UTILIZATION OF CRISPR INTERFERENCE IN IDENTIFICATION OF NOVEL ANTIBIOTIC TARGET IN *BURKHOLDERIA PSEUDOMALLEI*

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ABSTRACT

Melioidosis is a life-threatening disease caused by *Burkholderia pseudomallei*. In the absence of vaccines and the limitation of antibiotic therapy due to the inherent antibiotic resistance of this bacterium, new drugs are needed to improve treatment and prevention. Since *B. pseudomallei* is classified as a select agent, genetic tools for genetic manipulations of this pathogen are limited. In the characterization of gene functions, gene deletion mutation using an allelic exchange is among the most common genetic manipulation tools available to this microorganism, but not all genes can be deleted, especially an essential gene. Essential gene is a gene encoding bacterial components essential for growth and survival which is an attractive target for the development of novel antibiotics. In this study, the CRISPR interference (CRISPRi) technology was used to identify the novel essential genes in *B. pseudomallei* strain 1026b. *BP1026B_I0557*, a gene encoding putative exported proteins with unknown functions, was selected as a candidate essential gene in this study. We confirmed that *BP1026B_I0557* is essential for *B. pseudomallei* growth and survival by depleting its expression using CRISPRi. Unfortunately, no growth defect was observed in strains expressing sgRNA targeting *BP1026B_I0557*, which indicated that *BP1026B_I0557* might not be essential.

Keywords: *Burkholderia pseudomallei*, CRISPR interference, essential gene, gene knockdown

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INTRODUCTION

Melioidosis is a life-threatening infectious disease caused by *Burkholderia pseudomallei*, a gram-negative bacterium frequently found in the soil and surface of groundwater. This bacterium can infect both humans and animals in various ways. There are a variety of clinical symptoms of melioidosis ranging from asymptomatic, abscesses, pneumonia, and acute septicemia, which can be fatal within 24-48 hours following infection (Wiersinga et al., 2018). The melioidosis treatment consists of two phases, the initial intensive or acute phase treatment which involves giving patients intravenous ceftazidime or meropenem in order to prevent death from severe sepsis for at least 10 to 14 days, the eradication phase, this phase consists of taking trimethoprim-sulfamethoxazole orally for 3-5 months in order to prevent relapse (Wiersinga et al., 2018; Dance, 2014).

There is currently no vaccine available against melioidosis and the treatment options for the disease are limited. *B. pseudomallei* has intrinsic resistance to a variety of antibiotics, including aminoglycosides and macrolides, because of the efflux pumps and reduction of outer membrane permeability (Wiersinga et al., 2018; Schweizer, 2012). It is therefore imperative to find new therapeutic targets to develop alternative melioidosis therapies. Genes encoding essential bacterial components have been identified as attractive targets for the development of new antibiotics. Thus, it is crucial to understand the proteins responsible for essential processes involving *B. pseudomallei* growth and survival. A gene deletion mutation is one of the most common genetic manipulation tools available to this pathogen for the identification and characterization of gene function (Lopez et al., 2009). However, not every gene can be deleted, especially an essential one that is involved in bacterial growth and survival (Juhas et al., 2011). Moreover, the functions and contributions of these genes in this organism still remain unclear.

The clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) system is a rapidly developing genome engineering technique for effectively editing DNA targets in both humans and animals. In 2018, a CRISPR interference (CRISPRi) system for gene depletion in the genus *Pseudomonas* was developed by using *Streptococcus pasteurianus* dCas9 (dCas9-SpaS) which can easily repress a specific gene expression in *Pseudomonas* spp. (Tan et al., 2018). The CRISPRi is composed of the nuclease-inactive version of Cas9, dead Cas9 (dcas9), and single-guide RNA (sgRNA) which is complementary to the target sequence. CRISPRi, in contrast to CRISPR-Cas9, can only suppress gene expression when targeted at particular genes. (Zhang et al., 2021). The present study used CRISPRi (dCas9-Spas) technology to identify and characterize the candidate essential gene (*BP1026B_I0557*) in *B. pseudomallei* strain 1026b.

