niumsup, Pannika, and Vanaporn Wuthiekanun. 2002. Cloning of the class D beta-lactamase gene from *Burkholderia pseudomallei* and studies on its expression in ceftazidime-susceptible and -resistant strains. The Journal of Antimicrobial Chemotherapy 50, no. 4 (October): 445-55. doi:12356787.

Ohtsubo, Yoshiyuki, Hiroyuki Genka, Harunobu Komatsu, Yuji Nagata, and Masataka Tsuda. 2005. High-Temperature-Induced Transposition of Insertion Elements in *Burkholderia multivorans* ATCC 17616. Applied and Environmental Microbiology 71, no. 4 (April). doi:10.1128/AEM.71.4.1822-1828.2005.

Oie, S, and A Kamiya. 1996. Microbial contamination of antiseptics and disinfectants. American Journal of Infection Control 24, no. 5 (October): 389-95. doi:8902114.

O'Sullivan, L A, and E Mahenthiralingam. 2005. Biotechnological potential within the genus *Burkholderia*. Letters in Applied Microbiology 41, no. 1: 8-11. doi:LAM1758.

O'Sullivan, Louise A, Andrew J Weightman, T Hefin Jones, et al. 2007. Identifying the genetic basis of ecologically and biotechnologically useful functions of the bacterium *Burkholderia vietnamiensis*. Environmental Microbiology 9, no. 4 (April): 1017-34. doi:EMI1228.

Ou, Keli, Catherine Ong, Shze Yung Koh, et al. 2005. Integrative Genomic, Transcriptional, and Proteomic Diversity in Natural Isolates of the Human Pathogen *Burkholderia pseudomallei*. Journal of Bacteriology 187, no. 12 (June). doi:10.1128/*JB*. 187.12.4276-4285.2005.

Parke, J L, and D Gurian-Sherman. 2001. Diversity of the *Burkholderia cepacia* complex and implications for risk assessment of biological control strains. Annual Review of Phytopathology 39: 225-58. doi:11701865.

Partida-Martinez, Laila P, Ingrid Groth, Imke Schmitt, et al. 2007. *Burkholderia rhizoxinica* sp. nov. and *Burkholderia endofungorum* sp. nov., bacterial endosymbionts of the plant-pathogenic fungus *Rhizopus microsporus*. International Journal of Systematic and Evolutionary Microbiology 57, no. Pt 11 (November): 2583-90. doi:57/11/2583.

Pecota, Douglas C, George Osapay, Michael E Selsted, and Thomas K Wood. 2003. Antimicrobial properties of the *Escherichia coli* R1 plasmid host killing peptide. Journal of Biotechnology 100, no. 1 (January 9): 1-12. doi:12413781.

Pelicic, V, M Jackson, J M Reyrat, et al. 1997. Efficient allelic exchange and transposon mutagenesis in *Mycobacterium tuberculosis*. Proceedings of the National Academy of Sciences of the United States of America 94, no. 20 (September 30): 10955-60. doi:9380741.

Peñaloza-Vazquez, A, G L Mena, L Herrera-Estrella, and A M Bailey. 1995. Cloning and sequencing of the genes involved in glyphosate utilization by *Pseudomonas pseudomallei*. Applied and Environmental Microbiology 61, no. 2 (February): 538-43. doi:7574593.

Pilatz, Sabine, Katrin Breitbach, Nadine Hein, et al. 2006. Identification of *Burkholderia pseudomallei* genes required for the intracellular life cycle and *in vivo* virulence. Infection and Immunity 74, no. 6 (June): 3576-86. doi:74/6/3576.

Posfai, G, V Kolisnychenko, Z Bereczki, and FR Blattner. 1999. Markerless gene replacement in *Escherichia coli* stimulated by a double- strand break in the chromosome. Nucleic Acids Research. 27, no. 22 (November 15): 4409-4415. doi:10.1093/nar/27.22.4409.

