รายงานวิจัยฉบับสมบูรณ์

โครงการ บทบาทของปัจจัยที่ยับยั้งวงจรของเซลล์จากเชื้อเบอโคลเดอเรีย สูโดมัลลิไอ ต่อการแสดงออกของโปรตีนภายในโฮสต์เซลล์ และความแพร่หลายของปัจจัยที่ยับยั้งวงจรของเซลล์ในเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ

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Pornpan Pumirat

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ระยะเวลาโครงการ : 2 ปี

Cycle inhibiting factor (Cif หรือ ปัจจัยที่ยับยั้งวงจรของเซลล์) คือ bacterial effector ที่พบในเชื้ออีโคไล ชนิด enteropathogenic และ enterohaemorrhagic (EPEC และ EHEC) ซึ่งเมื่อเชื้อแบคทีเรียฉีดโปรตีนนี้เข้าไปในโฮสต์เซลล์ชนิด HeLa โดย Type III secretion system (T3SS) แล้วมีผลทำให้ยับยั้งวงจรแบ่งตัวของโฮสต์เซลล์ อีกทั้งยังทำให้เกิดการสร้าง stress fibres ขึ้นภายในเซลล์ และกระทั่งเหนี่ยวนำให้เซลล์ตายในที่สุด ซึ่งต่อมามีรายงานว่ามี homologue ของ Cif ในเชื้อเบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์เคเก้าหกสองสี่สาม (CHBP) แต่ถึงกระนั้นยังไม่เคยมีการรายงานถึงหน้าที่ของ CHBP กับการติดเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ มาก่อน ดังนั้นในงานวิจัยหนึ่งของเราจึงสร้างเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์ที่ถูกปรับแต่งพันธุกรรมให้ผิดปกติไปจากเดิมบริเวณยีนปัจจัยที่ยับยั้งวงจรของเซลล์โฮสต์ขึ้น และทำการศึกษาจนพบว่าจะมีโปรตีน CHBP ปรากฏในไซโทรพลาสซึมของโฮสต์เซลล์ U937ที่มีการติดเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ โดยทั้งนี้พบว่า secretion ของโปรตีน CHBP ในโฮสตเซลล์ U937 ขึ้นอยู่กับ T3SS ด้วย ยิ่งไปกว่านั้น CHBP ยังอาจมีบทบาทเกี่ยวข้องกลไกก่อโรคของเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ เนื่องจากพบว่า เชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์ที่ถูกปรับแต่งพันธุกรรมให้ผิดปกติไปจากเดิมบริเวณยีนปัจจัยที่ยับยั้งวงจรของเซลล์โฮสต์ ลดความสามารถในการสร้าง plaque formation และ cytotoxicity ของโฮสต์ HeLa เมื่อเปรียบเทียบกับสายพันธุ์ดังเดิม

ส่วนในงานวิจัยนี้ผู้วิจัยทำการศึกษาต่อ โดยเริ่มต้นจากการทำ PCR เพื่อตรวจหายีน CHBP ในเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ จำนวน 32 สายพันธุ์ และยืนยันต่อด้วย Western blot analysis ทำให้ทราบว่าในกลุ่มเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ มีปัจจัยที่ยับยั้งวงจรของเซลล์โฮสต์ หรือ CHBP อยู่ หลังจากนั้นผู้วิจัยได้แบ่งกลุ่มเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ เป็น 2 กลุ่ม คือ กลุ่มที่มี CHBP และไม่มี CHBP แล้วนำไปทดสอบ Plaque-forming efficiency เพื่อศึกษาถึงความสำคัญของการมีปัจจัยที่ยับยั้งวงจรของเซลล์นี้ต่อการทำให้เกิดพยาธิสภาพของเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ แต่ผลการศึกษาพบว่าการมี CHBP ไม่ได้เกี่ยวข้องการการก่อพยาธิสภาพของเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ ดังนั้นผู้วิจัยจึงได้ทำการศึกษาต่อเพื่อที่จะค้นหาว่าแล้ว CHBP มีประโยชน์อะไรกับเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ โดยใช้เทคนิค proteomics เพื่อศึกษาโปรตีนโฮสต์เซลล์ที่ติดเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ ด้วยเทคนิคแมสสเปกโทรเมตรี จากการศึกษาพบว่าโฮสต์เซลล์ที่ติดเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์ที่ถูกปรับแต่งพันธุกรรมมีการปรับเปลี่ยนการสังเคราะห์โปรตีนแตกต่างไปจากโฮสต์เซลล์ที่ติดเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์ดั้งเดิม โดยพบโปรตีนที่มีการแสดงออกเพิ่มขึ้น 97 ชนิด และพบโปรตีนที่มีการแสดงออกลดลงจำนวน 166 ชนิด ในกลุ่มโปรตีนของโฮสต์เซลล์ที่ติดเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์ที่ถูกปรับแต่งพันธุกรรม ซึ่งโปรตีนที่มีการเปลี่ยนแปลงไปนี้มีความเกี่ยวข้องกับกระบวนการทำงานของเซลล์หลายด้าน รวมทั้งด้าน microtubule network และ stress fibre formation นอกจากนี้ผลการศึกษา Pathway analysis ทำให้ทราบว่ามีโปรตีนบางชนิดในกลุ่มนี้มีความเชื่อมโยงกับการก่อพยาธิสภาพในลักษณะเช่นเดียวกันกับเชื้อ EPEC ดังนั้นจากการศึกษานี้ถือได้ว่าเป็นข้อมูลที่สำคัญที่ช่วยทำให้ทราบถึงความสำคัญของ CHBP ที่มีผลต่อการทำงานของโฮสต์เซลล์ โดยอย่างน้อยมีผลในการปรับเปลี่ยนการแสดงออกของโปรตีนภายใน HeLa cells ที่ติดเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ เช่น cytoskeleton rearrangement เพื่อให้เหมาะสมต่อการก่อโรคของเชื้อแบคทีเรีย แต่ถึงอย่างไรยังจำเป็นจะต้องทำการศึกษาเพิ่มเติมเพื่อยืนยันต่อไป

คำหลัก : เบอโคลเดอเรีย สูโดมัลลิไอ, บทบาทของปัจจัยที่ยับยั้งวงจรของเซลล์, โฮสต์เซลล์, การแสดงออกของโปรตีน

Abstract

Project Code : MRG5580040

Project Title : Role of cycle inhibiting factor (Cif) in host protein expression and prevalence of Cif in *Burkholderia pseudomallei*

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Project Period : 2 years

A cycle-inhibiting factor (Cif) is a bacterial effector that present in enteropathogenic and enterohaemorrhagic *Escherichia coli*. Cif is known to be injected into HeLa cells by *E. coli* Type III secretion system (T3SS) and induces cell cycle arrest, stress fibre formation and delayed apoptosis. Subsequently, a homologue of Cif in *B. pseudomallei* (CHBP) was identified. However, the function of *B. pseudomallei* CHBP in pathogenesis of meliodosis disease is still unclear. In our previous study, we constructed *B. pseudomallei chbP* mutant to explore the role of CHBP in *B. pseudomallei* infection. Infection of U937 macrophage with *B. pseudomallei* revealed that host cell contact was required for the secretion of CHBP. Interestingly, CHBP was secreted into the host cell in a manner dependent on the Bsa T3SS. Moreover, the responsibility of CHBP is likely to be important for *B. pseudomallei* pathogenesis as we found that *B. pseudomallei chbP* mutant showed the significant defects in abilities to induce plaque formation and cytotoxicity of HeLa cells when compared to the wild type.

In this study, we found the existence of *cif* homologue gene encoding for Cif in *B. pseudomallei* populations. To investigate the requirement of CHBP for pathogenesis of *B. pseudomallei* infection, *B. pseudomallei* clinical isolates were investigated on their virulence in term of plaque formation efficiency. The result showed that CHBP was not really intended to involve with pathogenicity of *B. pseudomallei*. In addition, we further investigated the host

protein expression profiles of infected HeLa cells using mass spectrometry. Investigation by this technique, we identified 97 up-regulated and 166 down-regulated proteins that significantly altered in HeLa cells infected with *chbP* mutant compared with that of *B. pseudomallei* wild type strain. Among these, there are proteins associated with microtubule network and stress fibre formation. Pathway analysis revealed that some of these proteins are linked with infection of pathogenic *E. coli*. Thus, based upon our finding, it is suggested that CHBP is involved in modulation of host cell process, at least by disturbing the protein expression of host cell cytoskeleton rearrangement, which might be benefit for the pathogenesis of *B. pseudomallei*. However, additional studies are needed to confirm these findings.

Keywords : *Burkholderia pseudomallei*, host protein expression, cycle inhibiting factor (Cif)

Executive Summary

Burkholderia pseudomallei is a Gram-negative bacterium that causes a human infectious disease called melioidosis, which is recognized as a major public health problem in many tropical countries (White, 2003). The endemic areas of melioidosis include northeastern Thailand and northern Australia (Cheng and Currie, 2005). Rice farmers are considered as the high risk of exposure group during the monsoonal and rainy season (Currie and Jacups, 2003), particularly when planting and harvesting in mud and surface water in rice fields. The infection occurs by inoculation through skin abrasions or inhalation. Melioidosis may be presented as acute to chronic infection. Acute infection is often septicemia, resulting in death within days of exposure. Furthermore, *B. pseudomallei* exhibit resistance to diverse groups of antibiotics including third-generation cephalosporins, penicillins, rifamycins, macrolides, quinolones, and aminoglycosides. Owing to its aerosol infectivity, the severe course of infection, and the absence of vaccines and fully effective treatments, *B. pseudomallei* is classified as a hazard category three pathogen and considered a potential biothreat agent (Cheng and Currie, 2005). Currently, a high rate of relapse has been recognized (Frangoulidis *et al.*, 2008). Moreover, recommended antibiotic regimens are expensive, and in severe disease should be prolonged to 20 weeks to reduce the risk of relapse. Therefore, the mechanisms contributing to *B. pseudomallei* virulence need to be elucidated in order to understand the pathogenicity of this organism.

An essential feature of pathogenic *B. pseudomallei* is their ability to invade a number of cells in both phagocytic and non-phagocytic cells and their capability to stimulate a variety of host-cell responses (Jones *et al.*, 1996). After internalisation, *B. pseudomallei* can escape membrane bound phagosome into the cytoplasm (Wiersinga *et al.*, 2006). Inside the cells, *B. pseudomallei* can spread from one cell to another, induce cell-to-cell fusion which resulting in multinucleated giant cell (MNGC) formation (Kespichayawattana *et al.*, 2000). This unique ability also observed in the tissues of patients with melioidosis (Wong *et al.*; 1995). Nevertheless, the molecular mechanisms of *B. pseudomallei* pathogenesis remain unclear.

Several virulence factors of *B. pseudomallei* have been identified including both cell-associated and secreted products. The type III secreted proteins which secreted through the type III secretion systems (T3SSs) have been identified as one of the virulence of many

Gram negative pathogens including *B. pseudomallei* (Hueck, 1998). Type III secretion system (T3SSs) are molecular syringes/needles that inject bacterial virulence proteins directly into host cells. These injected effectors subvert host cellular processes and contribute to disease (Cornelis and Van, 2000). Recently, a new T3SS translocated effector molecule of *Enteropathogenic* and *enterohaemorrhagic Escherichia coli* (EPEC and EHEC) called “Cif” is identified (Marches *et al.*, 2003). This protein is a cycle inhibiting factor, which blocks cell cycle G2/M transition, induces the formation of stress fibres and provokes a delayed cell death (Samba-Louaka *et al*., 2008; Samba-Louaka *et al*., 2009). Interestingly, *E. coli* Cif shows 21% identity and 40% similarity with an ORF in *B. pseudomallei* strain K96243. It has been shown that *B. pseudomallei* Cif homologue (CHBP) can be injected by the EPEC T3SS and induces cell cycle arrest and stress fibre formation in HeLa cells in an identical manner to *E. coli* Cif (Jubelin *et al*., 2009). Sequenced genomes of 10 different *B. pseudomallei* clinical isolates all contain the Cif homolog gene with various % identities, whereas CHBP is absent from genomes of closely related *Burkholderia thailandensis* and *Burkholderia mallei* that usually do not cause human melioidosis (Yao *et al*., 2009). Crystal structure analysis revealed that CHBP possess a papain-like fold with a Cys-His-Gln catalytic triad similar to EPEC Cif (Crow *et al.,* 2009; Yao *et al.,* 2009). Furthermore, a recent study shows that CHBP is recognized strongly by melioidosis patient sera (Philip *et al.*, 2009). Thus, it is possible that CHBP might play a functional role in pathogenesis.

Very recently, *B. pseudomallei cif* knockout mutant has been constructed by our group. We observed that CHBP is expressed only in intra-host cell conditions (Pumirat *et al*., 2014); however, the essential function of CHBP in pathogenesis of meliodosis remains to be clarified. As *E. coli* Cif protein plays an important role in interfering host cell cycle progression (Marches *et al.*, 2003; Nougayrede *et al.*, 2005; Samba-Louaka *et al*., 2009). Therefore, we postulated that the CHBP might alter the expression of host proteins and facilitate the inhibition of host cell cycle. We used the proteomic analysis to compare the proteome patterns of host macrophage cells infected by *B. pseudomallei* wild type and isogenic *cif* mutant strains to achieve this goal. The differences of host protein expression profiles would reflect host cell processes that related to function of CHBP within host cells. In addition, we studied the occurrence and sequence diversity of *chbP* gene in clinical and environmental *B. pseudomallei* isolates. This information could provide the information about

the existence and/or diversity of Cif homologue in *B. pseudomallei* populations, which might required for pathogenesis of *B. pseudomallei* infection. Those isolates were further investigated on their virulence in term of plaque formation efficiency to screen capability of *B. pseudomallei* in invasion and intracellular survival. This data would provide more understanding on the impact of CHBP in virulence and pathology of infection by *B. pseudomallei*. Our findings might provide the key basis for future treatment and management of patients.

INTRODUCTION (บทนำ)

Burkholderia pseudomallei is a Gram-negative bacterium that causes a human infectious disease called melioidosis, which is recognized as a major public health problem in many tropical countries (White, 2003). The endemic areas of melioidosis include northeastern Thailand and northern Australia (Cheng and Currie, 2005). Rice farmers are considered as the high risk of exposure group during the monsoonal and rainy season (Currie and Jacups, 2003), particularly when planting and harvesting in mud and surface water in rice fields. The infection occurs by inoculation through skin abrasions or inhalation. Melioidosis may be presented as acute to chronic infection. Acute infection is often septicemia, resulting in death within days of exposure. Furthermore, *B. pseudomallei* exhibit resistance to diverse groups of antibiotics including third-generation cephalosporins, penicillins, rifamycins, macrolides, quinolones, and aminoglycosides. Owing to its aerosol infectivity, the severe course of infection, and the absence of vaccines and fully effective treatments, *B. pseudomallei* is classified as a hazard category three pathogen and considered a potential biothreat agent (Cheng and Currie, 2005). Currently, a high rate of relapse has been recognized (Frangoulidis *et al.*, 2008). Moreover, recommended antibiotic regimens are expensive, and in severe disease should be prolonged to 20 weeks to reduce the risk of relapse. Therefore, the mechanisms contributing to *B. pseudomallei* virulence need to be elucidated in order to understand the pathogenicity of this organism. An essential feature of pathogenic *B. pseudomallei* is their ability to invade a number of cells in both phagocytic and non-phagocytic cells and their capability to stimulate a variety of host-cell responses (Jones *et al.*, 1996). After internalisation, *B. pseudomallei* can escape membrane bound phagosome into the cytoplasm (Wiersinga *et al.*, 2006). Inside the cells, *B. pseudomallei* can spread from one cell to another, induce cell-to-cell fusion which resulting in multinucleated giant cell (MNGC) formation (Kespichayawattana *et al.*, 2000). This unique ability also observed in the tissues of patients with melioidosis (Wong *et al.*; 1995). Nevertheless, the molecular mechanisms of *B. pseudomallei* pathogenesis remain unclear.

Several virulence factors of *B. pseudomallei* have been identified including both cell associated and secreted products. The type III secreted proteins which secreted through the

type III secretion systems (T3SSs) have been identified as one of the virulence of many Gram negative pathogens including *B. pseudomallei* (Hueck, 1998). Type III secretion system (T3SSs) are molecular syringes/needles that inject bacterial virulence proteins directly into host cells. These injected effectors subvert host cellular processes and contribute to disease (Cornelis and Van, 2000). Recently, a new T3SS translocated effector molecule of *Enteropathogenic* and *enterohaemorrhagic Escherichia coli* (EPEC and EHEC) called “Cif” is identified (Marches *et al.*, 2003). This protein is a cycle inhibiting factor, which blocks cell cycle G2/M transition, induces the formation of stress fibres and provokes a delayed cell death (Samba-Louaka *et al*., 2008; Samba-Louaka *et al*., 2009). Interestingly, *E. coli* Cif shows 21% identity and 40% similarity with an ORF in *B. pseudomallei* strain K96243. It has been shown that *B. pseudomallei* Cif homologue (CHBP) can be injected by the EPEC T3SS and induces cell cycle arrest and stress fibre formation in HeLa cells in an identical manner to *E. coli* Cif (Jubelin *et al*., 2009). Sequenced genomes of 10 different *B. pseudomallei* clinical isolates all contain the Cif homolog gene with various % identities, whereas CHBP is absent from genomes of closely related *Burkholderia thailandensis* and *Burkholderia mallei* that usually do not cause human melioidosis (Yao *et al*., 2009). Crystal structure analysis revealed that CHBP possess a papain-like fold with a Cys-His-Gln catalytic triad similar to EPEC Cif (Crow *et al.,* 2009; Yao *et al.,* 2009). Furthermore, a recent study shows that CHBP is recognized strongly by melioidosis patient sera (Philip *et al.*, 2009). Thus, it is possible that CHBP might play a functional role in pathogenesis. Very recently, *B. pseudomallei cif* knockout mutant has been constructed by our group. We observed that CHBP is expressed only in intra-host cell conditions (Pumirat *et al*., 2014); however, the essential function of CHBP in pathogenesis of meliodosis remains to be clarified. As *E. coli* Cif protein plays an important role in interfering host cell cycle progression (Marches *et al.*, 2003; Nougayrede *et al.*, 2005; Samba-Louaka *et al*., 2009). Therefore, we postulated that the CHBP might alter the expression of host proteins and facilitate the inhibition of host cell cycle. We used the proteomic analysis to compare the proteome patterns of host macrophage cells infected by *B. pseudomallei* wild type and isogenic *cif* mutant strains to achieve this goal. The differences of host protein expression profiles would reflect host cell processes that related to function of *B. pseudomallei* Cif within host cells. In addition, we studied the occurrence and sequence diversity of *cif* gene in clinical and environmental *B.*

pseudomallei isolates. This information would provide the information about the existence and/or diversity of Cif homologue in *B. pseudomallei* populations, which might required for pathogenesis of *B. pseudomallei* infection. Those isolates were further investigated on their virulence in term of plaque formation efficiency to screen capability of *B. pseudomallei* in invasion and intracellular survival. This data would provide more understanding on the impact of CHBP in virulence and pathology of infection by *B. pseudomallei*. Our findings might provide the key basis for future treatment and management of patients.

OBJECTIVES (วัตถุประสงค์)

1. To compare protein expression profiles of human macrophage HeLa cell line infected with *B. pseudomallei* wild type K96243 and *chbP* mutant strains.
2. To examine the occurrence and sequences of *chbP* gene from clinical and environmental *B. pseudomallei* isolates.
3. To study *B. pseudomallei* isolates in aspect of the intracellular survival capability using plague formation assay.