LITERATURE REVIEWS

Burkholderia pseudomallei, Pathogenesis, and Drug Resistance Mechanisms

B. pseudomallei is a gram-negative, facultative anaerobic bacterium, found in soil, groundwater, and plant roots. This pathogen is categorized as a tier 1 select agent because it has the potential to be used in biological warfare. *B. pseudomallei* is a causative agent of melioidosis, an infectious disease found in both humans and animals. A person can be infected by touching contaminated soil or water, eating contaminated food, or inhaling contaminated air. Tropical and subtropical nations have been reported to have *B. pseudomallei* predominance. Melioidosis is known as "the great mimicker" because its symptoms can mimic a variety of diseases, such as tuberculosis. With an incubation period of approximately 1 to 21 days, the infection may be acute, chronic, or latent (Wiersinga et al., 2018). Many virulence factors in this bacterium make it effective at infecting and surviving in host tissues. *B. pseudomallei* can proliferate in epithelial cells after entering the body through the site of infection. (Wiersinga et al, 2018). As an intracellular pathogen, *B. pseudomallei* can infect both phagocytic and nonphagocytic cells. Following internalization into the host cell by phagocytosis, *B. pseudomallei* is able to escape from the phagosome, enter the cytoplasm and remain there.

Moreover, the bacteria can disseminate to nearby cells, leading to the formation of multinucleated giant cells (MNGC). Furthermore, this bacterium can also spread by cell-to-cell contact into the lymphatic system, circulation, and other internal organs (Wiersinga et al., 2018). There is a limitation to the treatment of melioidosis due to the inherent antibiotic resistance of this organism to aminoglycosides and macrolides via the reduction of outer membrane permeability and efflux pump (Kostyanev & Can, 2017). The major efflux pump is the RND family that is widely spread in gram-negative organisms including *B. pseudomallei*. The other antibiotic resistance mechanisms include enzymatic inactivation such as the β-lactamases enzyme which is involved in the degrading β-lactam antibiotics (Schweizer, 2012).

Essential Genes and Novel Target of Melioidosis Treatment

Despite the fact that *B. pseudomallei* is naturally resistant to various antibiotics, the risks of relapse and failure with the current treatment are also high since there are few medicines available for the treatment and no new antibiotic classes have been discovered in recent years. It has become more critical to find new antibiotic targets and alternative therapeutics for melioidosis. Since essential genes are absolutely necessary for an organism to survive, it may be probable to utilize *B. pseudomallei* essential genes as potential antibacterial targets for the development of antibiotics. There are two main categories of essential genes including the core essential genes, which are present in almost all members of the group and require for every living cell, and additional essential genes which are required for the survival of particular cell types (Juhas et al., 2011). There are two chromosomes in *B. pseudomallei*, the larger one, chromosome 1, which mainly contains genes involved in bacterial growth and primary metabolism, and the smaller one, chromosome 2, which contains genes involved in bacterial adaptation in some environmental conditions (Juhas et al., 2011). *BP1026B_I0557* gene is a gene located on chromosome 1 encoding for hypothetical protein with unknown function. *BP1026B_I0557* protein has a subunit homolog to *P. aeruginosa* Type IVA minor pilin protein (Boddey, 2006). A type IV pili is a hair-like structure on the surface of the bacterial cell which can facilitate attachment to the host organism, motility, and biofilm formation (Leighton et al., 2018). The previous study demonstrated that type IV major pilin, pilA deletion in *Burkholderia pseudomallei* reduced attachment to the host cell, decreased virulence in BALB/c mice and nematode worms model, and significantly prolonged living time of animals when compared to the wild-type strain. (Essex-Lopresti et al., 2005). Moreover, the prediction of essential genes using transposon-directed insertion site sequencing (TraDIS) from previous study showed that the Type IV major pilin, PilA, was identified as an essential gene required for *Burkholderia cenocepacia* growth (Wong et al., 2016). Bacterial surface structures are also potential targets for therapeutic intervention. The *BP1026B_I0557* gene and the related genes in this system might be a candidate essential gene for the novel drug targets.