Poteete, A R. 2001. What makes the bacteriophage lambda Red system useful for genetic engineering: molecular mechanism and biological function. FEMS Microbiology Letters 201, no. 1 (July 10): 9-14. doi:11445160.

Radua, S, O W Ling, S Srimontree, et al. 2000. Characterization of *Burkholderia pseudomallei* isolated in Thailand and Malaysia. Diagnostic Microbiology and Infectious Disease 38, no. 3 (November): 141-5. doi:11109011.

Reckseidler, S L, D DeShazer, P A Sokol, and D E Woods. 2001. Detection of bacterial virulence genes by subtractive hybridization: identification of capsular polysaccharide of *Burkholderia pseudomallei* as a major virulence determinant. Infection and Immunity 69, no. 1 (January): 34-44. doi:11119486.

Reeder, T, and R Schleif. 1991. Mapping, sequence, and apparent lack of function of araJ, a gene of the *Escherichia coli* arabinose regulon. Journal of Bacteriology 173, no. 24 (December): 7765-71. doi:1744033.

Reyrat, Jean-Marc, Vladimir Pelicic, Brigitte Gicquel, and Rino Rappuoli. 1998. Counterselectable Markers: Untapped Tools for Bacterial Genetics and Pathogenesis. Infect. Immun. 66, no. 9 (September 1): 4011-4017.

Rocha, Eduardo P. C, Emmanuel Cornet, and Bénédicte Michel. 2005. Comparative and Evolutionary Analysis of the Bacterial Homologous Recombination Systems . PLoS Genetics 1, no. 2: e15. doi:10.1371/journal.pgen.0010015.

Ruiz-Echevarría, M J, A Berzal-Herranz, K Gerdes, and R Díaz-Orejas. 1991. The kis and kid genes of the *parD* maintenance system of plasmid R1 form an operon that is autoregulated at the level of transcription by the co-ordinated action of the Kis and Kid proteins. Molecular Microbiology 5, no. 11 (November): 2685-93. doi:1779758.

Sabaty, M, and S Kaplan. 1996. mgpS, a complex regulatory locus involved in the transcriptional control of the *puc* and *puf* operons in *Rhodobacter sphaeroides* 2.4.1. Journal of Bacteriology 178, no. 1 (January): 35-45. doi:8550440.

Sambrook, Joseph, and David W. Russell. 2001. Molecular Cloning: A Laboratory Manual. CSHL Press.

Sander, P, A Meier, and E C Böttger. 1995. *rpsL*+: a dominant selectable marker for gene replacement in mycobacteria. Molecular Microbiology 16, no. 5 (June): 991-1000. doi: 7476195.

Sanford, J P, and W L Moore. 1971. Recrudescent melioidosis: a southeast asian legacy. The American Review of Respiratory Disease 104, no. 3 (September): 452-3. doi:5098680.

Sarkar-Tyson, M., J. E. Thwaite, S. V. Harding, et al. 2007. Polysaccharides and virulence of *Burkholderia pseudomallei.* J Med Microbiol 56, no. 8 (August 1): 1005-1010. doi: 10.1099/jmm.0.47043-0.

Schell, Mark A, Lyla Lipscomb, and David Deshazer. 2008. Comparative Genomics and an Insect Model Rapidly Identify Novel Virulence Genes of *Burkholderia mallei.* J Bacteriol (January 25).

Schell, Mark A, Ricky L Ulrich, Wilson J Ribot, et al. 2007. Type VI secretion is a major virulence determinant in *Burkholderia mallei.* Molecular Microbiology 64, no. 6 (June): 1466-85. doi:MMI5734.

Schell, Mark A., Lyla Lipscomb, and David DeShazer. 2008. Comparative Genomics and an Insect Model Rapidly Identify Novel Virulence Genes of *Burkholderia mallei* . J. Bacteriol. 190, no. 7 (April 1): 2306-2313. doi:10.1128/JB.01735-07.