LITERATURE REVIEW (งานวิจัยที่เกี่ยวข้อง)

B. pseudomallei and melioidosis

B. pseudomallei is a soil saprophyte and the causative agent of melioidosis, a potentially fatal disease in both human and animal (White, 2003; Cheng and Currie, 2005). This pathogen is classified as a category B bioterrorism agent by Centers for Disease Control and Prevention (www.bt.cdc.gov/agent/agentlist.asp). *B. pseudomallei* is visualized as a Gram negative bacillus that have bipolar staining characteristic, vacuolated, slender and rounded ends, which is often described as a “safety pin” appearance. The bacteria are small approximately 0.4-0.8 μm in width and 2.0-5.0 μm in length. *B. pseudomallei* have either a single polar flagellum or a tuft of polar flagella (Inglis *et al*., 2003).

Melioidosis, is a serious disease caused by the bacterium *B. pseudomallei*, also called Whitmore’s disease or Nightcliff gardener's disease. In the late half of the 20th century, melioidosis emerged as an infectious disease of major public health importance in

Southeast Asia and northern Australia. In Ubon Ratchathani, Thailand, *B. pseudomallei* accounts for up to approximately 20% of community-acquired bacteremias (Chaowagul *et al.*, 1989). At the Royal Darwin Hospital, Australia, it has been the most common cause of fatal community-acquired bacteremic pneumonia (Currie, 2003; Heng *et al.*, 1998). The most important risk factor for developing severe melioidosis is diabetes mellitus (Currie *et al.*, 2000). Other risk factors include thalassaemia, kidney disease, cystic fibrosis and occupation such as rice paddy farmers (Cheng and Currie, 2005).

Humans can be infected by exposure to *B. pseudomallei* present in soil and surface water in endemic locations (Currie and Jacups, 2003; Currie, 2003). Three modes of acquisition, i.e., inhalation, ingestion, and inoculation, are recognized for *B. pseudomallei*. Infection by inoculation is the major mode of acquisition. Minor wounds to the feet of rice farmers are common during the planting and harvesting seasons, when farmers spend most of the working day wading in mud and surface water. Besides, person-to-person transmission of *B. pseudomallei* is very unusual (Currie, 2003). Zoonotic transmission to humans is extremely unusual, but possible three epizoonotic human infections have been implicated in Australia (Cheng and Currie, 2005; Cheng *et al.*, 2005)

Clinical manifestations of melioidosis are varied from localized infection to fulminant septic melioidosis (Wiersinga *et al.*, 2006). In all series, pneumonia is the most common presentation and is involved in approximately half of all cases. Skin and soft tissue infections are a common manifestation of melioidosis, while bone and joint infections are uncommon and may be difficult to differentiate from other causes of infection (Wang *et al.*, 2003). However, the most severe clinical feature is septic shock, which often associated with bacterial dissemination to distant sites such as the lung, liver and spleen (Wiersinga *et al.*, 2006).

Relapse after apparently successful treatment is well described and is associated with mortality similar to that for the initial episode. In the majority of cases, relapse is due to reactivation of the original infecting strain. Poor adherence to therapy was discussed as important factors associated with a higher risk of relapse (Limmathurotsakul *et al.*, 2011).

In 2004, the genome sequence of *B. pseudomallei* strain K96243 was completed (Holden *et al.*, 2004). *B. pseudomallei* K96243 genome consists of two

chromosomes of 4.07 Mb and 3.17 Mb with an average G+C content of 67.7% and 68.5%, respectively (Nierman *et al.*, 2004), which included at least 5,854 predicted coding sequences (CDSs) within the two chromosomes. The large chromosome carries many genes associated with core functions such as cell metabolism and growth, whereas the smaller chromosome carries more genes encoding accessory functions that trend to require for survival and virulence factors (Holden *et al*., 2004). However, very little is known about the genes associated with *B. pseudomallei* virulence and pathogenicity.

B. pseudomallei is resistant to diverse groups of antibiotics including third-generation cephalosporins, penicillins, rifamycins, macrolides, quinolone, and aminoglycosides, but is usually susceptible to trimethoprim-sulphmethoxazole, chloramphenicol, amoxicillin-clavalanate, doxycycline, ureidopenicillins, ceftazidime and carbapenems (Cheng and Currie, 2005). The treatment is required for 20 weeks to reduce the risk of relapse and is divided into intravenous and oral phases. Cetazidime is often used as a parental antibiotic treatment. Moreover, intravenous amoxicillin-clavulanate is also widely used in Thailand for the treatment of empirical sepsis and melioidosis (Dance *et al.*, 1989). Currently, there is no vaccine against meliodosis. Therefore, the best way to handle with melioidosis may be to prevent *B. pseudomallei* infection.

Host immune responses to *B. pseudomallei* infection are divided into cell-mediated and humoral immune responses. Cell-mediated responses are usually adaptive against intracellular pathogens, however; the mechanisms of immunity to *B. pseudomallei* in humans are poorly understood. In addition, macrophages exposed to *B. pseudomallei* do not appear to respond in the same way as they do to other pathogens; in one study, lower levels and slower production of inducible nitric oxide synthase and tumor necrosis factor alpha were seen in a macrophage cell line compared to those after exposure to *Escherichia coli* and *Samonella enterica* serovar Typhi (Utaisincharoen *et al.*, 2000; Utaisincharoen *et al.*, 2001).

Cycle inhibiting factor (Cif)

The cycle inhibiting factor or Cif belongs to a family of bacterial toxins “cyclomodulins”, which is a repertoire of proteins that use the T3SS to be injected into the

host cell (Marches *et al.*, 2003). Cif was identified as a novel T3SS translocated effector molecule of *Enteropathogenic* and *enterohaemorrhagic Escherichia coli* (EPEC and EHEC) that modulate host cell cycle (Marches *et al.*, 2003). Cif consists of a 282-amino–acid protein with predicted molecular mass of 32 kDa. This protein is the first effector molecule not encoded on the locus of enterocyte effacement (LEE) but on a lambdoid phage that present in most EPEC and EHEC strains (Marches *et al.*, 2003).

Upon injection into the host cell by the T3SS of EPEC, Cif effector protein blocks cell cycle G2/M transition and induces the formation of stress fibres through the recruitment of focal adhesions (Nougayrede *et al.*, 2005). Samba-Louaka *et al*. (Samba-Louaka *et al*., 2008) reported that bacterial cyclomodulin Cif blocks the host cell cycle by stabilizing the cyclin-dependent kinase inhibitors p21 and p27. Moreover, EPEC Cif not only induces cell cycle arrest but also eventually provokes a delayed cell death (Samba-Louaka *et al*., 2009). Recently, the effector Cif has been demonstrated to interfere with the eukaryotic cell cycle by inhibiting the function of cullin RING E3 ubiquitin ligases, which associated with Nedd8-Induced conformational control (Morikawa *et al.,* 2010; Boh *et al.*, 2011).

Interestingly, recent study revealed a family of Cif homologues from *Yersinia*, *Photorhabdus,* and *Burkholderia* (Jubelin *et al*., 2009). All members of Cif family can be injected by the EPEC T3SS and harbor a sufficient and potent G2/M arrest activity (Yao *et al.,* 2009). Cif family of T3SS effectors inhibits the eukaryotic ubiquitination pathway by specific deaminase activity toward ubiquitin and NEDD8 (Cui *et al.,* 2009). Furthermore, crystal structure analysis revealed that *B. pseudomallei* Cif (CHBP) possess a papain-like fold with a Cys-His-Gln catalytic triad similar to EPEC Cif (Crow *et al.,* 2009; Yao *et al.,* 2009).

The putative *cif* homolog (*chbP*; locus_tag BPSS1385) of *B. pseudomallei* strain K96243 was identified on chromosome 2. The putative 987 base pairs of *B. pseudomallei chbP* gene encoded the predicted CHBP of 328 amino acids. Interestingly, sequenced genomes of 10 different *B. pseudomallei* clinical isolates showed the presence Cif homolog gene with various % identities, whereas CHBP is absent from genomes of closely related *Burkholderia thailandensis* and *Burkholderia mallei* that usually do not cause human melioidosis (Yao *et al*., 2009). There is an evidence shows that CHBP is recognized

strongly by melioidosis patient sera (Philip *et al.*, 2009). However, the underlying mechanism of Cif family remains to be deciphered.

Study of *B. pseudomallei* by proteomics

Proteomics is the systematic study of proteome expressed by the genome of the cell tissue, or organism (Yates *et al.*, 2009). Whereas genome of an organism (or cell) is constant and static, proteome is dynamic and specific. The major goal of proteomics is to understand the protein complement of cells, including their identification, modification, quantification and localization. Several methods are currently available to assist these goals and the most recent technique for proteomic strategies is mass spectrometry (MS) (Yates *et al.*, 2009). With high-resolution of MS, thousands of proteins and protein fragments on the basis of their molecular weights and electrical charges can be sorted out.

The achievements of proteomics allow us to understand different biological processes and basis for several pathogens (Karvunidis *et al.*, 2009; Windle *et al.*, 2010). Likewise, the use of proteomic tools has increased gradually to determine the underlying pathology caused by *B. pseudomallei* infection. For example, Wongtrakoongate *et al.* (Wongtrakoongate *et al.*, 2007) compared protein profiles of *B. pseudomallei* and *B. thailandensis* in order to identify virulent characters of *B. pseudomallei* by used 2D (2-dimentional) gel electrophoresis. They found 12 out of 14 protein spots are detected in *B. pseudomallei* that they might be involved in virulence of *B. pseudomallei*. In addition, to identify immunogenic surface proteins of *B. pseudomallei*, Harding *et al.* (Harding *et al.*, 2007) have also employed proteomic-based approaches. Their study demonstrated that this detection of surface located proteins identified 35 proteins, while screening with human sera identified 12 immunogenic proteins.

Thongboonkerd *et al.* (Thongboonkerd *et al.*, 2007) have used a 2D-based proteomics for comparison of the proteomes of *B. pseudomallei* wild-type with the rpoE operon knockout mutant. RpoE, is an alternative sigma factor sigma E, plays functional role in stress tolerance and survival of *B. pseudomallei*. Significantly differential expression of 52 proteins between two strains reflects the mechanisms of the rpoE operon of *B. pseudomallei* under stress conditions. Besides, Osiriphun *et al.* (Osiriphun *et al.*, 2009) identified the sigma factor RpoS regulon of *B. pseudomallei* using a proteomics approach, which revealed 70

differentially expressed proteins between rpoS(+) and rpoS(-) strains. Their data provide the information of RpoS regulation in *B. pseudomallei*, whose RpoS regulon differs from other Gram-negative bacteria.

Furthermore, one of my studies employed a classical proteomics approach to examine alterations in secreted proteins (secretome) of *B. pseudomallei* under a salt stress (Pumirat *et al.*, 2009). The secreted protein profile of *B. pseudomallei* in salt-rich medium demonstrated that *B. pseudomallei* express several proteins that involved with several metabolic enzymes, stress response proteins, beta-lactamase like proteins and potential virulence factors. This finding showed that salinity has the prospective to influence the virulence of *B. pseudomallei*. Recently, comparison of the outer membrane proteome of *B. mallei* and *B. pseudomallei* has been studied (Schell *et al.*, 2011). This study showed the differences of proteins that perhaps reflect the evolution and adaptation of these two strains.

Although *B. pseudomallei* research during the past few decades provides a lot of information on exploring pathogenic and molecular mechanisms of this bacterial infection, little is known about informative research in term of *B. pseudomallei*-infected host cells. So, focusing on *B. pseudomallei* and host interaction would be crucial for successful prevention and/or treatment of this infectious disease.

MATERIALS AND METHODS (วัสดุและวิธีการทดลอง)

1. Bacterial strains, cell line and growth condition

B. pseudomalli and isogenic *cif* mutant were cultured on Luria-Bertani medium and grown at 37^{o}C. The HeLa cells (human cervical carcinoma) were routinely grown and maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco-BRL, NY, USA) and incubated at 37°C in a humidified incubator in the presence of 5% CO_2. The DMEM medium was supplemented with 10% heat-inactivated (30 min, 56°C) fetal bovine serum (FBS; HyClone, Logan, UT, USA).

2. Proteomic study

2.1 Infection condition

The HeLa cells were used as host model in this study. Briefly, HeLa cells were infected with *B. pseudomallei* wild type and isogenic *chbP* mutant strain. Two hrs after infection at 37°C with 5% CO_2 for 2 h, the infected cell monolayers were washed. Thereafter, the extracellular bacteria were killed with kanamycin (250 μg/ml) in the overlay for another 2 hrs. The infected cells were washed with prewarmed PBS and incubated in the culture medium containing a lower concentration of kanamycin (20 μg/ml) to inhibit the growth of residual extracellular bacteria. After 6 hrs of infection, the infected HeLa cells were lysed with Triton-X reagent. The lysates of HeLa cells were used for proteomic analysis.

2.2 In solution digestion

The sample was resuspended in 50 mM ammonium bicarbonate. The cystine was reduced and alkylated using 5 mM dithiothreitol (DTT) and 20 mM iodoacetamide, respectively. Excess iodoacetamide was quenched with the addition of DTT to a final concentration of 10 mM. Trypsin was added at a ratio of 1/50 (w/w) and incubated at 37 ºC overnight. The sample was collected and ready to be used for further process.

2.3 Strong cation exchange chromatography (SCX)

Tryptic peptides were fractionated by the strong cation exchange chromatography using a polysulfoethyl column (PolyLC, MD, USA). Fractions were typically collected over a 30 min linear gradient from 100% A, 0% B to 10% A, 90% B using the following mobile phases: solution A, 20% (v/v) acetonitrile, 0.1% (v/v) formic acid; solution B, 20% (v/v) acetonitrile, 0.1% (v/v) formic acid, 1 M potassium chloride.

2.4 Mass spectrometry analysis

A QToF was used to analysed protein digests; it was coupled to a reversed phase liquid chromatography capillary column. Solution A consists of 2% (v/v) acetonitrile, 0.1% (v/v) formic acid in HPLC grade water. Solution B consists of 0.1% (v/v) formic acid in HPLC grade acetonitrile. The 58 min gradient was performed. The eluent was sprayed from an emitter using a capillary voltage of 22 to 28 kV into the Z-spray™ nano-electrospray source of

the QToF. The cone was at 100 V. Source temperature is at 85°C. Microchannelplates detector (MCP) was 2300 V. The survey scan mode covered the mass range of *m/z* 400-2000. Three most abundant precursors were selected to fragment for 3 s. The MS/MS spectra was covered the mass range of *m/z* 50-1500.

2.5 Data analysis

The LC-MS/MS data file was converted into smoothed and centroided mascot generic file (.mgf) using DataAnalysis™ software. The .mgf file contained *m/z* of precursor ion, *m/z* of corresponding fragment ions, signal intensities and charge state. The file was then searched against the in-house Mascot version 2.2 to obtain the identification and quantification of proteins.

A decoy algorithm was used to estimate the number of false positives. Missed cleavages were set to 2 with peptide tolerance set to 100 ppm and tandem MS tolerance set to 0.3 Da. Variable modifications were set to include methionine oxidation. Only peptides identified above 95% confidence were reported in this research. Each identified peptide was searched against BLAST (Basic Local Alignment Search Tool) for considering isoforms of proteins (www.ncbi.nlm.nih.gov/BLAST). Normalization was introduced for abundance of proteins in the sample. Quantification was performed using emPAI values which are provided by the Mascot.

3. Detection of *cif* gene

A polymerase chain reaction (PCR) technique was used to investigate the presence of *cif* gene in *B. pseudomallei* populations isolated from clinical and environmental samples. The amplification condition of the *cif* gene was optimized using specific pair primers, which are the Cif-forward primer (5'ATGCTACTATTGTTGGAGCACG3') and Cif-reverse primer (5'ACATCTGCTGCGGTCTCAC3'). The reaction mixture was separated by electrophoresis on agarose gel. Negative and positive controls were used in all assays. The amplicons of *cif* gene from those isolates were sequenced to detect the presence of mutation.

4. Plaque formation assay

The HeLa cells were infected with *B. pseudomallei*. Two hrs after infection, the infected cell monolayers were washed and overlaid with a 0.5% agarose medium containing D-glucose (4.5 mg/ml) and kanamycin (250 μg/ml). The plates were incubated at 37°C in a humidified 5% CO_2 atmosphere for overnight. To enhance visualization of the plaques, another similar agarose overlay containing in addition 0.01% neutral red was added, and the plaques will be observed 4 hrs later.

Plaque-forming efficiency = number of plaques/bacterial CFU added per well

RESULTS (ผลการดำเนินงาน)

ส่วนที่ 1 การศึกษาความแพร่หลายของปัจจัยที่ยับยั้งวงจรของเซลล์ในเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ

1. ออกแบบและสังเคราะห์ oligonucleotide primers สำหรับ *chbP* gene

ผู้วิจัยได้ออกแบบ oligonucleotide primers สำหรับยีนที่ encoded for CHBP หรือ ปัจจัยที่ยับยั้งวงจรของเซลล์ของเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ (ตารางที่ 1) จาก gene sequence ของ เชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์ K96243 ที่มีอยู่ในฐานข้อมูล (รูปที่ 1) ผู้วิจัยพบว่า full length gene ดังกล่าวจะมีขนาด 1,059 base pairs (bp)

ตารางที่ 1 แสดง oligonucleotide primers สำหรับ amplify Cycle-inhibiting factor (Cif) หรือ ปัจจัยที่ยับยั้งวงจรของเซลล์ของเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ ในการศึกษาครั้งนี้

Primer name	Oligonucleotide sequence (5'-3')
Cif-forward primer	ATGCTACTATTGTTGGAGCACG3
Cif-reverse primer	ACATCTGCTGCGGTCTCAC3

>NC_006351.1:c1894828-1893842 Burkholderia pseudomallei K96243 chromosome 2
TTGTTGGAGCACGGCGTCATGAAAATCCCGGGTATTAACAACGTCGGAAAAACTGGACAGGCCGGCGGAG
AAACCGAGCGTATACCATCGACCGAACCTTTGGGGTCCAGCGCCGCGACCAGTCCCGCCGGTCCACTTGG
TGGGCTGCCAGCACGTTCGTCGAGCATCTCGAACACTAACCGAACTGGCGAAAATCCTATGATAACGCCG
ATCATTTCATCGAACCTCGGGTTGAAGCATAGAGTTACCTTGCGCAAAGCTACGCTAGCCAGCCTAATGC
AGTCCCTGAGCGGGGAAAGCAGTAACCGCGTAATGTGGAACGACCGATACGACACGCTGCTTATTGCGCG
CGACCCGAGAGAAATAAAAAACGCCATTGAGAAAAGCGTCACCGACTTTGGTGGGTTGGAAAACTATAAG
GAACTTACGGGGGGCGCAGATCCATTTGCTCTGATGACGCCCGTGTGCGGACTTTCGGCCAACAACATCT
TCAAACTCATGACAGAGAAGGACGTTCCGATCGATCCAACGTCTATTGAATACCTGGAGAATACATCATT
CGCCGAACACGTGAATACGCTTGATTCGCATAAAAATTACGTGGTAATAGTCAATGACGGTCGACTTGGG
CATAAATTCTTGATCGACTTGCCCGCCCTGACCCAGGGGCCTCGCACGGCATATATCATTCAGTCTGATC
TCGGCGGTGGAGCACTGCCGGCAGTCAGGGTAGAGGACTGGATAAGCCGCCGCGGCAGTGACCCAGTGTC
GCTCGACGAACTAAATCAGCTATTGTCGAAGGATTTTTCAAAAATGCCCGACGATGTGCAGACGCGTTTG
CTAGCTTCCATTCTGCAAATCGACAAGGATCCACATAAAGTCGATATCAAAAAATTGCACCTCGATGGGA
AACTGAGATTCGCATCGCACGAATACGATTTTCGTCAATTTCAACGGAATGCACAATACGTCGCCGGCCT
TGGCTAG

รูปที่ 1 แสดง nucleotide sequence ของ *chbP* หรือ ปัจจัยที่ยับยั้งวงจรของเซลล์ของเชื้อ เบอโคล-เดอเรีย สูโดมัลลิไอ K96243 ที่มีในฐานข้อมูล (NCBI Reference Sequence: NC_006351.1)