CRISPR-Cas9 and CRISPR Interference (CRISPRi) System

The clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) or CRISPR-Cas9 system is an acquired immune system in prokaryotes that protects them from foreign genetic material, such as bacteriophages. CRISPR-Cas9 has three important components including (1) Cas enzymes for foreign target DNA cleavage, (2) guide RNA (gRNA) that guide Cas protein to target sequences, and (3) a PAM sequence, usually consists of two to six base pairs downstream of the target sequence and is known to prevent cleavage of non-target sequences (Strich & Chertow, 2019; de la Fuente-Nunez & Lu, 2017). There are three steps of the CRISPR-Cas9 adaptive immune system including adaptation, maturation, and interference (Strich & Chertow, 2019). CRISPR interference (CRISPRi), a modified version of CRISPR-Cas9 technology, is composed of dCas9 or dead Cas9, a nuclease-inactive version of Cas9 and sgRNA, a short nucleotide sequence that is complementary to the target sequence which plays a role in guiding dCas9 to target sequence. The CRISPRi system only down-regulates the expression of the targeted genes and does not make a deletion of the chromosome, which can be used to study the functions of essential and non-essential genes. The CRISPRi system was used in gene depletion in the genus

Pseudomonas, which contains high genomic GC content as *B. pseudomallei*. In this system, the *Streptococcus pasteurianus* dCas9 (dCas9-SpaS) was used to suppress a specific protein in *Pseudomonas* spp. (Tan et al., 2018). So, the CRISPRi (dCas9-SpaS) is a simple method to repress the expression of a specific gene in *Pseudomonas* spp. Based on this significance, the following hypothesis is developed: H1: CRISPR interference (dCas9-Spas) can be used in the identification and characterization of the survival function of selected essential genes (*BP1026B_I0557*) in *B. pseudomallei*.

RESEARCH METHODOLOGY

Bacterial strains and culture conditions

Escherichia coli strain DH5α was used for cloning. Avirulent *B. pseudomallei*, Bp1026bΔ*purM*Δ(*amrAB-oprA*) or SCBP27 was used in all experiments as a parental strain. All bacterial strains were grown in Lennox Luria-Bertani (LB) agar or LB broth.

B. pseudomallei SCBP27 was cultured in LB broth or agar supplemented with 40 µg/ml or 80 µg/ml adenine, respectively. The antibiotics were used as the following concentration, 35 µg/ml kanamycin (Km), 15 µg/ml gentamycin (Gm), and 25 µg/ml zeocin (Zeo) for both *E. coli* and *B. pseudomallei* SCBP27. One millimolar IPTG was used in the induction of dCas9 expression.

CRISPR interference (CRISPRi) knockdown system construction

Two plasmids were used in the CRISPRi system including the pUC18-mini-Tn7T-Lac-dCas9-Gm^r, an integrating plasmid containing bacterial Tn7 with *Streptococcus pasteurianus* dCas9 expressed from the P_{tac} promoter, and the pBx-SpaS-sgRNA-Km^r, a plasmid containing *S. pasteurianus* single-guide RNA (sgRNA) with no 20-base pair target sequence (Tan et al., 2018). The *S. pasteurianus* dCas9 was then integrated into *B. pseudomallei* SCBP27 by co-electroporation of the pUC18-mini-Tn7T-Lac-dCas9-Gm^r with the helper plasmid pTNS3 and subsequent selection on 15 µg/ml gentamycin. The strain thus obtained was SCBP67, *B. pseudomallei* SCBP27::dCas9 (Figure 1A). The sgRNA plasmid construction was performed as described previously (Tan et al., 2018). Briefly, the 20-base pair sgRNAs complementary to the target sequence in the *BP1026B_I0557* gene was designed to include 4 base pairs overhangs complementary to the overhangs in *BbsI*-digested pBx-SpasS-sgRNA plasmid. The annealed oligonucleotides sgRNA were then ligated to the *BbsI*-digested pBx-Spas-sgRNA-Km^r plasmid and then electroporated into *B. pseudomallei* SCBP27::dCas9 (SCBP67) and selected on Lennox LB agar supplemented with 35 µg/ml kanamycin (Figure 1B). Two sgRNAs were designed to target the different regions within *BP1026B_I0557* gene. *B. pseudomallei* SCBP67 harboring sgRNA targeting of *BP1026B_I0557* thus obtained was SCBP110 and SCBP122 for sgRNA 1 and 2, respectively.