Scherlach, Kirstin, Laila P Partida-Martinez, Hans-Martin Dahse, and Christian Hertweck. 2006. Antimitotic rhizoxin derivatives from a cultured bacterial endosymbiont of the rice pathogenic fungus *Rhizopus microsporus.* Journal of the American Chemical Society 128, no. 35 (September 6): 11529-36. doi:10.1021/ja062953o.

Schweizer, H P. 1992. Allelic exchange in *Pseudomonas aeruginosa* using novel ColE1-type vectors and a family of cassettes containing a portable *oriT* and the counter-selectable *Bacillus subtilis sacB* marker. Molecular Microbiology 6, no. 9 (May): 1195-204. doi: 1588818.

Schweizer, H P, and T T Hoang. 1995. An improved system for gene replacement and *xylE* fusion analysis in *Pseudomonas aeruginosa.* Gene 158, no. 1 (May 26): 15-22. doi:7789804.

Serre, L, E C Verbree, Z Dauter, A R Stuitje, and Z S Derewenda. 1995. The *Escherichia coli* malonyl-CoA:acyl carrier protein transacylase at 1.5-A resolution. Crystal structure of a fatty acid synthase component. The Journal of Biological Chemistry 270, no. 22 (June 2): 12961-4. doi:7768883.

Sharma, Manjuli R., Ch, ana Barat, et al. 2005. Interaction of Era with the 30S Ribosomal Subunit: Implications for 30S Subunit Assembly. Molecular Cell 18, no. 3 (April): 319-329. doi:10.1016/j.molcel.2005.03.028.

Shulman, M J, L M Hallick, H Echols, and E R Signer. 1970. Properties of recombination-deficient mutants of bacteriophage lambda. Journal of Molecular Biology 52, no. 3 (September 28): 501-20. doi:4923748.

Simpson, Andrew J. H., Nicholas J. White, and Vanaporn Wuthiekanun. 1999. Aminoglycoside and Macrolide Resistance in *Burkholderia pseudomallei.* Antimicrobial Agents and Chemotherapy 43, no. 9 (September 1): 2332.

Skorupski, K, and R K Taylor. 1996. Positive selection vectors for allelic exchange. Gene 169, no. 1 (February 22): 47-52. doi:8635748.

Sokol, Pamela A, Rebecca J Malott, Kathrin Riedel, and Leo Eberl. 2007. Communication systems in the genus *Burkholderia*: global regulators and targets for novel antipathogenic drugs. Future Microbiology 2, no. 5 (October): 555-63. doi:10.2217/17460913.2.5.555.

Srinivasan, A, C N Kraus, D DeShazer, et al. 2001. Glanders in a military research microbiologist. The New England Journal of Medicine 345, no. 4 (July 26): 256-8. doi: 11474663.

Steinmetz, M, D Le Coq, H B Djemia, and P Gay. 1983. Genetic analysis of *sacB*, the structural gene of a secreted enzyme, levansucrase of *Bacillus subtilis* Marburg. Molecular & General Genetics : MGG 191, no. 1: 138-44. doi:6412036.

Stevens, Mark P, Andrea Friebel, Lowrie A Taylor, et al. 2003. A *Burkholderia pseudomallei* type III secreted protein, BopE, facilitates bacterial invasion of epithelial cells and exhibits guanine nucleotide exchange factor activity. Journal of Bacteriology 185, no. 16 (August): 4992-6. doi:12897019.

Stevens, Mark P, Ashraful Haque, Timothy Atkins, et al. 2004. Attenuated virulence and protective efficacy of a *Burkholderia pseudomallei bsa* type III secretion mutant in murine models of melioidosis. Microbiology (Reading, England) 150, no. Pt 8 (August): 2669-76. doi:15289563.

Stevens, Mark P, Joanne M Stevens, Robert L Jeng, et al. 2005. Identification of a bacterial factor required for actin-based motility of *Burkholderia pseudomallei.* Molecular Microbiology 56, no. 1 (April): 40-53. doi:MMI4528.