2. การสำรวจความแพร่หลายของปัจจัยที่ยับยั้งวงจรของเซลล์โดยเทคนิค PCR

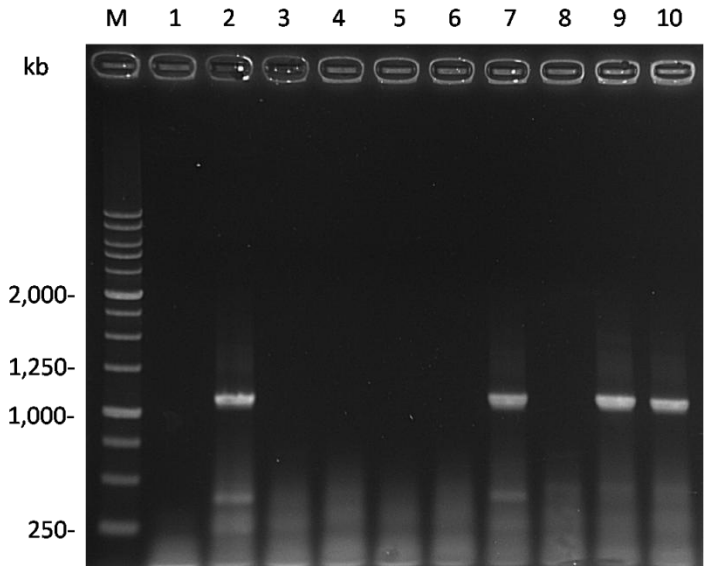
เพื่อสำรวจความแพร่หลายของปัจจัยที่ยับยั้งวงจรของเซลล์ในเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ ด้วยเทคนิค PCR ได้เริ่มต้นทำการศึกษาโดยทำการสกัด Genomic DNA จากเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์ K96243 และสายพันธุ์อื่นๆที่คัดแยกได้จากคนไข้อีกจำนวน 8 สายพันธุ์ เพื่อใช้เป็น DNA ต้นแบบโดยใช้ชุดสกัด DNA (Qiagen) จากนั้นทำ PCR ด้วย primers ที่ได้ออกแบบไว้ คือ Cif-forward primer และ Cif-reverse primer (ตารางที่ 1) ใน PCR mixture และ Thermal cycles ดังระบุไว้ในตารางที่ 2 และ 3 ซึ่งทำให้ผู้วิจัยพบชิ้นส่วน DNA จากการ amplify ที่มีขนาด 1,059 base pairs ดังแสดงในรูปที่ 2 และจากการทำ PCR นี้ในเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ จำนวน 32 สายพันธุ์ ทำให้ทราบว่าบางสายพันธุ์เท่านั้นที่มียีน *chbP* ซึ่งสายพันธุ์เหล่านั้นได้แก่ K96243, 1530, 1026b, 1634, 576, H2820, H2613, H2148, H2144, H1882, H1248, NR9923, NR9910, AUS640, AUS387, AUS373 และ AUS44 และสายพันธุ์ที่ตรวจไม่พบยีน *chbP* ได้แก่ 1106, 1066, 406, 164, H2747, H2644, H2454, H1244, NR9922, NR9921, NR9914, AUS668, 33DM, BA30 และ BA14

ตารางที่ 2 แสดง PCR mixture (25 μl) สำหรับ amplify ยีน *chbP* หรือ ปัจจัยที่ยับยั้งวงจรของเซลล์ของเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ ในการศึกษาครั้งนี้

Ingredient	Volume (μl)	Final concentration
Sterile ultra-pure distilled water	15.8	-
PCR buffer (10x)	2.5	1x
PCR enhancer (10x)	2.5	1x
50 mM $MgCl_2$	0.75	1.5 mM
10 mM dNTP (2.5 mM each)	0.25	0.2 mM
Cif-forward primer (10 μM)	1	0.4 μM
Cif-reverse primer (10 μM)	1	0.4 μM
DNA polymerase	0.2	1.0 unit
DNA template	1	20 μg

ตารางที่ 3 แสดง Thermal cycles สำหรับ amplify ยีน *chbP* หรือ ปัจจัยที่ยับยั้งวงจรของเซลล์ของเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ ในการศึกษาครั้งนี้

Step	Temperature (°C)	Duration
First Denaturation	95	5 minutes
35 cycle of Denaturation,	95	45 seconds
Annealling,	50	45 seconds
and Extension	72	1 minutes
Final Extension	72	7 minutes



รูปที่ 2 การเพิ่มจำนวนของยีนปัจจัยที่ยับยั้งวงจรของโฮสต์เซลล์จากเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ โดยวิธี PCR ที่ถูกแยกโดยกระแสไฟฟ้า บน 1% agarose gel

M คือ DNA มาตรฐาน

1 คือ negative control

2 คือ เชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์ดั้งเดิม K96243

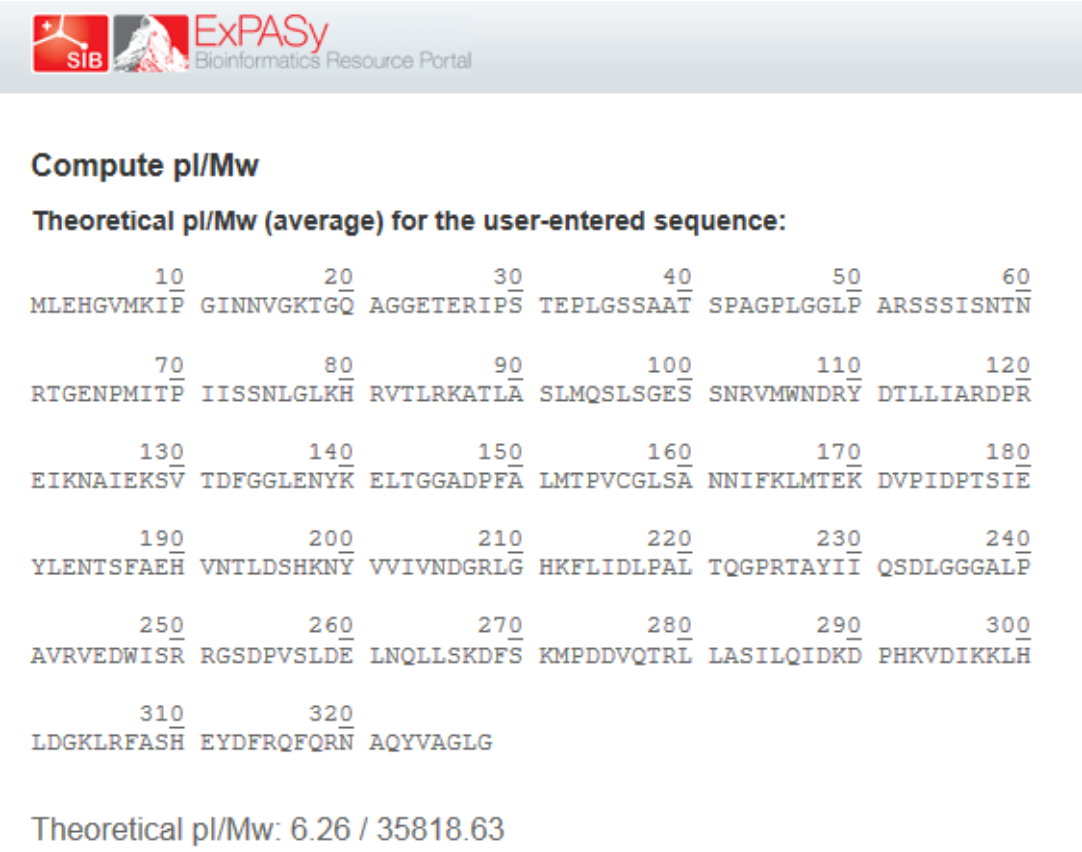
3-10 คือ เชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์อื่นๆ

3. การตรวจหาโปรตีน CHBP โดยเทคนิค Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) และ Western blot analysis

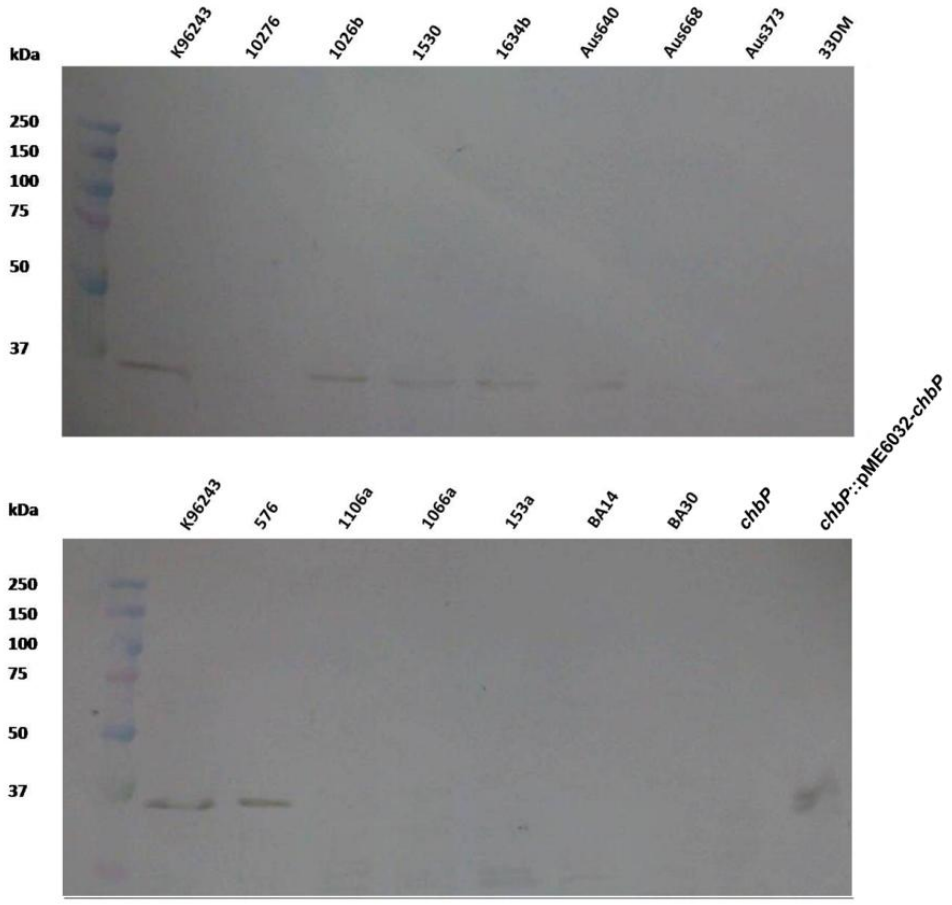
ขั้นตอนแรกเริ่มจากทำการเตรียม Protein lysate ของเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์ K96243 และสายพันธุ์อื่นๆที่คัดแยกได้จากคนไข้อีกจำนวน 14 สายพันธุ์ ด้วย B-PER® II Reagent (Pierce, Rockford, USA) จากนั้นทำการคัดแยกโปรตีนด้วยเทคนิค SDS-PAGE แล้วย้ายโปรตีนจากเจล (Acrylamide gel) ลงบนแผ่นไนโตรเซลลูโลส (Nitrocellulose, NC) ด้วยสนามไฟฟ้า แล้วทำการ block พื้นที่ว่างบน NC ด้วยสารละลาย phosphate buffered saline (PBS) ที่มีส่วนผสมของ 5% Boline serum albumin (BSA) แล้วทำการตรวจหาโปรตีน CHBP โดยใช้ rabbit anti-CHBP polyclonal antibody (Pumirat et al. 2014) ที่เจือจาง 1:500 ตามด้วยการตรวจติดตาม bound antibody ด้วย Horseradish peroxidase (HRP)-conjugated mouse anti-rabbit IgG (DAKO, USA) ที่เจือจาง 1:500 1:3,000 และใช้ substrate เป็น chromogenic substrate-3, 3'-diaminobenzidine (DAB; Sigma Chemical Co., USA)

เบื้องต้นจากการวิเคราะห์ Protein sequence ของโปรตีน CHBP ทำให้ทราบโปรตีนจะมีขนาดประมาณ 35.8 kDa ดังแสดงในรูปที่ 3 และจากการทดสอบด้วย SDS-PAGE และ Western blot analysis โดยใช้ *chbP* mutant เชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ กลายพันธุ์ที่ไม่สามารถผลิตโปรตีน CHBP เป็น negative control และใช้ *chbP*::pME6032-*chbP* เชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ กลายพันธุ์ที่สามารถผลิตโปรตีน CHBP ในปริมาณมากเป็น positive control ผู้วิจัยพบว่าเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์ K96243 ผลิตโปรตีน CHBP ขนาดที่คาดไว้คือประมาณ 35 kDa นอกจากนี้ยังพบเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ อื่นๆอีก 7 สายพันธุ์ อันได้แก่ 1026b, 1530, 1634b, 576, Aus640, Aus668 และ Aus373 ที่สามารถผลิตโปรตีน CHBP ได้ แต่ไม่สามารถตรวจพบโปรตีนดังกล่าวในเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์ 10276, 33DM, 1106a, 153a และ BA14 ดังแสดงในรูปที่ 4

จากกิจกรรมนี้ยืนยันได้ถึงความแพร่หลายของปัจจัยที่ยับยั้งวงจรของเซลล์ในเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ ในบางสายพันธุ์ที่มียีน *chbP* ซึ่งตรวจด้วยเทคนิค PCR นอกจากนี้แสดงให้เห็นว่าเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์ที่มียีน *chbP* สามารถผลิตโปรตีน CHBP ได้จริง



รูปที่ 3 แสดง Amino acid sequence and Theoretical pI/MW of CHBP



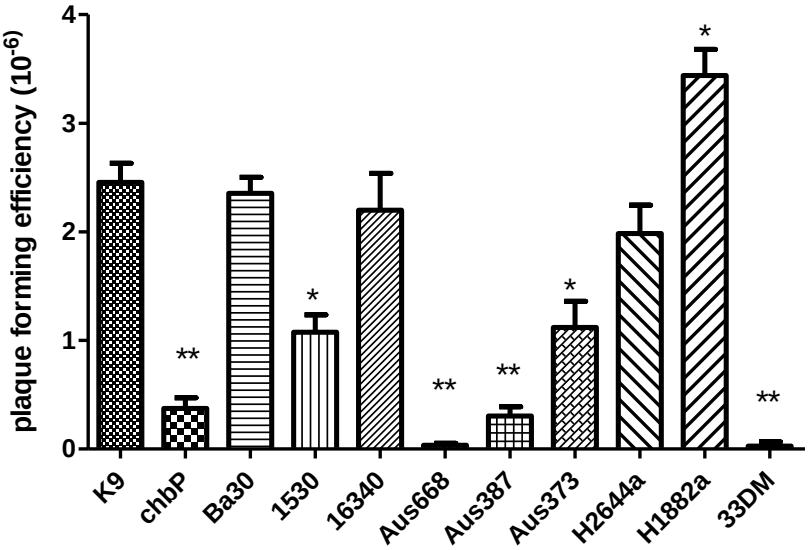
รูปที่ 4 Western blot analysis เพื่อวิเคราะห์โปรตีน CHBP ของเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ Left lane คือ Pre-stained SDS-PAGE Standard Broad Range โดยที่ตัวเลขด้านซ้ายมือคือขนาดโปรตีน เป็น kDa

ส่วนที่ 2 การศึกษาบทบาทของปัจจัยที่ยับยั้งวงจรของเซลล์ต่อ plaque-forming efficiency ในเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ

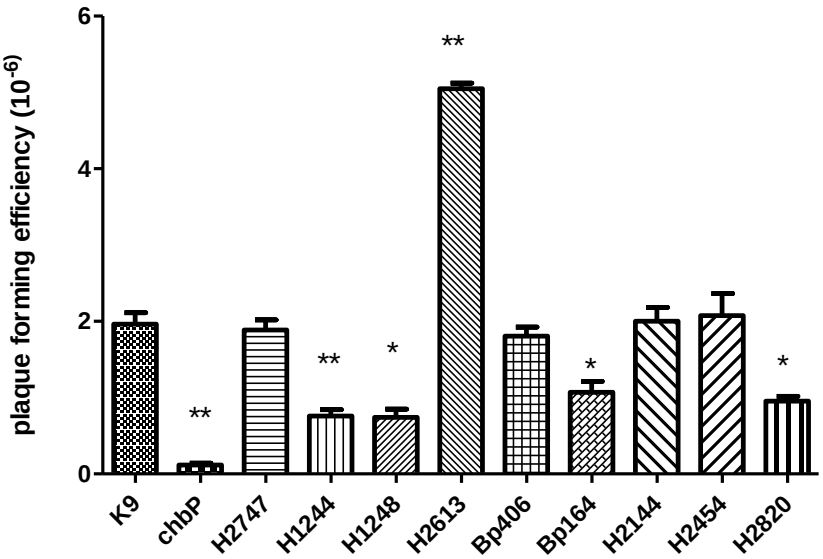
เนื่องจากการศึกษานี้พบว่าเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ บางสายพันธุ์เท่านั้นที่มีปัจจัยที่ยับยั้งวงจรของเซลล์ (หรือ CHBP) ผู้วิจัยจึงทำศึกษาถึงความสำคัญของการมีปัจจัยที่ยับยั้งวงจรของเซลล์นี้ ต่อการทำให้เกิดพยาธิสภาพของเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ โดยการทดสอบความสามารถในการสร้าง plaque ของเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ ระหว่างกลุ่มที่มี CHBP และ ไม่มี CHBP และผลการทดสอบเป็นดังรูปที่ 5 (A-F) ซึ่งแสดงให้เห็นว่าการมีปัจจัยที่ยับยั้งวงจรของเซลล์ (หรือ CHBP) ไม่เกี่ยวข้องกับการสร้าง plaque ของเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ

A

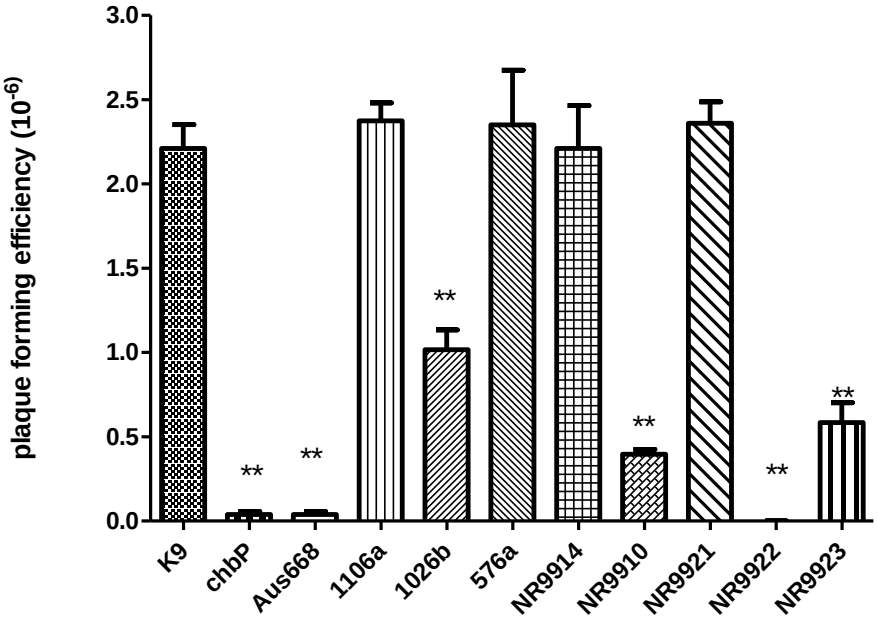
B



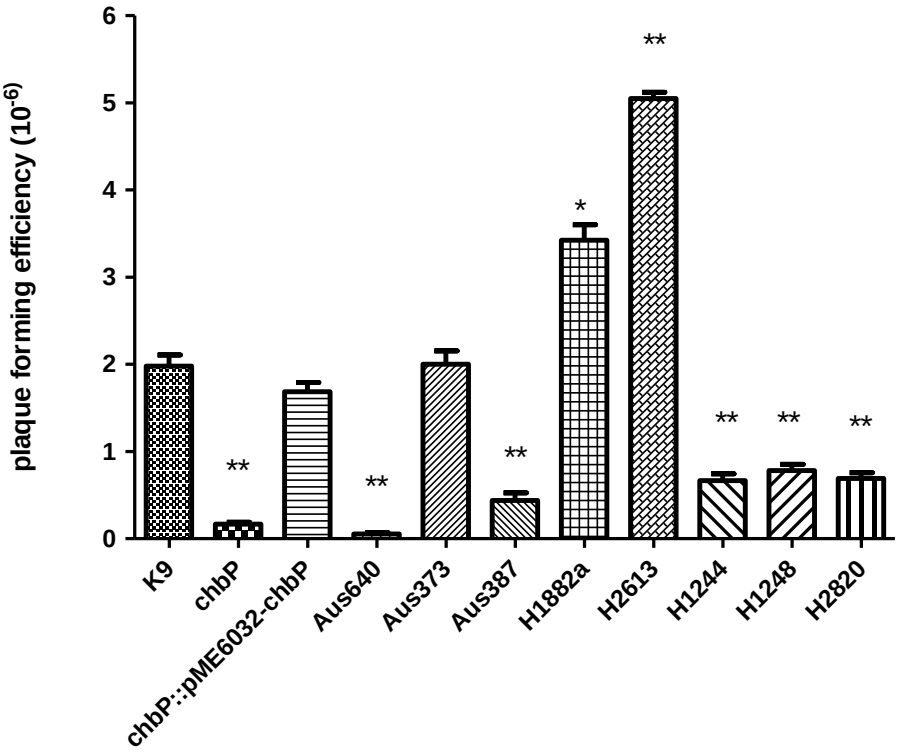
C


plaque forming efficiency (10-6)
6
4
2
0
**
**
**
*
*
*
K9
chbP
H2747
H1244
H1248
H2613
Bp406
Bp164
H2144
H2454
H2820