Bacterial growth assay

The *B. pseudomallei* containing sgRNA plasmids were cultured in LB agar supplemented with appropriate antibiotics at 37 °C for 24 hrs. The single colony was picked and inoculated into LB broth and incubated at 37 °C, 200 rpm agitation, overnight. The overnight culture was then sub-culture into fresh LB broth (1% inoculum) and incubated at 37 °C, 200 rpm until reached the mid-log phase ($OD_{600nm} \cong 0.5$). The mid-log culture was then 10-fold serial diluted and dropped 10 µl each dilution on LB agar with and without 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for induction of dCas9 expression. The plates were incubated for 24 hrs at 37 °C. Moreover, the bacterial growth curve analysis was also performed. Two microliters of overnight culture were inoculated into a sterile 96-well plate containing 200 µl fresh LB broth supplemented with or without 1 mM IPTG. Bacterial growth at 37 °C was recorded every 30 minutes for up to 30 hours using a microplate reader (BioTek Epoch 2 Instrument).

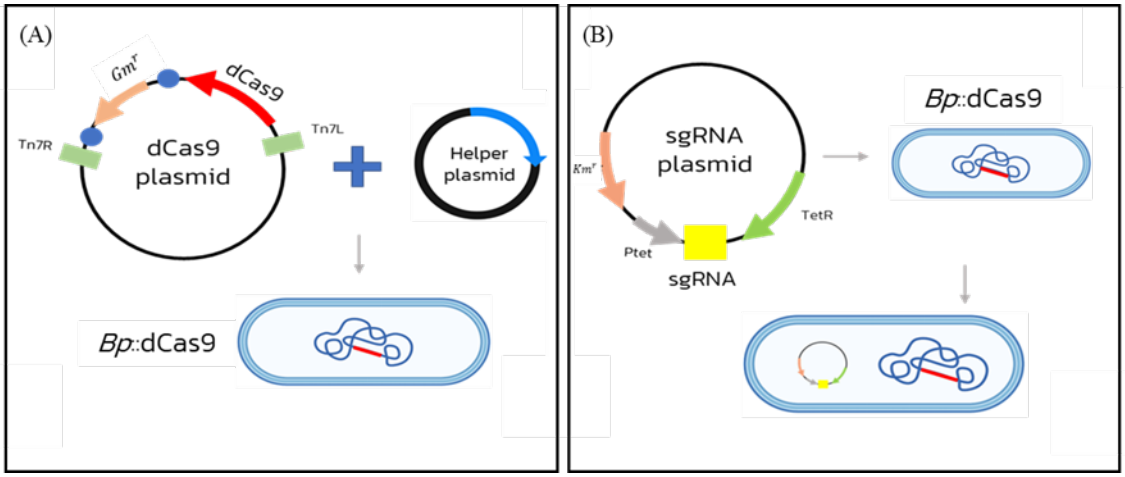


Figure 1 The dCas9 insertion and the introduction of sgRNA plasmid into *B. pseudomallei* SCBP27.