Stevens, Mark P, Michael W Wood, Lowrie A Taylor, et al. 2002. An Inv/Mxi-Spa-like type III protein secretion system in *Burkholderia pseudomallei* modulates intracellular behaviour of the pathogen. Molecular Microbiology 46, no. 3 (November): 649-59. doi: 12410823.

Suputtamongkol, Y, A J Hall, D A Dance, et al. 1994. The epidemiology of melioidosis in Ubon Ratchatani, northeast Thailand. International Journal of Epidemiology 23, no. 5 (October): 1082-90. doi:7860160.

Takada, Ayako, Kazuo Nagai, and Masaaki Wachi. 2005. A decreased level of FtsZ is responsible for inviability of RNase E-deficient cells. Genes to cells : devoted to molecular & cellular mechanisms 10, no. 7 (July): 733-41. doi:GTC872.

Tamura, Glen S., Debra S. Bratt, Harry H. Yim, and Aphakorn Nittayajarn. 2005. Use of *glnQ* as a Counterselectable Marker for Creation of Allelic Exchange Mutations in Group B Streptococci. Applied and Environmental Microbiology 71, no. 1 (January). doi: 10.1128/AEM.71.1.587-590.2005.

Thomas, A D. 1981. Prevalence of melioidosis in animals in northern Queensland. Australian Veterinary Journal 57, no. 3 (March): 146-8. doi:7259663.

Thomas, A D, and J C Forbes-Faulkner. 1981. Persistence of *Pseudomonas pseudomallei* in soil. Australian veterinary journal 57, no. 11 (November): 535-6. doi:7342941.

Thomas, Christopher M, and Kaare M Nielsen. 2005. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. Nature Reviews. Microbiology 3, no. 9 (September): 711-21. doi:nrmicro1234.

Thomas, Mark S. 2007. Iron acquisition mechanisms of the *Burkholderia cepacia* complex. Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine 20, no. 3-4 (June): 431-52. doi:10.1007/s10534-006-9065-4.

Thongdee, Metawee, Larry A Gallagher, Mark Schell, et al. 2008. Targeted mutagenesis of *Burkholderia thailandensis* and *Burkholderia pseudomallei* through natural transformation of PCR fragments. Applied and Environmental Microbiology. May, 74(10): 2985-9.

Tischer, B Karsten, Jens von Einem, Benedikt Kaufer, and Nikolaus Osterrieder. 2006. Two-step red-mediated recombination for versatile high-efficiency markerless DNA manipulation in *Escherichia coli*. BioTechniques 40, no. 2 (February): 191-7. doi:16526409.

Tsilibaris, Virginie, Geneviève Maenhaut-Michel, Natacha Mine, and Laurence Van Melderen. 2007. What is the benefit to *Escherichia coli* of having multiple toxin-antitoxin systems in its genome? Journal of Bacteriology 189, no. 17 (September): 6101-8. doi:*JB*. 00527-07.

Tuanyok, Apichai, Marina Tom, John Dunbar, and Donald E Woods. 2006. Genome-wide expression analysis of *Burkholderia pseudomallei* infection in a hamster model of acute melioidosis. Infection and Immunity 74, no. 10 (October): 5465-76. doi:74/10/5465.

Tumapa, Sarinna, Matthew Holden, Mongkol Vesaratchavest, et al. 2008. *Burkholderia pseudomallei* genome plasticity associated with genomic island variation. BMC Genomics 9, no. 1: 190. doi:10.1186/1471-2164-9-190.

Truder P, Provost S, Massie B, Chartrand P, and Wall L. 1996. pGATA: a positive selection vector based on the toxicity of the transcription factor GATA-1 to bacteria. Biotechniques Apr;20(4):684-93.

Ulrich, Ricky L, David Deshazer, Ernst E Brueggemann, et al. 2004. Role of quorum sensing in the pathogenicity of *Burkholderia pseudomallei*. Journal of medical microbiology 53, no. Pt 11 (November): 1053-64. doi:15496380.