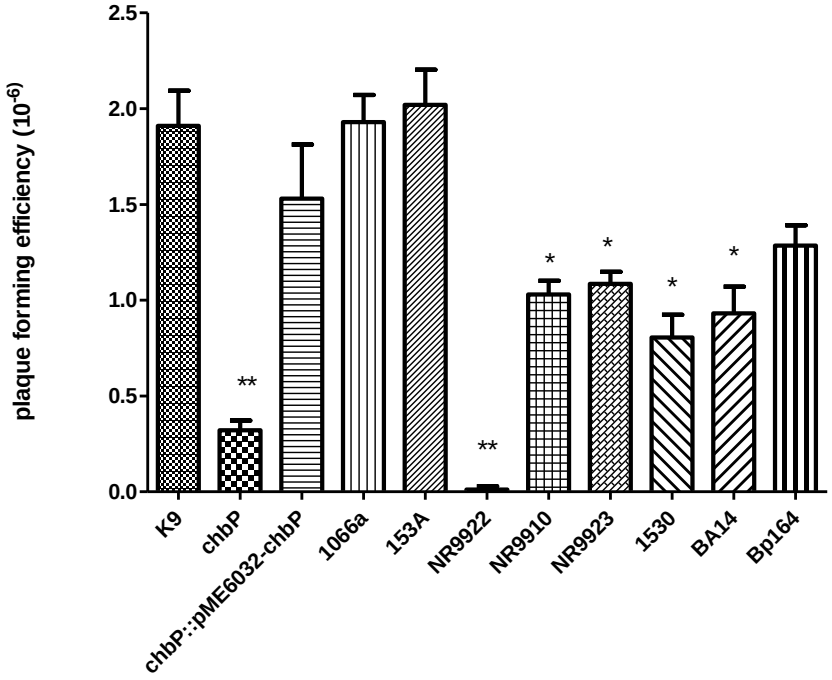
D


plaque forming efficiency (10-6)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
**
**
**
**
**
K9
chbP
Aus668
1106a
1026b
576a
NR9914
NR9910
NR9921
NR9922
NR9923

E


plaque forming efficiency (10^{-6})
6
5
4
3
2
1
0
**
**
*
**
**
**
**
**
K9
chbP
chbP::pME6032-chbP
Aus640
Aus373
Aus387
H1882a
H2613
H1244
H1248
H2820

F


plaque forming efficiency (10^{-6})
2.5
2.0
1.5
1.0
0.5
0.0
**
**
*
*
*
*
K9
chbP
chbP::pME6032-chbP
1066a
153A
NR9922
NR9910
NR9923
1530
BA14
Bp164

รูปที่ 5 แสดงผล Plaque-forming efficiency ของเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ

A: ภาพตัวอย่างการทดลอง Plaque-forming efficiency ของเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์ K96243 และ สายพันธุ์ *chbP* เทียบกับ uninfection control

B-F: ค่าเฉลี่ย Plaque-forming efficiency ของเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอสายพันธุ์ต่างๆ โดยใช้เชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ กลายพันธุ์ (*chbP*) ที่ไม่สามารถผลิตโปรตีน CHBP เป็น negative control และใช้เชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์ K96243 และสายพันธุ์กลายพันธุ์ที่สามารถผลิตโปรตีน CHBP ในปริมาณมาก (*chbP*::pME6032-*chbP*) เป็น positive control

ส่วนที่ 3 การศึกษาบทบาทของปัจจัยที่ยับยั้งวงจรของเซลล์จากเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ ต่อการแสดงออกของโปรตีนภายในโฮสต์เซลล์ ด้วยเทคนิค proteomic analysis

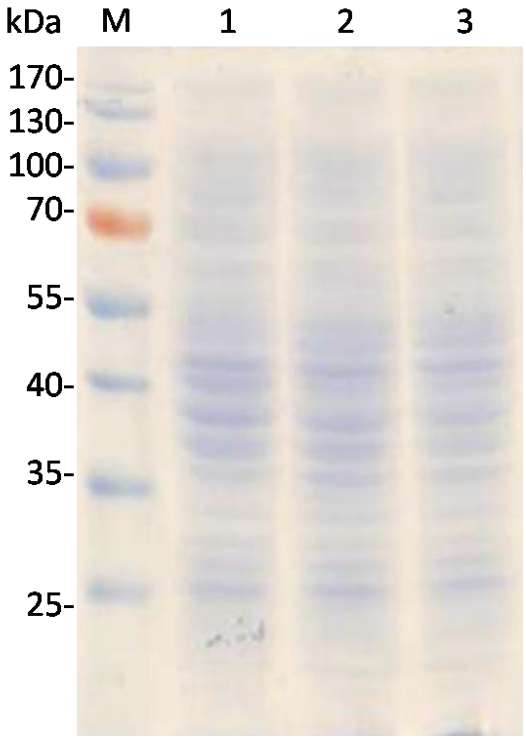
1. การเตรียม lysate ของโฮสต์เซลล์ และการวัดปริมาณโปรตีน

เนื่องจากหลายการศึกษาก่อนหน้าได้รายงานกลไกการทำงานของปัจจับที่ยับยั้งวงจรในโฮสต์เซลล์ของเชื้ออีโคไล โดยใช้โฮสต์ที่เป็น HeLa เซลล์ ดังนั้นเพื่อให้การวิเคราะห์ผลสอดคล้องกับงานวิจัยดังกล่าว การทดลองครั้งนี้จึงเลือกใช้ HeLa เซลล์ เพื่อวิเคราะห์ถึงบทบาทของปัจจัยที่ยับยั้งวงจรของเซลล์จากเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ ต่อการแสดงออกของโปรตีนจากโฮสต์ ซึ่งข้อมูลที่ได้นอกจากจะทำให้ทราบถึงการทำงานของปัจจัยที่ยับยั้งวงจรโฮสต์ HeLa cells โดยเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ แล้ว ยังจะสามารถเปรียบเทียบกลไกการทำงานของปัจจัยที่ยับยั้งวงจรของโฮสต์เซลล์ระหว่างเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ และเชื้ออีโคไลได้อีกด้วย

โดยการทดลองนี้ HeLa เซลล์ได้ถูกเตรียมขึ้นเป็น 3 แบบ คือ1) HeLa เซลล์ที่ปราศจากเชื้อ 2) HeLa เซลล์ที่ติดเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์ดั้งเดิม และ 3) HeLa เซลล์ที่ติดเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์ที่ถูกปรับแต่งพันธุกรรม หลังจากนั้นเซลล์โฮสต์ถูกทำให้แตกเพื่อปลดปล่อยโปรตีนภายในเซลล์ออกมาด้วย 1% SDS และ ป้องกันโปรตีนถูกย่อยสลายด้วยการเติม protease inhibitor จากนั้น lysate ของเซลล์โฮสต์ถูกนำไปวัดปริมาณโปรตีนโดยวิธี bicinchoninic acid (BCA) assay ซึ่งผู้วิจัยพบว่าโปรตีนที่เตรียมได้มีความเข้มข้นประมาณ 5 μg/μl

5. การแยกโปรตีนของโฮสต์เซลล์ด้วยวิธีอิเล็กโทรโฟรีซิส

โปรตีนจาก lysate ของโฮสต์เซลล์ที่เตรียมไว้ ถูกทำให้เสียสภาพโดยการเติม sample buffer ซึ่งประกอบด้วย Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 1% (v/v) β-mercaptoethanol และ bromophenol blue แล้วนำไปต้มที่อุณหภูมิ 95 องศาเซลเซียส เป็นเวลา 5 นาที จากนั้นถูกนำไปแยกด้วยวิธี อิเล็กโทรโฟรีซิส และย้อมด้วย Coomassie Brilliant Blue R-250 ได้ผลดังรูปที่ 6



รูปที่ 6 แสดงแถบโปรตีนของโฮลต์ 10 ไมโครกรัม ที่ถูกแยกด้วยอิเล็กโทรโฟรีซิส โดยใช้ 12% separating gel และ 5% stacking gel ที่กระแสไฟฟ้าคงที่ ที่ 60A

M คือ แถบโปรตีนมาตรฐาน

1 คือ เซลล์ HeLa ที่ปราศจากเชื้อ

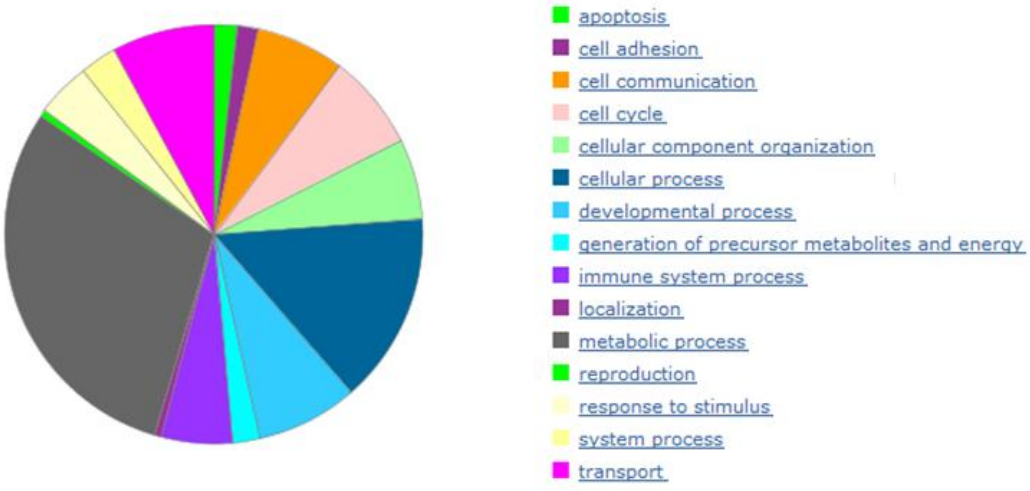
2 คือ เซลล์ HeLa ที่ติดเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์ดั้งเดิม

3 คือเซลล์ HeLa ที่ติดเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์ที่ถูกปรับแต่งพันธุกรรมให้ผิดปกติไปจากเดิมบริเวณยีนปัจจัยที่ยับยั้งวงจรของเซลล์โฮสต์

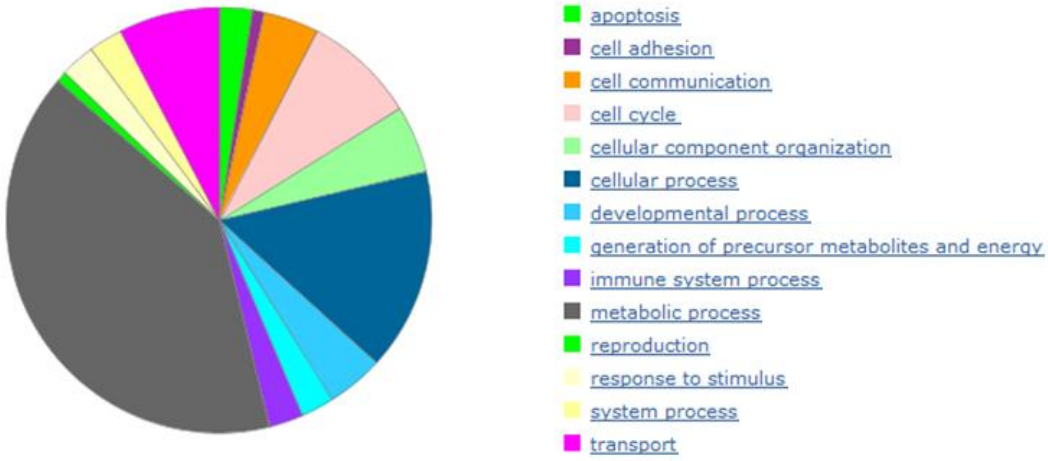
หลังจากนั้นเจลในแต่ละเลนถูกตัดตลอดความยาวเป็นชิ้นย่อยๆจำนวนทั้งหมด 20 ชิ้นแล้วนำไป reduce cysteine และเติมหมู่ alkyl เพื่อ ป้องกันการย้อนกลับไปสร้างพันธะ disulfide อีกจากนั้นทำการย่อยโปรตีนภายในชิ้นเจลด้วย trypsin และนำ peptides ที่ได้ไปวิเคราะห์ด้วยแมสสเปกโทรเมตรี

2. การวิเคราะห์โปรตีนโฮสต์เซลล์ด้วยเทคนิคแมสสเปกโทรเมตรี

ผลการวิเคราะห์โปรตีนของโฮสต์จาก 20 ชิ้นเจลที่ได้จากเจลอิเล็กโทรโฟรีซิส ด้วยเทคนิคแมสสเปกโทรเมตรี (LC/MS/MS) พบว่าโฮสต์เซลล์ที่ติดเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์ที่ถูกปรับแต่งพันธุกรรมมีการปรับเปลี่ยนการสังเคราะห์โปรตีนแตกต่างไปจากโฮสต์เซลล์ที่ติดเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์ดั้งเดิม การเปลี่ยนแปลงโปรตีนของโฮสต์แสดงลักษณะดังนี้พบโปรตีนที่มีการแสดงออกเพิ่มขึ้น 97 ชนิด และพบโปรตีนที่มีการแสดงออกลดลงจำนวน 166 ชนิด ในกลุ่มโปรตีนของโฮสต์เซลล์ที่ติดเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์ที่ถูกปรับแต่งพันธุกรรม ซึ่งโปรตีนที่มีการเปลี่ยนแปลงไปนี้มีความเกี่ยวข้องกับกระบวนการทำงานของเซลล์หลายด้าน ดังแสดงในรูปที่ 7 และ 8 ตัวอย่างเช่น ความเกี่ยวข้องในด้านเมตาบอลิซึมของโฮสต์เซลล์ โปรตีนกลุ่มนี้มีการลดการแสดงออกลงคิดเป็น 68.7% จากโปรตีนที่ลดการแสดงออกทั้งหมดและมีโปรตีนในกลุ่มนี้บางตัวเพิ่มการแสดงออกขึ้นคิดเป็น 66.2% จากโปรตีนที่เพิ่มการแสดงออกทั้งหมด



รูปที่ 7 สัดส่วนของโปรตีนของ HeLa เซลล์ที่ลดลงเมื่อติดเชื้อบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์ที่ถูกปรับแต่งพันธุกรรม (ข้อมูลนี้ได้จากการวิเคราะห์ด้วย panther classification system)

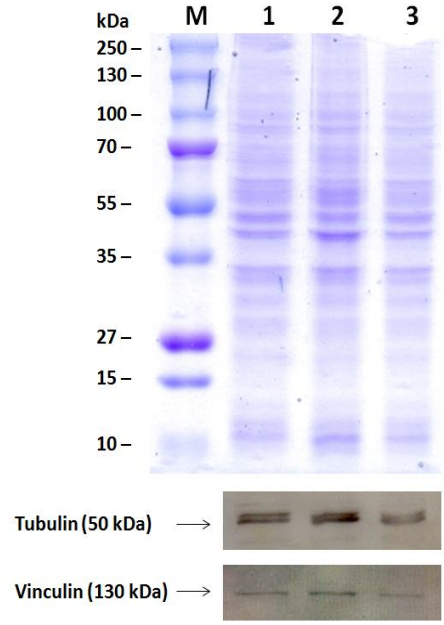


รูปที่ 8 สัดส่วนของโปรตีน HeLa เซลล์ที่เพิ่มขึ้นเมื่อติดเชื้อบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์ดั้งเดิม (ข้อมูลนี้ได้จากการวิเคราะห์ด้วย panther classification system)

นอกจากนี้ยังพบการเปลี่ยนแปลงของโปรตีนในกลุ่ม ปฏิกิริยาของเซลล์ (catalytic activity) และการสื่อสารภายในเซลล์ (signaling pathway) ตลอดจน ubiquitin proteasome system ซึ่งสอดคล้องกับรายงานก่อนหน้าที่พบความเกี่ยวข้องของโปรตีนในกลุ่ม ubiquitin กับการทำงานของปัจจัยที่ยับยั้งวงจรของโฮสต์เซลล์

3. การพิสูจน์ยืนยันการแสดงออกของโปรตีนที่แตกต่างที่พบด้วยวิธีเวสเทิร์นบลอต (Western blot)

จากผลการศึกษาโปรตีโอมิกส์ที่พบว่าโฮสต์เซลล์ที่ติดเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์ที่ถูกปรับแต่งพันธุกรรม CHBP นั้นมีการเปลี่ยนแปลงของการสังเคราะห์โปรตีน และหนึ่งในการเปลี่ยนแปลงนี้ คือการเพิ่มขึ้นของโปรตีน vinculin และ tubulin ที่เกี่ยวข้องกับ host cell cytoskeleton rearrangement ซึ่งผู้วิจัยได้ทำการพิสูจน์ยืนยันการเพิ่มขึ้นด้วยเทคนิค Western blot analysis ดังแสดงในรูป 9



รูปที่ 9 SDS-PAGE และ Western blot analysis ต่อโปรตีน tubulin and vinculin ในเซลล์ HeLa โดยใช้ตัวอย่าง HeLa lysates ที่มีปริมาณโปรตีนเท่ากัน แล้วนำมาแยกด้วย SDS-PAGE และย้อมสี Coomassie blue หรือนำ The blotted proteins มา probed ด้วยแอนติบอดีต่อโปรตีน tubulin หรือแอนติบอดีต่อโปรตีน vinculin