Note: (A) The mini-Tn7T-Lac-dCas9 insertion in *B. pseudomallei* SCBP27. The pUC18-mini-Tn7T-Lac-dCas9-Gm^r plasmid was co-introduced with helper plasmid, pTNS3, into *B. pseudomallei* SCBP27. The mini-Tn7T-Lac-dCas9 was then integrated into the *B. pseudomallei* SCBP27 chromosome at specific *att*Tn7 sites and created the *B. pseudomallei* SCBP67 (*Bp*::dCas9). (B) The introduction of sgRNA plasmid into *B. pseudomallei* SCBP67. The sgRNA plasmid (pBx-SpaS-sgRNA-Km^r) containing 20base pair sgRNA complementary to the target sequence in the *BP1026B_I0557* gene, was introduced into *B. pseudomallei* SCBP67 by electroporation.

RESEARCH RESULTS

Growth assay

To demonstrate whether the *BP1026B_I0557* is an essential gene which plays an important role in *B. pseudomallei* growth and survival. We designed two sgRNA to target the region inside *BP1026B_I0557* gene. All strains including *B. pseudomallei*::dCas9 (Empty vector control), and SCBP27::dCas9//sgRNA targeting *BP1026B_I0557* (I0557 g1, I0557 g2). The result showed that there was no difference in growth between the bacterial cells spotted on the plates with and without 1 mM IPTG induction (Figure 2). To confirm this, the growth curve analysis of all strains was performed, and the result showed that repressing *BP1026B_I0557* gene expression by CRISPRi does not affect growth (Figure 3A, B).

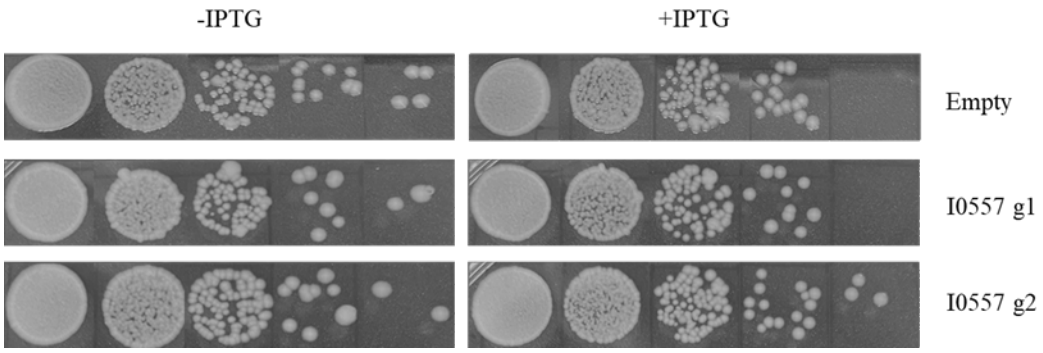
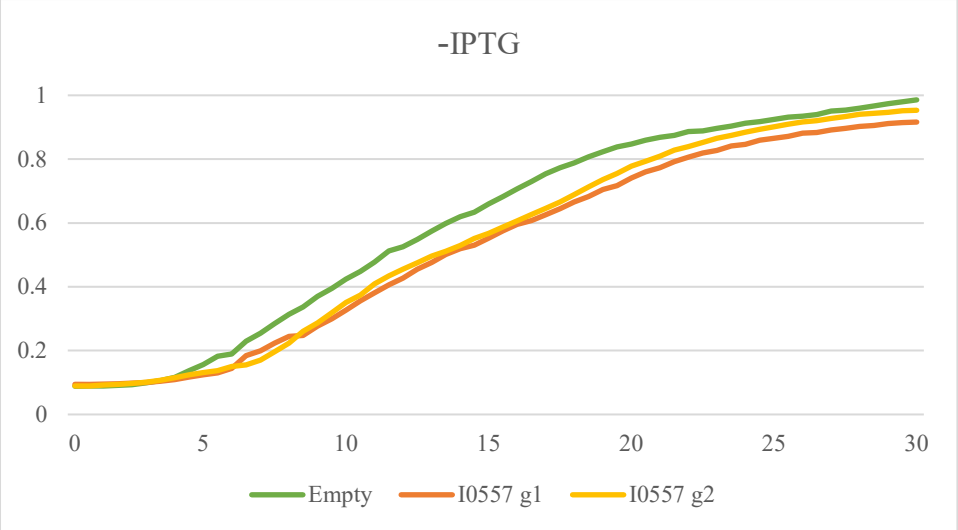