Utsumi, R, T Yagi, S Katayama, et al. 1991. Molecular cloning and characterization of the fusaric acid-resistance gene from *Pseudomonas cepacia*. Agricultural and biological chemistry 55, no. 7 (July): 1913-8. doi:1370369.

Valade, E, F M Thibault, Y P Gauthier, et al. 2004. The PmlI-PmlR quorum-sensing system in *Burkholderia pseudomallei* plays a key role in virulence and modulates production of the MprA protease. Journal of Bacteriology 186, no. 8 (April): 2288-94. doi:15060030.

Vandamme, Peter, Deborah Henry, Tom Coenye, et al. 2002. *Burkholderia anthina* sp. nov. and *Burkholderia pyrrocinia*, two additional *Burkholderia cepacia* complex bacteria, may confound results of new molecular diagnostic tools. FEMS Immunology and Medical Microbiology 33, no. 2 (June 3): 143-9. doi:12052570.

Vandamme, Peter, Barry Holmes, Tom Coenye, et al. 2003. *Burkholderia* ceno*cepacia* sp. nov.--a new twist to an old story. Research in microbiology 154, no. 2 (March): 91-6. doi: 12648723.

Van Oevelen, Sandra, Rupert De Wachter, Peter Vandamme, Elmar Robbrecht, and Els Prinsen. 2002. Identification of the bacterial endosymbionts in leaf galls of *Psychotria* (Rubiaceae, angiosperms) and proposal of 'Candidatus *Burkholderia kirkii*' sp. nov. International Journal of Systematic and Evolutionary Microbiology 52, no. Pt 6 (November): 2023-7. doi:12508863.

Vermis, K., P.a.r. Vandamme, and H.j. Nelis. 2003. *Burkholderia cepacia* Complex Genomovars: Utilization of Carbon Sources, Susceptibility to Antimicrobial Agents and Growth on Selective Media. Journal of Applied Microbiology 95, no. 6 (December): 1191-1199. doi:10.1046/j.1365-2672.2003.02054.x.

Verwoert, I I, E F Verhagen, K H van der Linden, et al. 1994. Molecular characterization of an *Escherichia coli* mutant with a temperature-sensitive malonyl coenzyme A-acyl carrier protein transacylase. FEBS Letters 348, no. 3 (July 18): 311-6. doi:8034061.

Viallard, V, I Poirier, B Cournoyer, et al. 1998. *Burkholderia graminis* sp. nov., a rhizospheric *Burkholderia* species, and reassessment of [*Pseudomonas*] *phenazinium*, [*Pseudomonas*] *pyrrocinia* and [*Pseudomonas*] *glathei* as *Burkholderia*. International Journal of Systematic Bacteriology 48 Pt 2 (April): 549-63.

Vial, Ludovic, Marie-Christine Groleau, Valérie Dekimpe, and Eric Déziel. 2007. *Burkholderia* diversity and versatility: an inventory of the extracellular products. Journal of Microbiology and Biotechnology 17, no. 9 (September): 1407-29. doi:18062218.

Wagner, E G, and R W Simons. 1994. Antisense RNA control in bacteria, phages, and plasmids. Annual Review of Microbiology 48: 713-42. doi:7826024.

Walhout, A J, G F Temple, M A Brasch, et al. 2000. GATEWAY recombinational cloning: application to the cloning of large numbers of open reading frames or ORFeomes. Methods in Enzymology 328: 575-92. doi:11075367.

Wang, Shusheng, Dan Liu, Xu Zhang, et al. 2007. Study on glycosylated prodrugs of toxoflavins for antibody-directed enzyme tumor therapy. Carbohydrate Research 342, no. 9 (July 2): 1254-60. doi:S0008-6215(07)00130-9.

Warming, Søren, Nina Costantino, Donald L Court, Nancy A Jenkins, and Neal G Copeland. 2005. Simple and highly efficient BAC recombineering using *galK* selection. Nucleic Acids Research 33, no. 4: e36. doi:33/4/e36.