Lane M: Protein marker

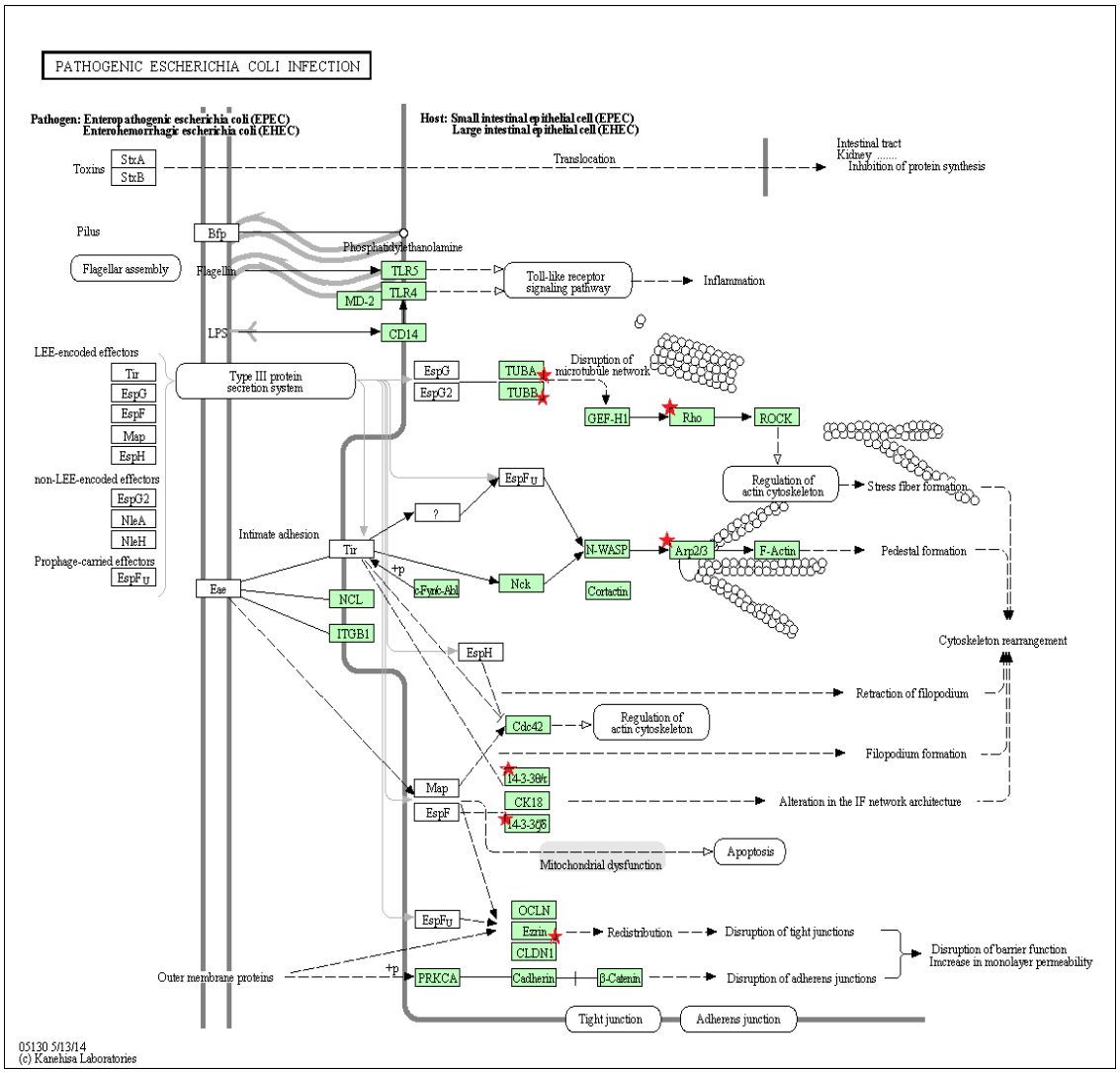
lane 1: HeLa cells

lane 2: HeLa cells infected with *B. pseudomallei*

lane 3: HeLa cells infected with *chbP* mutant

4. การวิเคราะห์เส้นทางการทำงานของโปรตีน (Pathway analysis)

ในการศึกษานี้ใช้ฐานข้อมูลของ Kyoto Encyclopedia of Genes and Genomes (KEGG) เพื่อวิเคราะห์เส้นทางการทำงานของโปรตีนที่ได้จากการศึกษา Proteomics ระหว่าง HeLa เซลล์ที่ติดเชื้อเบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์ดั้งเดิม และ HeLa เซลล์ที่ติดเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์ที่ถูกปรับแต่งพันธุกรรม ซึ่งทำให้ทราบข้อมูลที่น่าสนใจว่ามีบางโปรตีนเชื่อมโยงกับการก่อพยาธิสภาพของเชื้อ EPEC ดังแสดงในรูปที่ 10



รูปที่ 10 แสดง pathogenic *E. coli* infection pathway according to the KEGG database โดย key targets จะถูกติดดาวสีแดง (red stars) เพื่อชี้ให้เห็นว่าเป็นโปรตีนที่เปลี่ยนแปลงไปใน HeLa cells infected with *B. pseudomallei chbP* mutant เมื่อเปรียบเทียบกับ HeLa cells infected with wild type strain

DISCUSSION AND CONCLUSION (วิจารณ์ผลการทดลองและสรุปผลการทดลอง)

เชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ เป็นเชื้อแบคทีเรียแกรมลบ รูปแท่ง เคลื่อนที่ได้ และก่อให้เกิดโรคเมลิออยโดสิส (White, 2003) ซึ่งเป็นโรคที่พบมากในประเทศที่อยู่ในเขตร้อนโดยเฉพาะเอเชียตะวันออกเฉียงใต้ และตอนเหนือของประเทศออสเตรเลีย (Cheng and Currie, 2005) ผู้ป่วยส่วนใหญ่เป็นชาวไร่ชาวนาที่มีโอกาสสัมผัสกับเชื้อได้สูง และพบอัตราการเสียชีวิตสูงหากได้รับการรักษาที่ไม่เหมาะสม (Currie and Jacups, 2003) นอกจากเชื้อเบอโคลเดอเรีย สูโดมัลลิไอ จะสามารถก่อโรคได้ในคน แต่เชื้อยังสามารถก่อโรคได้ในสัตว์หลายชนิดอีกด้วย (Currie et al., 2014) และยังสามารถติดต่อเข้าสู่ร่างกายได้ในสองช่องทางหลัก ได้แก่ ทางการหายใจ ทางแผลที่ผิวหนัง นอกจากนี้ยังสามารถติดเชื้อได้ผ่านทางการกิน แต่พบได้น้อย ผู้ป่วยที่ติดเชื้อนี้มีทั้งไม่แสดงอาการและแสดงอาการแบบเฉียบพลันหรือเรื้อรัง ทั้งนี้หากแสดงอาการ ผู้ป่วยจะมีอาการแสดงที่หลากหลายไม่จำเพาะ เช่น ปอดอักเสบ แผลฝีที่ผิวหนังหรืออวัยวะภายใน ติดเชื้อในกระแสเลือด ติดเชื้อในสมอง นอกจากนี้เชื้อยังสามารถสร้าง virulence factors ได้หลายชนิด เช่น adhesins, quorum sensing, type III secretion system (T3SS), surface polysaccharide เช่น capsule polysaccharide และ lipopolysaccharide, type VI secretion system (T6SS), flagella และ pili

ในแง่การก่อโรค ตั้งแต่อดีตจนถึงปัจจุบันมีการค้นคว้าวิจัยเกี่ยวกับกลไกการก่อโรคของเชื้อนี้อย่างกว้างขวางในแง่มุมต่างๆ มีการศึกษาว่าเมื่อเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ เข้าสู่ร่างกายแล้วเชื้อสามารถรุกรานเซลล์โฮสต์ อยู่รอด และเพิ่มจำนวนภายในเซลล์โฮสต์ได้ทั้งในเซลล์ phagocyte และไม่ใช่ phagocyte (Jones *et al.*, 1996) สำหรับเซลล์ที่ไม่ใช่ phagocyte เช่น เซลล์ epithelium การเข้าเซลล์จะอาศัยการเกาะ ซึ่ง type 4 pili จัดเป็นหนึ่งในหลายชนิดของ adhesins ที่เชื้อใช้ในการเกาะเซลล์โฮสต์ เช่นเดียวกับ flagella เมื่อเกาะแล้วจะมีการบุกรุกเข้าไปในเซลล์โฮสต์ อยู่ใน endosome และสามารถหลบหนีจากการถูกทำลายในจากเอนไซม์ใน endosome ออกมาที่อยู่ในไซโตพลาสซึม โดยอาศัยการทำงานของ T3SS เมื่อออกมาอยู่ในไซโตพลาสซึม เชื้อสามารถเพิ่มจำนวนและเคลื่อนที่ภายในเซลล์ไปยังเซลล์ข้างเคียงโดยอาศัยขบวนการที่เรียกว่า actin polymerization เมื่อเชื้อไปถึงเซลล์ข้างเคียง จะทำให้เยื่อหุ้มเซลล์โฮสต์ยืดตัวออกร่วมกับกลไก actin-based motility ร่วมกับการทำงานของ T6SS จะเกิดสภาวะการรวมตัวของนิวเคลียสของเซลล์โฮสต์ (multinucleated giant cells หรือ MNGC) นำไปสู่การสลายตัว (cell lysis) และตายของเซลล์โฮสต์ (cell death) ตามมา (Allwood et al., 2011)

สำหรับในงานวิจัยนี้ผู้วิจัยมีความสนใจ virulence factor ของ T3SS ตัวหนึ่งที่ชื่อว่า Cycle inhibiting factor หรือ Cif หรือ ปัจจัยที่ยับยั้งวงจรของเซลล์ เนื่องจากมีการศึกษาในเชื้ออีโคไล EPEC ว่า Cif (Marches *et al.*, 2003) สามารถยับยั้งวงจรการเปลี่ยนระยะ G2/M ของเซลล์ อีกทั้งยัง

ทำให้เกิดการสร้าง stress fibres ขึ้นภายในเซลล์ และกระทั่งเหนี่ยวนำให้เซลล์ตายในที่สุด (Samba-Louaka *et al*., 2008; Samba-Louaka *et al*., 2009) และที่สำคัญ Cif ในเชื้ออีโคไล มีความเหมือนกันกับ CHBP หรือ Cif ของเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์เคเก้าหกสองสี่สาม โดยมีค่า identity = 21% และ similarity = 40% ซึ่งนอกจากนี้ CHBP พบว่าสามารถถูกฉีดเข้าไปในเซลล์โฮสต์โดย T3SS ของอีโคไล EPEC ได้ แล้วส่งผลทำให้เกิดการยับยั้งวงจรการเปลี่ยนระยะของเซลล์ ตลอดจนการสร้าง stress fibres ในลักษณะเดียวกันกับ Cif ของเชื้ออีโคไล (Jubelin *et al*., 2009)

มีรายงานเกี่ยวกับการวิเคราะห์ลำดับเบสของจีโนมในเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ 10 สายพันธุ์ที่แยกได้จากตัวอย่างของคนไข้ ทำให้ทราบว่าทั้ง 10 สายพันธุ์มี CHBP แต่มี identities ที่แตกต่างกัน ซึ่งในขณะที่เชื้อ เบอโคลเดอเรีย ไทยแลนเด็นซิส และเชื้อ เบอโคลเดอเรีย มัลลิอาย ซึ่งเป็นเชื้อแบคทีเรียที่มีความใกล้ชิดกันแต่ปกติไม่ได้ก่อโรคเมลิออยโดสิสกลับไม่มี Cif (Yao *et al*., 2009) นอกจากนี้มีการทำการศึกษา Crystal structure ของ CHBP ในเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ จึงทำให้ทราบว่ามี papain-like fold ซึ่งบรรจุ Cys-His-Gln catalytic triad คล้ายกันกับ Cif ในเชื้ออีโคไล EPEC (Crow *et al.,* 2009; Yao *et al.,* 2009) ยิ่งไปกว่านั้นยังมีรายงานว่าพบแอนติบอดีต่อ CHBP ในซีรั่มของผู้ป่วยโรคเมลิออยโดสิส ซึ่งบ่งบอกให้เห็นว่า CHBP ของเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ นั้นอาจมีความสำคัญในการก่อพยาธิสภาพของเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ (Philip *et al*., 2009)

และเมื่อเร็วๆนี้ผู้วิจัยและทีมได้สร้างเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์ที่ถูกปรับแต่งพันธุกรรมให้ผิดปกติไปจากเดิมบริเวณยีนปัจจัยที่ยับยั้งวงจรของเซลล์โฮสต์ขึ้น (Pumirat et al., 2014) และได้นำไปศึกษาความแพร่หลายของยีน *chbP* ใน genome sequences ของเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ จำนวน 43 สายพันธุ์ ด้วย Bioinformatic tool พบว่า 33 genomes (76.7%) มียีน *chbP* และในการทดลอง Western blot analysis ด้วยแอนติบอดีต่อ CHBP พบว่าเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ ที่คัดแยกได้จากคนไข้ในบริเวณที่การระบาดของโรคมีโปรตีน CHBP ประมาณ 46.6% (7/15) นอกจากนี้เมื่อศึกษา secretion ของโปรตีน CHBP เชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ พบว่าไม่เป็นไปอย่างโปรตีน BopE (T3SS effector ที่ใช้ Bsa T3SS) ที่เคยมีรายงานเอาไว้ แต่พบว่ามีโปรตีน CHBP ปรากฏในไซโทรพลาสซึมของโฮสตเซลล์ U937ที่มีการติดเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ โดยทั้งนี้พบว่า secretion ของโปรตีน CHBP ในโฮสตเซลล์ U937 ขึ้นอยู่กับ BsaQ ด้วย (Pumirat et al., 2014)

ส่วนในงานวิจัยนี้ผู้วิจัยได้นำเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์ที่ถูกปรับแต่งพันธุกรรมให้ผิดปกติไปจากเดิมบริเวณยีนปัจจัยที่ยับยั้งวงจรของเซลล์โฮสต์ มาศึกษาต่อ โดยเริ่มต้นจากการทำ PCR เพื่อตรวจหายีน *chbP* ของเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ จำนวน 32 สายพันธุ์

และยืนยันต่อด้วย Western blot analysis ทำให้ทราบว่า 53.1% (17/32) มีปัจจัยที่ยับยั้งวงจรของเซลล์โฮสต์ หรือ CHBP อยู่ หลังจากนั้นผู้วิจัยได้แบ่งกลุ่มเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ เป็น 2 กลุ่ม คือ กลุ่มที่มี CHBP และไม่มี CHBP แล้วนำไปทดสอบ Plaque-forming efficiency เพื่อศึกษาถึงความสำคัญของการมีปัจจัยที่ยับยั้งวงจรของเซลล์นี้ต่อการทำให้เกิดพยาธิสภาพของเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ แต่ผลการศึกษาพบว่าการมี CHBP ไม่ได้เกี่ยวข้องการการก่อพยาธิสภาพของเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ ดังนั้นผู้วิจัยจึงได้ทำการศึกษาต่อเพื่อที่จะค้นหาว่าแล้ว CHBP มีประโยชน์อะไรกับเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ โดยใช้เทคนิค proteomics เพื่อศึกษาโปรตีนโฮสต์เซลล์ที่ติดเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ ด้วยเทคนิคแมสสเปกโทรเมตรี จากการศึกษาพบว่าโฮสต์เซลล์ที่ติดเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์ที่ถูกปรับแต่งพันธุกรรมมีการปรับเปลี่ยนการสังเคราะห์โปรตีนแตกต่างไปจากโฮสต์เซลล์ที่ติดเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์ดั้งเดิม โดยพบโปรตีนที่มีการแสดงออกเพิ่มขึ้น 97 ชนิด และพบโปรตีนที่มีการแสดงออกลดลงจำนวน 166 ชนิด ในกลุ่มโปรตีนของโฮสต์เซลล์ที่ติดเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ สายพันธุ์ที่ถูกปรับแต่งพันธุกรรม ซึ่งโปรตีนที่มีการเปลี่ยนแปลงไปนี้มีความเกี่ยวข้องกับกระบวนการทำงานของเซลล์หลายด้าน ได้แก่ ด้านเมตาบอลิซึมของโฮสต์เซลล์ (metabolism) ด้านวงจรของเซลล์ (cell cycle) ด้านการทำงานของเซลล์ (cellular process) ด้านการขนส่ง (transport system) และการสื่อสารภายในเซลล์ (signaling pathway)

กล่าวโดยสรุปการศึกษานี้ได้ศึกษาการมีอยู่ของยีนและโปรตีนที่เกี่ยวข้องกับปัจจัยที่ยับยั้งวงจรของเซลล์ หรือ CHBP ในกลุ่มเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ และวิเคราะห์ถึงการมีปัจจัยที่ยับยั้งวงจรของเซลล์ของเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ ต่อการแสดงออกของโปรตีนจากโฮสต์ และการสร้าง plaque ซึ่งจากผลการศึกษานี้ถือได้ว่าเป็นข้อมูลที่สำคัญที่ช่วยทำให้ทราบถึงความสำคัญของการมีปัจจัยที่ยับยั้งวงจรโฮสต์ในการปรับเปลี่ยนการแสดงออกของโปรตีนภายใน HeLa cells ที่ติดเชื้อ เบอโคลเดอเรีย สูโดมัลลิไอ เพื่อให้เหมาะสมต่อการก่อโรคของเชื้อแบคทีเรียชนิดนี้

OUTPUT (ผลลัพธ์จากโครงการวิจัย)

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

1.1 Pumirat P, Reamtong O, Chantratita N and Korbsrisate S. Role of cycle inhibiting factor (Cif) in host protein expression and prevalence of Cif in *Burkholderia pseudomallei.* (อยู่ในขั้นตอนการเขียน Manuscript คาดว่าจะส่งตีพิมพ์ในวารสาร Journal of Bacteriology หรือเทียบเท่า)

1.2 Pumirat P, Vanaporn M, Boonyuen U, Indrawattana N, Rungruengkitkun A, and Chantratita N. The Effects of NaCl on Heat Resistance, Oxidative Susceptibility, Motility and Biofilm Formation of *Burkholderia pseudomallei***.** MicrobiologyOpen. 2017;e493.

1.3 Vanaporn M, Sarkar-Tyson M, Kovacs-Simon A, Ireland PM, Pumirat P, Korbsrisate S, et al. Trehalase plays a role in macrophage colonization and virulence of *Burkholderia pseudomallei* in insect and mammalian hosts. Virulence. 2017 Jan 02;8(1):30-40.

1.4 Pinweha P, Pumirat P, Cuccui J, Jitprasutwit N, Muangsombut V, Boonyuen U, Thiennimitr P, Vattanaviboon P, Cia F, Willcocks S, Bancroft GJ, Wren BW, and Korbsrisate S. Nitrate Acquisition under Anaerobic Condition by the BPSL1039-1040 ATP-Binding Cassette Transporter Contributes to Biofilm Formation, Intracellular Survival and Attenuation in the Murine Model of *Burkholderia pseudomallei* Infection. (Submitted to PlosOne; Manuscript is pending revision)

1.5 Pumirat P, Broek CV, Juntawieng N, Muangsombut V, Kiratisin P, Pattanapanyasat K, et al. Analysis of the prevalence, secretion and function of a cell cycle-inhibiting factor in the melioidosis pathogen *Burkholderia pseudomallei*. PLoS One. 2014;9(5):e96298.