Figure 2 The plates of growth assay of *B. pseudomallei* containing sgRNA targeting *BP1026B_I0557* and empty control were dropped as 10-fold serial dilutions on LB agar with or without 1 mM IPTG



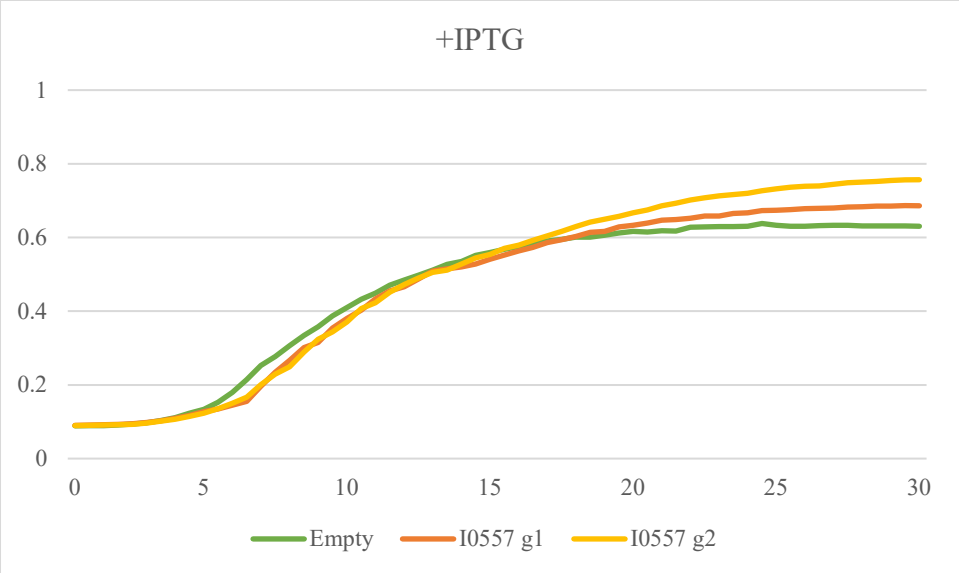


Figure 3 Growth curve analysis of SCBP27::dCas9 (Empty vector control, SCBP67), and SCBP27::dCas9 containing sgRNA targeting *BP1026B_I0557*, I0557 g1 (SCBP110) and I0557 g2 (SCBP122) in Lennox LB broth
Note: After CRISPRi depletion with 1mM IPTG, The *B. pseudomallei* containing sgRNA targeting *BP1026B_I0557* show no growth defect.

DISCUSSION & CONCLUSION

This study aims to discover novel essential genes which encode proteins necessary for *B. pseudomallei* growth and survival and open a new way to the development of new drug targets and alternative therapeutics approach to prevent melioidosis. The *BP1026B_I0557*, the candidate essential gene, was chosen. However, the results showed no growth defect after the gene repression by the CRISPRi system. It might be possible that *BP1026B_I0557* is a non-essential gene. Moreover, the CRISPRi system has limitation factors including the sgRNA designing, the genome editing in high GC-rich bacteria is a challenging process because designing sgRNA for this group of bacteria must be a high GC percentage of sgRNA. More than 60% of GC content sgRNA resulted in low efficiency (Liu et al., 2016). The multiple sgRNA may be required for each gene (Doench, 2017) since the activity of sgRNA can be erratic. Furthermore, the sgRNA targeted of *BP1026B_I0557* should be designed for a multiple site and qRT-PCR should be performed to confirm that the cause of normal growth results after

target gene depletion is *BP1026B_I0557* is a non-essential gene, or the gene can still express after CRISPRi depletion.

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