Welch, John J, Jason A Watts, Christopher R Vakoc, et al. 2004. Global regulation of erythroid gene expression by transcription factor GATA-1. Blood 104, no. 10 (November 15): 3136-47. doi:15297311.

Wheelis, M. 1998. First shots fired in biological warfare. Nature 395, no. 6699 (September 17): 213. doi:9751039.

White, N J. 2003. Melioidosis. Lancet 361, no. 9370 (May 17): 1715-22. doi:12767750.

Whitlock, Gregory C, D Mark Estes, and Alfredo G Torres. 2007. Glanders: off to the races with *Burkholderia mallei*. FEMS microbiology letters 277, no. 2 (December): 115-22. doi:FML949.

Wiersinga, W. Joost, Tom van der Poll, Nicholas J. White, Nicholas P. Day, and Sharon J. Peacock. 2006. Melioidosis: insights into the pathogenicity of *Burkholderia pseudomallei*. Nat Rev Micro 4, no. 4 (April): 272-282. doi:10.1038/nrmicro1385.

Wong, S M, and J J Mekalanos. 2000. Genetic footprinting with mariner-based transposition in *Pseudomonas aeruginosa*. Proceedings of the National Academy of Sciences of the United States of America 97, no. 18 (August 29): 10191-6. doi:10963681.

Wuthiekanun, V, M D Smith, and N J White. Survival of *Burkholderia pseudomallei* in the absence of nutrients. Transactions of the Royal Society of Tropical Medicine and Hygiene 89, no. 5: 491. doi:8560519.

Yabuuchi, E, Y Kawamura, T Ezaki, et al. 2000. *Burkholderia* uboniae sp. nov., L-arabinose-assimilating but different from *Burkholderia thailandensis* and *Burkholderia vietnamiensis*. Microbiology and Immunology 44, no. 4: 307-17. doi:10832977.

Yabuuchi, E, Y Kosako, H Oyaizu, et al. 1992. Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) *comB.* nov. Microbiology and immunology 36, no. 12: 1251-75.

Yu, Yiting, H Stanley Kim, Hui Hoon Chua, et al. 2006. Genomic patterns of pathogen evolution revealed by comparison of *Burkholderia pseudomallei*, the causative agent of melioidosis, to avirulent *Burkholderia thailandensis*. BMC Microbiology 6: 46. doi: 1471-2180-6-46.

Zhang, Youming, Joep Muyrers, Jeanette Rientjes, and A Francis Stewart. 2003. Phage annealing proteins promote oligonucleotide-directed mutagenesis in *Escherichia coli* and mouse ES cells. BMC Molecular Biology 4, no. 1: 1. doi:10.1186/1471-2199-4-1.

Zhong, Jin, Michael Karberg, and Alan M Lambowitz. 2003. Targeted and random bacterial gene disruption using a group II intron (targetron) vector containing a retrotransposition-activated selectable marker. Nucleic acids research 31, no. 6 (March 15): 1656-64. doi: 12626707.

Zhou, Hongwei, Fude Yao, Daniel P Roberts, and Thomas G Lessie. 2003. AHL-deficient mutants of *Burkholderia* ambifaria BC-F have decreased antifungal activity. Current microbiology 47, no. 3 (September): 174-9. doi:14570265.

Zilles, J L, T J Kappock, J Stubbe, and D M Downs. 2001. Altered pathway routing in a class of Salmonella enterica serovar Typhimurium mutants defective in aminoimidazole ribonucleotide synthetase. Journal of Bacteriology 183, no. 7 (April): 2234-40. doi:11244062.

Zolg, W, and J C Ottow. 1975. *Pseudomonas* glathei sp. nov., a new nitrogen-scavening rod isolated from acid lateritic relicts in Germany. The Journal of Comparative Neurology 164, no. 1 (November 1): 287-99. doi:1176650.