2. การนำเสนอผลงานในที่ประชุมวิชาการ

มีการนำเสนอผลงานในที่ประชุมวิชาการแห่งชาติและนานาชาติ ดังนี้

2.1 Pumirat P, Reamtong O, Chantratita N and Korbsrisate S. เรื่อง “The role of *Burkholderia pseudomallei* cycle-inhibiting factor on host protein expression” ในการประชุมวิชาการนานาชาติ European Melioidosis Congress 2015 ณ University of Cambridge ประเทศอังกฤษ วันที่ 26-27 มี.ค. 2558

2.2 Pumirat P, Vanaporn M, Boonyuen U and Chantratita N. เรื่อง “Effects of NaCl on heat Resistance, oxidative Susceptibility, motility and biofilm formation of a potential biothreat

agent *Burkholderia pseudomallei*" ในการประชุมวิชาการนานาชาติ ASM Biodefense and Emerging Diseases Research Meeting ณ Washington Marriott Wardman Park ประเทศสหรัฐอเมริกา วันที่ 9-11 ก.พ. 2558

2.3 Pumirat P, Reamtong O, Chantratita N, and Korbsrisate S เรื่อง "The role of cycle-inhibiting factor in *Burkholderia pseudomallei*" ในการประชุมวิชาการนานาชาติ Learning from each other- improving the clinical management and prevention of melioidosis and tuberculosis in the UK and South East Asia ณ Pullman Hotel จังหวัดขอนแก่น วันที่ 6-7 ม.ค. 2558

2.4 Pumirat P, Reamtong O, Chantratita N, and Korbsrisate S เรื่อง "The role of cycle-inhibiting factor in *Burkholderia pseudomallei*–host cell interaction" ในการประชุมวิชาการนานาชาติ Joint International Tropical Medicine Meeting 2013 ณ Centara Grand & Bangkok Convention Centre กรุงเทพฯ วันที่ 2-4 ธ.ค. 2557

2.5 Pumirat P, Reamtong O, Chantratita, N, Korbsrisate, S. เรื่อง "A study of the prevalence of *Burkholderia pseudomallei* cycle inhibiting factor and its role on host protein expression" ในการประชุม "นักวิจัยรุ่นใหม่พบเมธีวิจัยอาวุโส สกว." ครั้งที่ 14 ในวันที่ 23 ตุลาคม 2557 ณ โรงแรมแอมบาสซาเดอร์ ซิตี้ จอมเทียน พัทยา จ. ชลบุรี

2.6 Vanaporn M, Pumirat P, Tyson MS, Harding S, Korbsrisate S, Titball R. เรื่อง "Trehalase Regulates Growth and Virulence in *Burkholderia pseudomallei*" ในการประชุม "นักวิจัยรุ่นใหม่พบเมธีวิจัยอาวุโส สกว." ครั้งที่ 13 ประจำปี 2556 วันที่ 16-18 ต.ค. 2556

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ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

1. Pumirat P, Vanaporn M, Boonyuen U, Indrawattana N, Rungruengkitkun A, and Chantratita N. The Effects of NaCl on Heat Resistance, Oxidative Susceptibility, Motility and Biofilm Formation of *Burkholderia pseudomallei***.** MicrobiologyOpen. 2017;e493.

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1. Pumirat P, Reamtong O, Chantratita N and Korbsrisate S. เรื่อง “The role of *Burkholderia pseudomallei* cycle-inhibiting factor on host protein expression” ในการประชุมวิชาการนานาชาติ European Melioidosis Congress 2015 ณ University of Cambridge ประเทศอังกฤษ วันที่ 26-27 มี.ค. 2558

2. Pumirat P, Vanaporn M, Boonyuen U and Chantratita N. เรื่อง “Effects of NaCl on heat Resistance, oxidative Susceptibility, motility and biofilm formation of a potential biothreat agent *Burkholderia pseudomallei*” ในการประชุมวิชาการนานาชาติ ASM Biodefense and Emerging Diseases Research Meeting ณ Washington Marriott Wardman Park ประเทศสหรัฐอเมริกา วันที่ 9-11 ก.พ. 2558

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ORIGINAL RESEARCH

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Effects of sodium chloride on heat resistance, oxidative susceptibility, motility, biofilm and plaque formation of *Burkholderia pseudomallei*

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Abstract

Burkholderia pseudomallei is an environmental saprophyte and the causative agent of melioidosis, a severe infectious disease prevalent in tropical areas, including southeast Asia and northern Australia. In Thailand, the highest incidence of melioidosis is in the northeast region, where saline soil and water are abundant. We hypothesized that *B. pseudomallei* develops an ability to thrive in saline conditions and gains a selective ecological advantage over other soil-dwelling microorganisms. However, little is known about how an elevated NaCl concentration affects survival and adaptive changes in this pathogen. In this study, we examined the adaptive changes in six isolates of *B. pseudomallei* after growth in Luria-Bertani medium containing different concentrations of NaCl at 37°C for 6 hr. The bacteria were then investigated for resistance to heat at 50°C and killing by hydrogen peroxide (H_2O_2). In addition, flagellar production, biofilm formation, and the plaque formation efficiency of *B. pseudomallei* after culture in saline conditions were observed. In response to exposure to 150 and 300 mmol L^{-1} NaCl, all *B. pseudomallei* isolates showed significantly increased thermal tolerance, oxidative resistance, and plaque-forming efficiency. However, NaCl exposure notably decreased the number of *B. pseudomallei* flagella. Taken together, these results provide insight into the adaptations of *B. pseudomallei* that might be crucial for survival and persistence in the host and/or endemic environments with high salinity.

KEYWORDS
Burkholderia pseudomallei, melioidosis, salt stress, sodium chloride

1 | INTRODUCTION

Burkholderia pseudomallei is a Gram-negative pathogenic bacterium responsible for melioidosis in humans and animals. This saprophytic organism is found in soil, stagnant water, and rice paddies. Regions in which melioidosis is endemic include southeast Asia, particularly Thailand, and northern Australia (Cheng & Currie, 2005; Wuthiekanun, Smith, Dance, & White, 1995). Rice farmers are considered a high-risk group for exposure to *B. pseudomallei* especially during the monsoonal and rainy season when there is a lot of mud and surface water in the rice fields (Chaowagul et al., 1989; Cheng & Currie, 2005; Inglis & Sagripanti, 2006; Wiersinga, van der Poll, White, Day, & Peacock, 2006). Infection mainly occurs by inoculation through skin abrasions or inhalation. The clinical features of

melioidosis vary considerably, ranging from acute fulminant septicemia to chronic localized infection. In its acute form, death can occur within days of the onset of symptoms. However, the longest reported incubation period between initial acquisition of the organism and subsequent infection is a remarkable 62 years. Furthermore, a high rate of relapse has been recognized (Ngauy, Lemeshev, Sadkowski, & Crawford, 2005). Unfortunately, there is currently no effective vaccine available for the prevention of melioidosis. The treatment of melioidosis generally involves the antibiotics ceftazidime or carbapenem as *B. pseudomallei* exhibits resistance to several empiric antimicrobial therapies.

In Thailand, the highest prevalence of *B. pseudomallei* and the highest incidence of melioidosis are in the northeast region, where saline soil and water are plentiful. The electrical conductivity of soil samples from northeast Thailand ranges from 4 to 100 dS/m, which is higher than that of normal soil from other regions (approximately 2 dS/m) (Development Department of Thailand). We hypothesized that *B. pseudomallei* may develop an ability to adapt to saline conditions and gain cross-protection to other stress conditions. There is evidence of a link between high NaCl concentrations and an ability to survive in saline conditions in other closely related organisms, namely, the *Burkholderia cepacia* complex (BCC). These organisms are opportunistic pathogens of cystic fibrosis (CF) sufferers (Mahenthiralingam, Baldwin, & Vandamme, 2002; Vandamme et al., 1997) whose lung airways have an increased concentration of NaCl in the surface liquid (Widdicombe, 2001), approximately twofold higher than that of healthy lungs (Joris, Dab, & Quinton, 1993). The potential pathogenic role of *B. pseudomallei* in CF lung disease has also been reported (O'Carroll et al., 2003).

Several studies have shown that exposure to NaCl can influence the adaptive survival and virulence of pathogenic bacteria. The relevance of this has been shown in *Salmonella enterica* serovar Typhimurium (12), *Staphylococcus aureus* (Park et al., 2012), and *Listeria monocytogenes* (Garner, James, Callahan, Wiedmann, & Boor, 2006), whereby bacteria cultured in medium-containing high NaCl show increased heat tolerance (Park et al., 2012; Yoon, Park, Oh, Choi, & Yoon, 2013), antibiotic resistance (Yoon et al., 2013), and invasion ability into host cells (Garner et al., 2006; Yoon et al., 2013). Our previous study also showed that *B. pseudomallei* grown under salt stress displayed significantly greater resistance to the antibiotic ceftazidime (Pumirat et al., 2009). Salt-treated *B. pseudomallei* exhibited greater invasion efficiency into the lung epithelial cell line A549 (Pumirat et al., 2010). However, only one *B. pseudomallei* isolate was used in our previous study and adaptive responses of *B. pseudomallei* to high NaCl concentrations remain largely unknown.

In this study, we further investigated the adaptive response of six *B. pseudomallei* isolates grown in Luria–Bertani (LB) medium with different concentrations of NaCl for 6 hr at 37°C. The concentrations of NaCl used were 0, 150, and 300 mmol L^{-1} which are equivalent to 0, 15, and 30 dS/m, respectively. The bacteria under salt stress were then tested for heat resistance, oxidative susceptibility, swarm motility, flagellar production, and biofilm and plaque formation.

2 | METHODS

2.1 | Bacterial strains, growth, and salt treatment

Experiments were performed using six clinical isolates of *B. pseudomallei*: strains 153, 576, 1026b, 1530, 1634, and the reference strain K96243. All strains were obtained from clinical specimens of six patients presenting with melioidosis in northeast Thailand. The bacteria were generally maintained on LB agar at 37°C. To examine the effect of NaCl, *B. pseudomallei* was subcultured in NaCl-free LB broth and incubated at 37°C with shaking at 200 rpm overnight. The bacteria were then inoculated at a dilution of 1:10 into 10 ml of LB broth containing 0, 150, and 300 mmol L^{-1} NaCl and incubated at 37°C for 6 hr with shaking. The salt-treated and untreated *B. pseudomallei* were adjusted to an OD_{600} of 0.15. A serial dilution was performed to determine the number of colony-forming units (CFU) to obtain the starting number of bacteria.

2.2 | Heat resistance assay

A heat stress resistance assay was performed as described previously (Vanaporn, Vattanaviboon, Thongboonkerd, & Korbsrisate, 2008) with some modifications. Briefly, *B. pseudomallei* cultured in LB medium containing different salt concentrations (0, 150, and 300 mmol L^{-1} NaCl) at 37°C for 6 hr were washed with phosphate-buffered saline (PBS) and resuspended in PBS to an OD_{600} of 0.15. One milliliter of the bacterial suspension was then added into a prewarmed tube and incubated at 50°C for 15 min. Before and after heat challenge, bacterial survival was enumerated on LB agar plates after incubating at 37°C for 24 hr. The number of surviving bacteria was expressed as a percentage of the viable cells.

% Survival = CFU (heat exposure) × 100/CFU (without heat exposure)

2.3 | Oxidative stress assay

The survival of *B. pseudomallei* under oxidative conditions was determined by observing the number of viable bacteria after exposure to an oxidative agent. After 6 hr of culturing in LB medium containing different salt concentrations (0, 150, and 300 mmol L^{-1} NaCl), *B. pseudomallei* cells were harvested, washed, and resuspended in PBS. The bacterial concentration was adjusted to an OD_{600} of 0.15. Then, 100 μl of bacterial suspension was treated with H_2O_2 (at a final concentration of 1 μmol L^{-1}) or left untreated at room temperature for 15 min. A 10-fold dilution of treated and untreated bacteria was performed and plated on LB agar. After incubation at 37°C for 24 hr, colonies were counted. The number of colonies of treated bacteria was compared with that of untreated bacteria (without oxidant) and presented as the % bacterial survival.

% Survival = CFU (with oxidant) × 100/ CFU (without oxidant)

2.4 | Motility assay

A motility assay was undertaken using the swarm plate method as previously described (Deziel, Comeau, & Villemur, 2001). Briefly, *B.*

pseudomallei were grown in LB broth with 0, 150, or 300 mmol L^{-1} NaCl for 6 hr at 37°C. Bacterial pellets were collected, washed, and adjusted in PBS to approximately 10^8 CFU/ml. Swarm plates were inoculated by placing 2 μl of the prepared inoculum onto the agar surface at the center of the plate. The diameter of the swarming motility zone was measured from the point of inoculation after incubation at 37°C for 24 hr.

2.5 | Electron microscopic examination

The presence of *B. pseudomallei* flagella was examined using a transmission electron microscope. Fifty microliters of *B. pseudomallei* grown in LB broth with different salt concentrations was harvested and dropped onto parafilm. Formvar-coated carbon grids were placed on top of the parafilm for 10 min to transfer the bacterial cells. The liquid was then carefully removed with filter paper. The samples were stained with 1% uranyl acetate for 10 min, then the liquid was removed again. The grid was dried at room temperature overnight. Bacteria were observed under a Hitachi Electron Microscope H-7000 (Japan). The presence of bacterial flagella was recorded for 100 bacteria per condition.

2.6 | RNA preparation and real-time RT-PCR

RNA was isolated from 6 hr culture of *B. pseudomallei* grown at 37°C by adding 10 ml of RNAprotect bacterial reagent (QIAGEN) to 5 ml of bacteria culture and incubating for 5 min at room temperature. Subsequently, total RNA was extracted from bacterial pellets using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and treated with DNase (NEB, MA, USA) for 10 min at 37°C before use. Conventional PCR for 23S RNA gene was used to verify that there was no gDNA contamination in the DNase-treated RNA samples. Real-time RT-PCR was performed for six genes (*rpoE*, *groEL*, *htpG bopA*, *bopE*, and *bipD)* using Brilliant II SYBR® Green QPCR Master Mix, one step (Agilent Technologies, Santa Clara, CA, USA) with following conditions: reverse transcription at 50°C for 30 min, enzyme activation at 95°C for 10 min, then 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 1 min, and melting curve analysis at 72°C for 1 min in a CFX96 Touch™ Real-Time PCR Detection System (CA, USA). Real-time RT-PCR primers are listed in Table 1. Relative mRNA levels were determined by fold change in expression, calculated by $2^{-\Delta\Delta CT}$ using the relative mRNA level of 23S RNA, representing a house-keeping gene expression, as a baseline for comparison.

2.7 | Biofilm formation assay

Quantification of biofilm formation was performed using a microtiter plate assay as previously described (Leriche & Carpentier, 2000; Stepanovic, Vukovic, Dakic, Savic, & Svabic-Vlahovic, 2000). Briefly, biofilm formation of *B. pseudomallei* was induced in trypticase soy broth at 37°C for 24 hr. After incubation, the adherent bacteria were washed using deionized water three times and fixed with 99% methanol for 15 min at room temperature. The bacteria were stained for 15 min with 1% crystal violet and solubilized with 33% (v/v) glacial acetic acid. The quantity of biofilm was measured at 630 nm using a microplate reader (Bio-Rad). Each *B. pseudomallei* isolate was assayed in duplicate, using eight wells per experiment.

TABLE 1 Oligonucleotide primers used in this study

Primers	Sequences (5′–3′)	Sources
RpoE 36	CTCCAAATACCACCGCAAGAT	(Korbsrisate et al., 2005)
RpoE 37	TATCCCTTAGTTGGTCCG	
Gro1	AGGACGGCGACTTGCTTGT	(Vanaporn et al., 2008)
Gro2	TTCCAAGACCAGTCGACAAC	
Htp1	TACAGCAACAAGGAAATCT	
Htp2	CACTCCTCCTTCTTCATCA	
BopA F	GTATTTCGGTCGTGGGAATG	(Pumirat et al., 2010)
BopA R	GCGATCGAAATGCTCCTTAC	
BopE F	CGGCAAGTCTACGAAGCGA	
BopE R	GCGGCGGTATGTGGCTTC G	
BipD F	GGACTACATCTCGGCCAAAG	
BipD R	ATCAGCTTGTCCGGATTGAT	
23s F	TTTCCCGCTTAGATGCTTT	
23s R	AAAGGTACTCTGGGGATAA	

2.8 | Plaque formation assay

Plaque-forming efficiency was assessed as previously described (Pumirat et al., 2014). HeLa cells were infected with *B. pseudomallei* at a multiplicity of infection of 20 and incubated at 37°C with 5% CO_2 for 2 hr. Thereafter, the infected cell monolayers were washed and replaced with medium-containing kanamycin (250 μg/ml). The plates were incubated at 37°C in a humidified 5% CO_2 atmosphere for 20 hr. Plaques were stained with 1% (w/v) crystal violet in 20% (v/v) methanol and counted by microscopy. Plaque-forming efficiency was calculated by determining the number of plaques per CFU of bacteria added per well.

2.9 | Statistical analysis

All assays were conducted in triplicate, and an unpaired *t*-test of independent experiments was performed using the GraphPad Prism 6 program (STATCON). Results were considered significant at a $p \leq .05$.

3 | RESULTS

3.1 | NaCl stress induces cross-protection against heat and oxidative agents

Different growth rates may affect the number of viable bacteria under NaCl stress conditions. Therefore, prior to observing the effect of NaCl stress on cross-protection against heat and oxidative agents, the

individual growth of six clinical *B. pseudomallei* isolates (K96243, 153, 576, 1026b, 1530, and 1634) from six patients in northeast Thailand was compared in LB broth containing different NaCl concentrations. Strains K96243, 153, 576, and 1026b were selected as these have been used extensively as reference isolates, and sequence type data are available (K96253, ST10; 153, ST15, 576; ST 501 and 1026b; ST102). Strains 1530 and 1634 were isolated from blood samples of two cases in northeast Thailand and used for comparison. In our previous study, *B. pseudomallei* K96243 demonstrated growth impairment during culture in LB containing 470 mmol L^{-1} NaCl (Pumirat et al., 2010). In this study, we investigated the growth kinetics of six *B. pseudomallei* isolates in LB media containing 0, 150, or 300 mmol L^{-1} NaCl for 6 hr after incubation at 37°C. Similar growth curves were observed for the six isolates under conditions of 0, 150, and 300 mmol L^{-1} NaCl (Figure S1). Therefore, salt concentrations ranging from 0 to 300 mmol L^{-1} and a culture time of 6 hr were chosen for further investigations.

To evaluate the effect of NaCl on heat resistance in *B. pseudomallei*, six *B. pseudomallei* isolates were cultured in LB broth with different concentrations of NaCl for 6 hr to reach the log phase of bacterial growth, followed by heating at 50°C for 15 min. Figure 1 shows the percentage of surviving bacteria and demonstrates a significant difference in heat resistance between *B. pseudomallei* isolates cultured in NaCl-free medium and those cultured in LB with 150 mmol L^{-1} NaCl ($p = .014$ for K96243, $p = .011$ for 153, $p = .028$ for 576, $p = .027$ for 1026b, $p = .011$ for 1530, and $p = .040$ for 1634) or those cultured in LB with 300 mmol L^{-1} NaCl ($p = .020$ for K96243, $p = .004$ for 153, $p < .001$ for 576, $p < .001$ for 1026b, $p < .001$ for 1530, and $p = .002$ for 1634). In addition, the data also showed a significant difference in the percentage of bacterial survival between *B. pseudomallei* isolates cultured in LB supplemented with 150 and 300 mmol L^{-1} NaCl ($p = .038$ for K96243, $p = .002$ for 153, $p = .001$ for 576, $p < .001$ for 1026b, $p = .002$ for 1530, and $p = .008$ for 1634). The mean and standard deviation (SD) of bacterial survival in NaCl-free medium of the six *B. pseudomallei* isolates after heat treatment were 2.2 ± 0.5%. By contrast, the mean and SDs of bacterial survival of the six isolates in medium containing 150 mmol L^{-1} and 300 mmol L^{-1} NaCl were 18.2 ± 2.9% and 67.9 ± 8.9%, respectively. These data clearly revealed that salinity is associated with increased resistance of *B. pseudomallei* to heat stress.

Activation of the oxidative response during survival in salt stress has been reported for various bacteria (den Besten, Mols, Moezelaar, Zwietering, & Abee, 2009; Metris, George, Mulholland, Carter, & Baranyi, 2014). We investigated the effect of NaCl on oxidative susceptibility of six *B. pseudomallei* isolates grown in different NaCl concentrations. Equal numbers of salt-treated and untreated *B. pseudomallei* were exposed to 1 μmol L^{-1} H_2O_2 for 15 min, and their survival on LB agar was determined (Figure 2). The percentage of surviving bacteria among the *B. pseudomallei* isolates grown in salt-free medium in the presence of H_2O_2 was significantly lower than the bacteria exposed to salt at a concentration of 150 mmol L^{-1} NaCl ($p = .046$ for K96243, $p = .039$ for 153, $p = .019$ for 576, $p = .027$ for 1026b, $p = .043$ for 1530, and $p = .014$ for 1634), or those exposed to 300 mmol L^{-1} NaCl ($p = .004$ for K96243, $p = .004$ for 153, $p < .001$ for 576, $p = .010$ for 1026b, $p = .011$ for 1530, and $p < .001$ for 1634). These data also showed a significant difference in the percentage of bacterial survival between *B. pseudomallei* isolates cultured in LB medium supplemented with 150 mmol L^{-1} and 300 mmol L^{-1} NaCl under oxidative stress conditions ($p = .010$ for K96243, $p = .004$ for 153, $p = .005$ for 576, $p = .046$ for 1026b, $p = .049$ for 1530, and $p < .001$ for 1634). In the presence of H_2O_2, the mean survival rate of untreated *B. pseudomallei* isolates was 1.7 ± 0.6%, compared with 5.6 ± 1.2% for those exposed to 150 mmol L^{-1} NaCl and 12.7 ± 2.3% for those exposed to 300 mmol L^{-1} NaCl. These data indicated that preexposing bacteria to salt stress reduced susceptibility to H_2O_2 in *B. pseudomallei*.

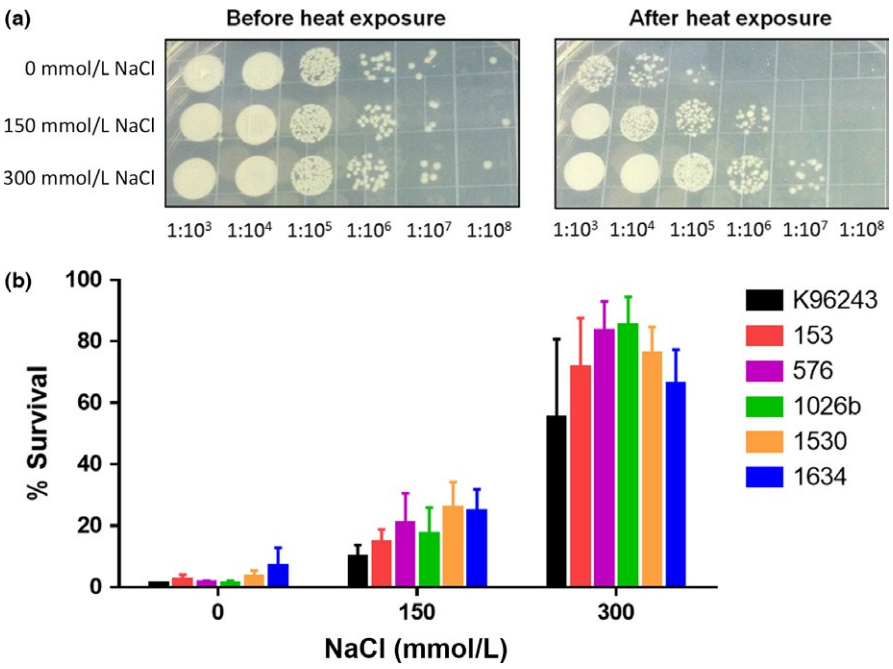

FIGURE 1 Resistance to heat of *Burkholderia pseudomallei* after growth in Luria–Bertani (LB) broth containing 0, 150, or 300 mmol L^{-1} NaCl. (a) Cell viability of *B. pseudomallei* K96243 before and after heat treatment at 50°C for 15 min. Colony-forming units were enumerated on LB agar plates after incubation at 37°C for 24 hr. (b) Percent survival of six *B. pseudomallei* isolates after heat treatment at 50°C for 15 min. 100% viability corresponds to the colony-forming unit count of unexposed bacteria. The data were obtained from at least three experiments. Error bars represent the standard deviation of the mean for experiments performed in triplicate

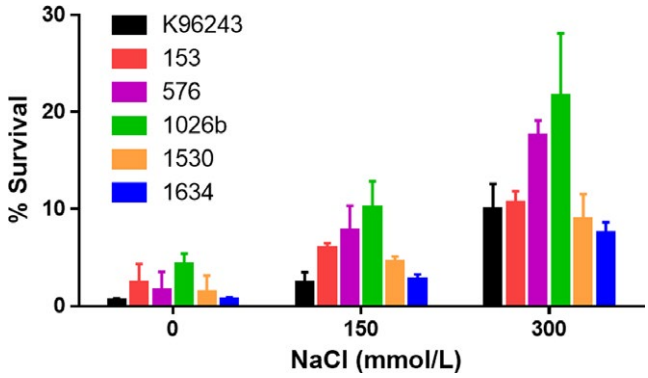

FIGURE 2 Susceptibility to oxidative stress of six *Burkholderia pseudomallei* isolates grown in Luria–Bertani (LB) broth containing 0, 150, and 300 mmol L^{-1} NaCl. Susceptibility to killing by 1 μmol L^{-1} H_2O_2 was determined at 15 min. Surviving bacteria were enumerated on LB agar plates after incubation at 37°C for 24 hr and were expressed as the % survival. The data were obtained from three experiments. Error bars represent the standard deviation of the mean for three experiments

The response of *B. pseudomallei* to heat and oxidative stress has been reported to be dependent on various cellular components, including transcription factors, heat shock proteins, and virulent proteins (Jitprasutwit et al., 2014; Korbsrisate et al., 2005; Vanaporn et al., 2008). We therefore investigated whether NaCl affects the expression of the *rpoE*, *groEL*, *htpG*, *bopA*, *bopE*, and *bipD*. The *rpoE*, *groEL*, and *htpG* genes were selected because they code transcription factors or heat shock proteins that have previously been reported to be involved in heat and oxidative stress (Jitprasutwit et al., 2014; Korbsrisate et al., 2005; Vanaporn et al., 2008). The *bopA*, *bopE*, and *bipD* were T3SS genes which may be important for cell invasion (Gong et al., 2011; Muangsombut et al., 2008; Stevens et al., 2003). Real-time RT-PCR results showed that *B. pseudomallei* K96243 when exposed to NaCl (150 and 300 mmol L^{-1}) exhibited increased expression of all tested genes, compared with bacteria grown under NaCl-free conditions (Figure 3). These data suggested that NaCl is involved in increasing the expression of stress response proteins, which might be responsible for the enhanced resistance of *B. pseudomallei* to heat and oxidative stress.

3.2 | NaCl decreases the expression of *B. pseudomallei* flagella

Motility is a crucial factor for bacterial pathogenesis. Using a microarray, we previously demonstrated that *B. pseudomallei* grown under high NaCl conditions exhibited downregulation of the flagella biosynthesis sigma factor gene "*fliA*" (*bpsl3291*) (Pumirat et al., 2010). Therefore, in this study, we further examined whether salt affects *B. pseudomallei* swarm motility. Six isolates of *B. pseudomallei* were grown in LB broth containing different concentrations of NaCl (0, 150, or 300 mmol L^{-1}) for 6 hr, then equal numbers of bacteria for each isolate were used to inoculate swarm agar medium. After incubation at 37°C for 24 hr, the

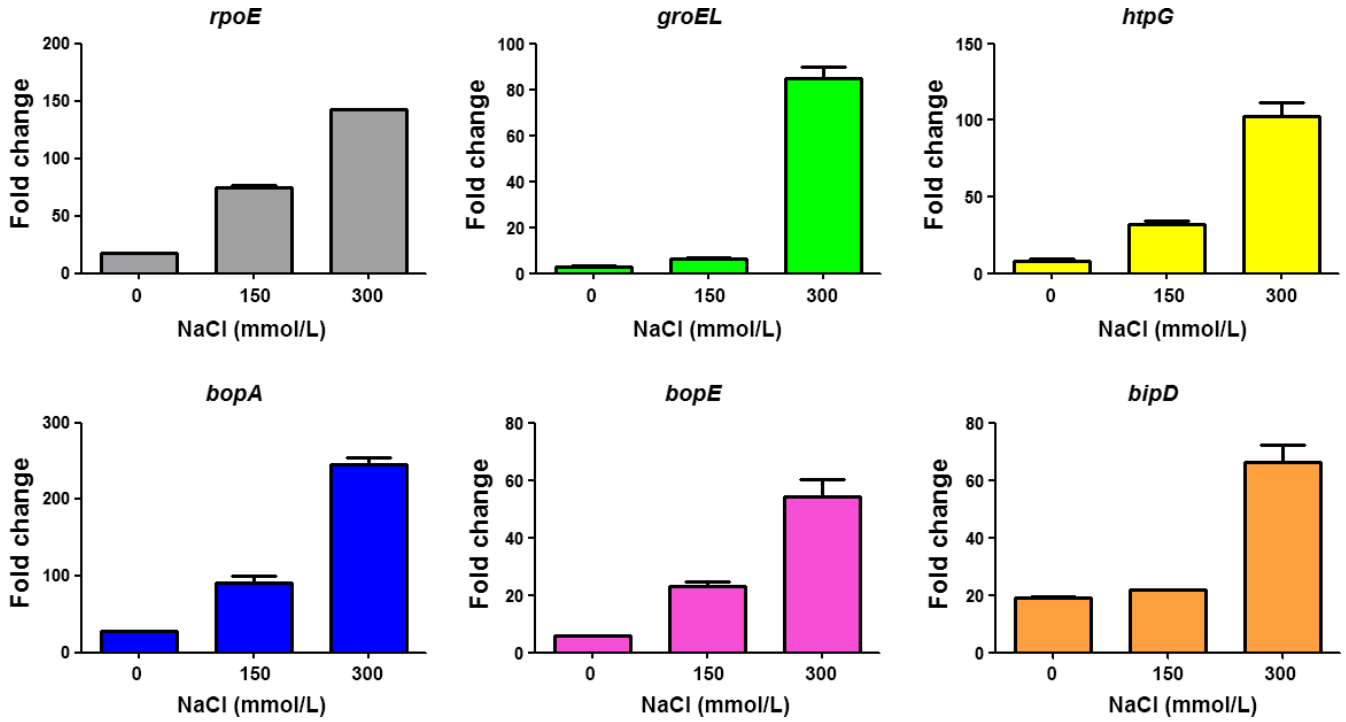

FIGURE 3 Fold change of *rpoE*, *groEL*, *htpG*, *bopA*, *bopE*, *and bipD* genes in *Burkholderia pseudomallei* K96243 grown in Luria–Bertani (LB) broth containing 0, 150, and 300 mmol L^{-1} NaCl. RNA of *B. pseudomallei* grown in LB broth with different NaCl concentrations for 6 hr was used for determination of gene expression by quantitative real-time RT-PCR using the Brilliant II SYBR® Green QPCR Master Mix, one step (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's recommendation. Relative mRNA levels were determined by fold changes in expression, calculated by $2^{-\Delta\Delta CT}$. 23S rRNA gene was used for normalization. Error bars represent the standard deviation of the means for experiments performed in triplicate

diameter of the swarming zone was measured (Figure S2). The mean and SDs of the swarming zone diameters of the six *B. pseudomallei* isolates were 23.7 ± 0.9, 21.8 ± 1.2, and 17.4 ± 1.6 mmol L^{-1} for bacteria exposed to 0, 150, and 300 mmol L^{-1} NaCl, respectively (Table 2).

To determine whether altered expression of the *fliA* gene affects bacterial flagella, we examined the number of flagella on the six *B. pseudomallei* isolates during growth under different salt conditions using an electron microscope. The results showed that the number of flagella decreased with increasing concentrations of NaCl (Figure S3). The number of flagella counted on 100 bacteria for each of the six isolates is shown in Table 3. The majority of *B. pseudomallei* isolates (70.7 ± 3.5%) grown in LB with 300 mmol L^{-1} NaCl showed no flagella. By contrast, only 38.0 ± 3.8% and 49.3 ± 4.3% of *B. pseudomallei* cultured in NaCl-free and 150 mmol L^{-1} NaCl-supplemented media, respectively, had no flagella. The number of unflagellated bacteria among the *B. pseudomallei* isolates grown in 300 mmol L^{-1} NaCl-supplemented medium was therefore significantly higher than among those grown in salt-free ($p < .001$) or 150 mmol L^{-1} NaCl-supplemented medium ($p = .003$, respectively). This phenomenon indicated that salinity affects flagella production in *B. pseudomallei*.

TABLE 2 Effect of NaCl on the swarming motility of *B. pseudomallei*

	Diameter of swarm zone (mmol L^{-1})		
B. pseudomallei isolates	0 mmol L^{-1} NaCl	150 mmol L^{-1} NaCl	300 mmol L^{-1} NaCl
K96243	24.0 ± 7.0	21.3 ± 6.7	17.7 ± 7.2
153	27.3 ± 4.6	26.3 ± 5.5	23.5 ± 4.4
576	24.7 ± 7.6	24.0 ± 8.2	16.0 ± 9.5
1026b	22.0 ± 7.0	21.7 ± 7.2	19.0 ± 8.2
1530	23.3 ± 9.1	18.3 ± 5.5	16.3 ± 6.4
1634	20.7 ± 2.3	19.3 ± 3.1	11.7 ± 8.1

Data represent the mean ± SD of three experiments each performed in triplicate.

TABLE 3 Effect of NaCl on the number of flagella expressed on *Burkholderia pseudomallei*

B. pseudomallei isolates	NaCl (mmol L^{-1})	% Bacteria with flagella		
		0	1–3	>3
K96243	0	36	50	14
	150	52	36	12
	300	76	24	0
153	0	36	52	12
	150	52	28	20
	300	60	40	0
576	0	24	42	24
	150	32	60	8
	300	76	24	0
1026b	0	36	56	8
	150	44	56	0
	300	80	20	0
1530	0	52	44	4
	150	52	44	4
	300	60	40	0
1634	0	44	52	4
	150	64	32	4
	300	72	24	4

Data represent the mean ± SD of three experiments each performed in triplicate. One hundred bacterial cells were counted to determine the number of flagella.

3.3 | Effect of NaCl on *B. pseudomallei* biofilm formation

B. pseudomallei can produce biofilm, which may offer protection against hostile conditions such as antibiotic treatment, salinity, and immune responses (Cheng & Currie, 2005; Inglis & Sagripanti, 2006; Kamjumphol, Chareonsudjai, Chareonsudjai, Wongratanacheewin, & Taweechaisupapong, 2013). We therefore tested whether *B. pseudomallei* biofilm formation is affected by salt stress. Six isolates of *B. pseudomallei* were grown in LB broth with different concentrations of NaCl for 6 hr at 37°C prior to the induction of biofilm formation. The results in Table 4 demonstrate the biofilm formation capacity of each of the *B. pseudomallei* isolates. The mean OD values and SDs of the biofilm formation capacity of the *B. pseudomallei* isolates increased from 0.19 ± 0.01 to 0.24 ± 0.03 and then to 0.31 ± 0.03 when bacteria were grown in the presence of 0, 150, and 300 mmol L^{-1} NaCl, respectively. Although, each of the *B. pseudomallei* isolates tended to show increased biofilm formation when grown in the presence of NaCl compared with those grown in 0 mmol L^{-1} NaCl, we could not detect a significant difference in biofilm formation when comparing bacteria grown in the presence of 0, 150, and 300 mmol L^{-1} NaCl.

3.4 | NaCl affects *B. pseudomallei* plaque formation

B. pseudomallei is a facultative intracellular bacteria that harbors the ability for cell-to-cell spread (Kespichayawattana,

TABLE 4 Effect of NaCl on biofilm formation of *Burkholderia pseudomallei*

	Corrected OD_{630} nm		
B. pseudomallei isolates	0 mmol L^{-1} NaCl	150 mmol L^{-1} NaCl	300 mmol L^{-1} NaCl
K96243	0.16 ± 0.03	0.21 ± 0.07	0.23 ± 0.07
153	0.24 ± 0.12	0.33 ± 0.18	0.35 ± 0.18
576	0.14 ± 0.01	0.16 ± 0.02	0.22 ± 0.04
1026b	0.20 ± 0.07	0.35 ± 0.22	0.45 ± 0.27
1530	0.17 ± 0.04	0.18 ± 0.04	0.23 ± 0.03
1634	0.21 ± 0.03	0.23 ± 0.03	0.25 ± 0.01

Data represent the mean ± SD of three experiments each performed in triplicate.

Rattanachetkul, Wanun, Utaisincharoen, & Sirisinha, 2000), which is an important characteristic for pathogenesis. Previously, NaCl was found to increase expression of the *Burkholderia* secretion apparatus (Bsa) type III secretion system (T3SS), which involved a virulence-associated interaction with the host cell (Pumirat et al., 2010). In particular, the translocon "BipB" and the secreted effector protein "Cif" homolog in *B. pseudomallei* were reported to induce cell-to-cell dissemination (Pumirat et al., 2014; Suparak et al., 2005). Hence, we investigated whether salt stress affects cell-to-cell spread of *B. pseudomallei*. Six *B. pseudomallei* isolates grown in LB with different concentrations of NaCl for 6 hr at 37°C were assessed for plaque formation. Figure 4a demonstrates plaque formation in the HeLa cell line induced by *B. pseudomallei* K96243 when grown in 0, 150, and 300 mmol L^{-1} NaCl. The mean and SD of the plaque-forming efficiency of *B. pseudomallei* isolates grown in 300 mmol L^{-1} NaCl were 59.8 ± 2.8, compared with 41.7 ± 2.2 for bacteria grown in 150 mmol L^{-1} NaCl and 35.0 ± 2.7 for those grown in NaCl-free LB. All *B. pseudomallei* isolates grown in the presence of 300 mmol L^{-1} NaCl showed significantly increased plaque formation relative to bacteria cultured in NaCl-free medium (p = .004 for K96243, p = .021 for 153, p = .002 for 576, p = .017 for 1026b, p = .032 for 1530, and p = .016 for 1634). Moreover, we also observed a significant difference in the plaque-forming capacity of all *B. pseudomallei* isolates cultured in LB supplemented with 300 mmol L^{-1} NaCl compared with those cultured in 150 mmol L^{-1} NaCl (p = .027 for K96243, p = .029 for 153, p = .019 for 576, p = .033 for 1026b, p = .048 for 1530, and p = .042 for 1634). This finding indicated the influence of NaCl on *B. pseudomallei* pathogenesis.

4 | DISCUSSION

B. pseudomallei is a saprophyte that can survive and multiply under different environmental conditions (Cheng & Currie, 2005; Dharakul & Songsivilai, 1999; White, 2003). It is a difficult microorganism to kill. It can inhabit harsh environments for many years, especially in endemic areas, including northeast Thailand (Wuthiekanun et al., 1995) where saline soil and water are abundant. *B. pseudomallei* was reported as potential opportunist pathogens of CF patients (Mahenthiralingam et al., 2002; O'Carroll et al., 2003; O'Sullivan et al., 2011; Vandamme et al., 1997), who have a high concentration of NaCl in their lung airway surface liquid. Adaptive responses of *Burkholderia* species, including *B. pseudomallei*, to high salt conditions have been investigated previously (Inglis & Sagripanti, 2006; O'Quinn, Wiegand, & Jeddeloh, 2001; Pumirat et al., 2009, 2010), however, the mechanisms underlying these remain poorly understood. This study demonstrated the adaptive phenotypes of six *B. pseudomallei* isolates to NaCl in various concentrations. The concentrations of NaCl used in our experiments were in the range of salt concentrations found in the soil and water in northeast Thailand. We showed that adaptations under salt stress conditions were associated with cross-protection against other environmental stresses, as well as increased pathogenicity.

Our present study verified that the growth rate of six *B. pseudomallei* isolates in LB containing 0, 150, and 300 mmol L^{-1} NaCl remained constant. We therefore conducted our experiments within this range of concentrations. Although high salinity seems to be a disadvantage for *B. pseudomallei*, as high salt (≥470 mmol L^{-1} NaCl) diminished bacterial growth (Pumirat et al., 2010; Wang-Ngarm, Chareonsudjai, & Chareonsudjai, 2014), *B. pseudomallei* would regularly encounter a

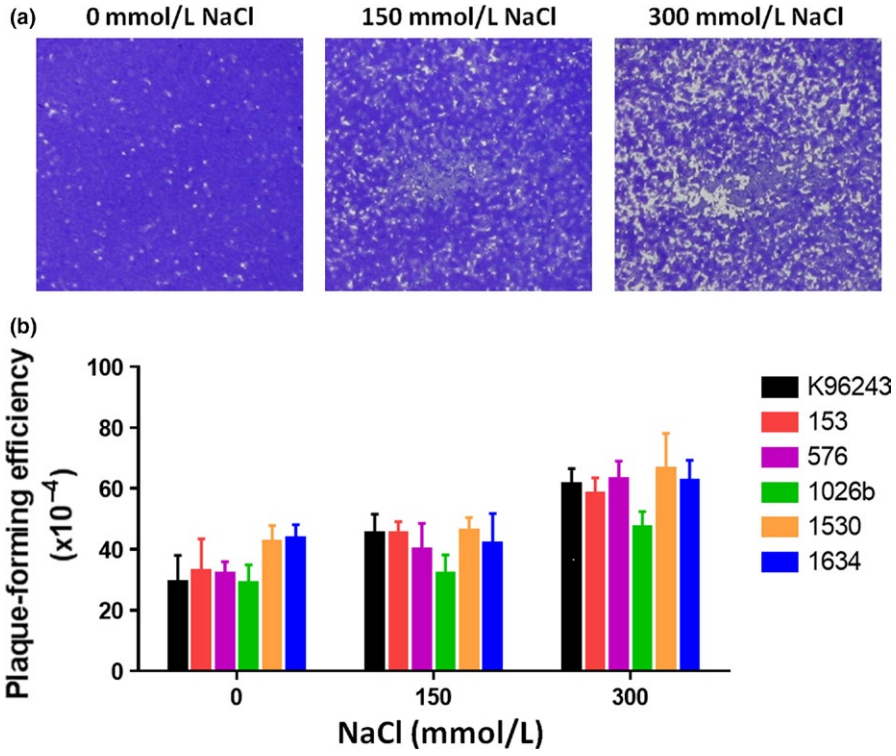

FIGURE 4 Plaque formation by *Burkholderia pseudomallei* after growth in Luria–Bertani (LB) broth containing 0, 150, and 300 mmol L^{-1} NaCl. (a) Images of plaques formed by *B. pseudomallei* K96243. Representative images of HeLa cell monolayers after infection with *B. pseudomallei* K96243, which had been grown in LB broth containing 0, 150, or 300 mmol L^{-1} NaCl for 20 hr. (b) Plaque-forming efficiency of six *B. pseudomallei* isolates. HeLa cells were infected with *B. pseudomallei* grown in LB broth containing 0, 150, or 300 mmol L^{-1} NaCl at a multiplicity of infection of 20. The infected cells were stained with crystal violet after 20 hr incubation. Plaque-forming efficiency was calculated as the number of plaques × 100/number of colony-forming units of bacteria added per well. Error bars represent the standard deviation of the means for experiments performed in triplicate

high salinity environment in its physiological habitat. In this study, we demonstrated that NaCl enhanced the ability of *B. pseudomallei* to survive under heat and oxidative stress. Several studies in other bacteria, such as *Bacillus cereus* (den Besten et al., 2009), *Bacillus subtilis* (Volker, Mach, Schmid, & Hecker, 1992), and *Escherichia coli* (Gunasekera, Csonka, & Paliy, 2008), have also reported that activation of the salt stress response conferred cross-protection against other stresses, that is, increased resistance to heat and H_2O_2. Recently, Yuan, Agoston, Lee, Lee, & Yuk, (2012) and Yoon et al., (2013) also showed that the heat resistance of *Salmonella enterica* was increased after exposure to NaCl. Moreover, it is evident that growing *Vibrio harveyi* in LB broth supplemented with 2% NaCl (34.2 mmol L^{-1}) resulted in increased resistance to menadione killing compared with the same organism grown in normal LB broth (Vattanaviboon, Panmanee, & Mongkolsuk, 2003). It is possible that the salt stress adaptation may reflect the ability of these bacteria, including *B. pseudomallei*, to survive under hostile environmental conditions, such as high temperature and oxidative stress.

As *B. pseudomallei* is an intracellular organism, it has the capability to survive in phagocytic cells (Allwood, Devenish, Prescott, Adler, & Boyce, 2011). While trafficking within macrophages, *B. pseudomallei* may be exposed to oxidative stress. Interestingly, Scott & Gruenberg (2011) reported that chloride and sodium ion channels play important roles in regulating the phagosomal environment through counter ion regulation and charge compensation of macrophages. Therefore, the salt content in the phagosome may promote bacterial resistance to oxidative stress and allow *B. pseudomallei* to survive within the host cell.

These oxidative and heat protective effects of NaCl could be a result of the increased expression of stress response cellular components. The increased expression of the *rpoE* and *groEL* genes detected in this study was in agreement with previous reports for the *B. pseudomallei* transcriptome (Pumirat et al., 2010) and secretome (Pumirat et al., 2009) under high salinity conditions. The expression of *groEL* (*bpss0477*) and *rpoE* (*bpsl2434*) was upregulated in *B. pseudomallei* cultured in LB containing 320 mmol L^{-1} NaCl, by approximately 1.2- and 1.4-fold, respectively, compared with *B. pseudomallei* cultured in 170 mmol L^{-1} NaCl at the 6-hr time point (Pumirat et al., 2010). Indeed, the secretomic profile confirmed the presence of GroEL in the culture supernatant only after exposure to 320 mmol L^{-1} NaCl (Pumirat et al., 2009). Moreover, our results were consistent with the observation that inactivation of the *rpoE* operon increased susceptibility of *B. pseudomallei* to killing by menadione and H_2O_2 and high osmolarity (Korbsrisate et al., 2005). Furthermore, it has been demonstrated that *rpoE* regulated a heat-inducible promoter of the *rpoH* gene in *B. pseudomallei* (Vanaporn et al., 2008). These data implied that RpoE plays an important role in the increased resistance of *B. pseudomallei* in response to heat and oxidative stress.

Among the salt-altered genes of *B. pseudomallei* K96243 (Pumirat et al., 2010), we previously detected downregulation of the flagella biosynthesis sigma factor *fliA* gene (*bpsl3291*), by approximately 1.5- and 1.2-fold (at 3 and 6 hr, respectively), when *B. pseudomallei* was grown in medium supplemented with 320 mmol L^{-1} NaCl compared with 170 mmol L^{-1} NaCl. This observation led us to examine whether growth of *B. pseudomallei* under high salt conditions affected the production of flagella. Under electron microscopic examination (Table 3), we found that most *B. pseudomallei* isolates grown under high salt conditions (300 mmol L^{-1} NaCl) did not produce flagella, whereas the majority of *B. pseudomallei* isolates grown under lower salt concentrations (0 and 150 mmol L^{-1} NaCl) presented at least one flagellum. The decreased expression of motility genes due to salt stress has also been documented for other bacteria such as *Sphingomonas* sp. strain LH128 (Fida et al., 2012) and *B. subtilis* (Hoper, Bernhardt, & Hecker, 2006; Steil, Hoffmann, Budde, Volker, & Bremer, 2003). All six *B. pseudomallei* isolates exhibited a smaller mean diameter for their motility zone when cultured under high salt conditions (300 mmol L^{-1} NaCl), compared with culturing under salt-free or low salt conditions (0 and 150 mmol L^{-1} NaCl). This observation implied that salt stress plays an important role in regulating the production of bacterial flagella. One possible explanation for this is that in order to cope with stressful environmental conditions the bacteria conserve energy by diminishing nonvital activities, such as motility, by reducing the production of flagella by decreasing the expression of the motility regulator gene.

The ability to form a biofilm is important for *B. pseudomallei* to gain resistance to numerous environmental factors, including certain antibiotics and stresses (Cheng & Currie, 2005; Inglis & Sagripanti, 2006; Kamjumphol et al., 2013). Our study detected the increased ability of *B. pseudomallei* to form a biofilm when bacterial isolates were grown in medium supplemented with NaCl, compared with salt-free medium (Table 4). This was consistent with the findings of Kamjumphol et al. who demonstrated that biofilm formation was increased when *B. pseudomallei* was grown in modified Vogel and Bonner's medium containing 0.85–1.7 mol L^{-1} NaCl (Kamjumphol et al., 2013). This indicated that *B. pseudomallei* responds to salt stress by producing a biofilm that could confer cross-protection against other environmental stresses.

Exposure to high salinity is likely to be associated with pathogenesis in *B. pseudomallei*. Previously, invasion of A549 cells was enhanced by culturing of *B. pseudomallei* K96243 in salt-supplemented LB medium (Pumirat et al., 2010). Our results showed that when grown in the presence of NaCl, all six *B. pseudomallei* isolates exhibited significantly increased plague formation in HeLa cells (Figure 4). The elevated rate of cellular invasion in response to NaCl may increase the load of intracellular bacteria, contributing to cell-to-cell spread or enhance cell cytotoxicity. Several studies have demonstrated the requirement of the Bsa T3SS and type VI secretion system (T6SS) for the intracellular pathogenicity of *B. pseudomallei* (Burtnick et al., 2008, 2011; Lim et al., 2015; Shalom, Shaw, & Thomas, 2007; Stevens et al., 2002; Warawa & Woods, 2005). We postulate that these systems may participate in the enhanced plaque formation of *B. pseudomallei* observed after exposure to NaCl. However, further experiments are required to investigate this possibility.

5 | CONCLUSIONS

In conclusion, our results demonstrated that high salt conditions modulate adaptive responses in *B. pseudomallei* isolates. These adaptive responses include increased thermal resistance, plaque formation, and

decreased flagella and oxidative susceptibility. Similar results were observed in all six isolates tested; suggesting that salt stress induces a general, conserved response in *B. pseudomallei*. Our findings provide insight into how these bacteria persist in endemic environments abundant in saline soil and water, and may indicate the link between the establishment and pathogenesis of *B. pseudomallei* infection in CF patients.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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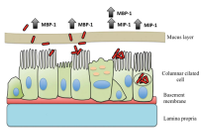

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RESEARCH PAPER

Trehalase plays a role in macrophage colonization and virulence of *Burkholderia pseudomallei* in insect and mammalian hosts

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ABSTRACT

Trehalose is a disaccharide formed from two glucose molecules. This sugar molecule can be isolated from a range of organisms including bacteria, fungi, plants and invertebrates. Trehalose has a variety of functions including a role as an energy storage molecule, a structural component of glycolipids and plays a role in the virulence of some microorganisms. There are many metabolic pathways that control the biosynthesis and degradation of trehalose in different organisms. The enzyme trehalase forms part of a pathway that converts trehalose into glucose. In this study we set out to investigate whether trehalase plays a role in both stress adaptation and virulence of *Burkholderia pseudomallei*. We show that a trehalase deletion mutant (*treA*) had increased tolerance to thermal stress and produced less biofilm than the wild type *B. pseudomallei* K96243 strain. We also show that the Δ*treA* mutant has reduced ability to survive in macrophages and that it is attenuated in both *Galleria mellonella* (wax moth larvae) and a mouse infection model. This is the first report that trehalase is important for bacterial virulence.

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Introduction

Trehalose is a disaccharide, composed from two glucose molecules, which can be found in many species of bacteria, fungi, plants and invertebrates.[1] It is a very stable molecule, typically requiring the presence of a trehalase enzyme for degradation into glucose. The properties of this molecule make it an atypical sugar with many varied functions and the stability of trehalose maybe central to many of its unique biological properties. Trehalose was first proposed to function as an energy storage molecule. This is certainly true in many insects where trehalose provides an easily mobilisable energy reserve, which is consumed during flight.[2] It is also thought that trehalose provides an energy reserve in fungal spores while spore germination is accompanied by trehalose hydrolysis.[3,4] In Mycobacteria and Corynebacteria, trehalose plays a role as a structural component of many cell wall glycolipids. In *Mycobacterium tuberculosis* the cell wall "cord factor" (trehalose 6,6-dimycolate) provides both impermeability to drugs and protection against phagocyte killing.[1,5] Additionally, high levels of trehalose are found in organisms that can survive desiccation. In this situation the trehalose serves to stabilize macromolecules and especially proteins and membrane lipids in the cell. By forming a glass-like structure around these molecules they are protected from damage caused by dehydration and oxidation.[1,6] The ability of trehalose to stabilize biological molecules is now being exploited widely to extend the shelf life of a wide range of products, including enzymes, antibodies and vaccines.[6] Finally, trehalose and trehalose derivatives such as trehalose-6-phosphate have been shown to regulate some biological processes such as metabolism and growth in plants and in fungi.[1,6,7]

The roles of trehalose in microbial virulence have been studied in fungal and bacterial pathogens. Mutations that abolish trehalose production in a range of fungi including *Candida albicans, Magnaporthe oryzae, Stagonospora nodorum, Cryptococcus neoformans*, and *Cryptococcus gattii* result in reduced virulence.[5] In *M. tuberculosis* the inactivation of the TreYZ pathway for trehalose biosynthesis reduced the ability of the bacterium to establish chronic disease.[8] The situation is also complicated by the findings that the role of trehalose in virulence may be

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host species dependent. For example, in *Pseudomonas aeruginosa* the disruption of trehalose biosynthesis reduced virulence toward plants, but not virulence toward *Caenorhabditis elegans, Drosophila melanogaster*, or mice.[9] Trehalose degradation, by endogenous trehalase, has been reported to play a role in virulence in some fungal pathogens including *M. oryzae* and *C. albicans*, but had no effect on virulence in *C. gattii*.[5] Trehalase has previously been identified as a possible virulence associated gene in bacteria, since it is present in bacterial pathogens but absent or uncommon in non-pathogens.[10] Orthologues have been identified in 7 pathogenic bacteria including *Burkholderia pseudomallei*,[10] [personal communications. In *B. pseudomallei* this gene is annotated as *treA* and the encoded protein TreA has sequence identity with proteins in *Salmonella typhimurium* (55%), *Klebsiella pneumoniae* (56%), *Shigella fexneri* (52%), *E. coli* 0157:H7 (56%) and *Pseudomonas aeruginosa* (60%). This has lead us to hypothesize that TreA could be important during mammalian infection.

Burkholderia pseudomallei is the causative agent of melioidosis, a serious and often fatal disease of humans. The disease is frequently reported in Southeast Asia and Northern Australia and there is increasing evidence that melioidosis occurs in many, and possibly all, tropical countries.[11] A recent study has found that the incidence of this disease is likely to have been significantly underestimated[12] and global deaths from melioidosis are comparable to those due to measles and much higher than fatalities due to leptospirosis or dengue fever.[12] In many of these countries the disease is currently mis-diagnosed as tuberculosis. Although melioidosis can occur in apparently healthy individuals, conditions such as diabetes predispose individuals.[13] The manifestations of disease range from an asymptomatic infection, to a fatal sepsis.[13] The bacterium has the ability to establish persistent but asymptomatic infections in humans for up to 60 y.[14] Therapy is not always successful because the bacterium is resistant to many antibiotics. Consequently, a protracted course of treatment with a combination of antibiotics is required and relapse following treatment is common.[13] Currently there is no licensed vaccine available for the prevention of disease. In endemic regions, *B. pseudomallei* can be readily isolated from soil and water. Melioidosis in humans is a consequence of exposure to this bacterium, via cuts and abrasions, inhalation or ingestion. The lifecycle of the bacterium in the environment is poorly understood but it is known that it can survive for years in hostile environments lacking nutrients and can survive exposure to a wide range of temperatures and dehydration.[15,16] In this study we have set out to investigate whether trehalase plays a role in virulence of *B. pseudomallei*.

Results

Construction of B. pseudomallei treA *mutant*

We identified a single trehalase gene, encoding a 565 amino acid protein, located on chromosome 2 (BPSS0671) in *B. pseudomallei* K96243. This gene was also present in all of the reported *B. pseudomallei* genome sequences. The *B. pseudomallei* trehalase was similar to the *E. coli* neutral trehalase (treA) with 56% amino acid identity. We constructed a *treA* deletion mutant using vector pMo130.[17] The mutant was genome sequenced to confirm that *treA* was deleted and we did not find additional mutations in the genome. To complement the mutant we cloned the *treA* gene downstream of the native promoterdownstream of a strong constitutive (*dhfr*) promoter or downstream of a regulatable promoter (*rhaB*). The plasmids (pBHRnat-*treA* pDA17-*treA* or pSCrhaB2-*treA* respectively) were transformed into *B. pseudomallei Δ*treA*. One or more of these plasmids were then used in subsequent assays.

In M9 glucose both wild type *B. pseudomallei* and *B. pseudomallei* Δ*treA* grew similarly (Fig. 1A). In contrast, when the strains were grown in M9 trehalose, the *ΔtreA* mutant failed to grow whereas the wild type strain reached a final optical density of OD_{590}nm 1.2 (Fig. 1B). The complemented strains *B. pseudomallei ΔtreA*/pBHR-nat*treA* and *B. pseudomallei ΔtreA*/pDA17-*treA* were able to grow in M9 trehalose, although it should be noted that this complementation assay was only performed once (Fig. 1C).

*Δ*treA *had increased tolerance to thermal stresses*

Bacterial trehalases have previously been shown to have a role in thermal stress.[18] As a consequence, we assayed the role of *B. pseudomallei treA* in survival after heat and cold stress. Overnight cultures of *B. pseudomallei, B. pseudomallei ΔtreA* or *B. pseudomallei ΔtreA*/pSCrhaB2-*treA* were standardized to OD_{590}nm 0.1 in LB broth before heating at 65°C for 2 hours and enumerating survivors on LB agar. The wild type and complemented mutant strains had survival frequencies of approximately 10^{-3} (Fig. 2A). In comparison the survival frequency of the Δ*treA* mutant was significantly higher at approximately 10^{-2} ($p = 0.015$ compared to WT). We also tested survival following exposure to cold stress. Overnight cultures of *B. pseudomallei, B. pseudomallei ΔtreA, B. pseudomallei ΔtreA*/pBHRnat-*treA* or *B.*

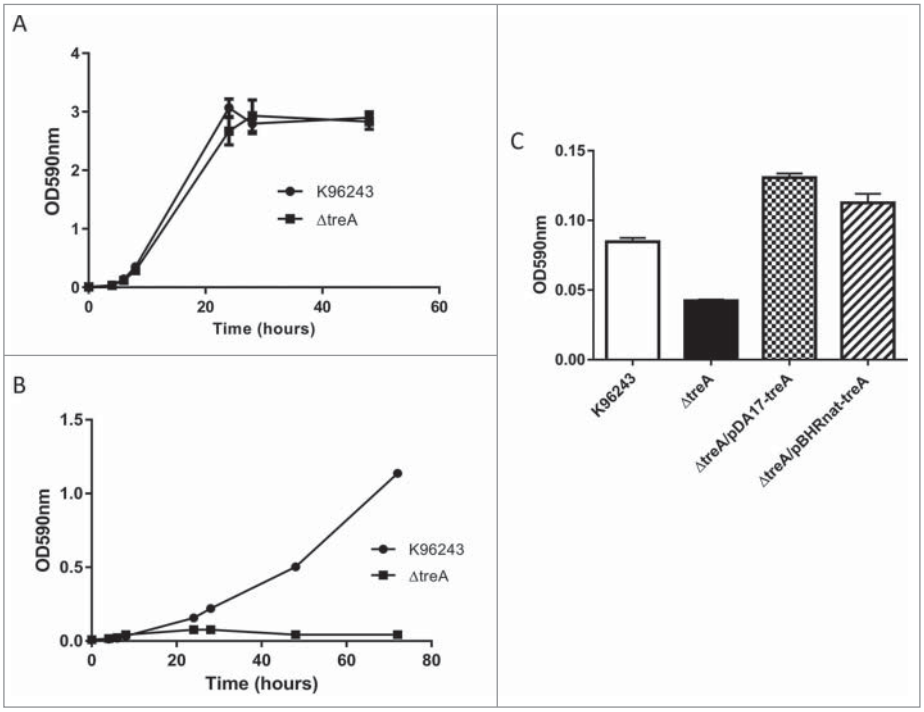

Figure 1. Growth of *B. pseudomallei* wild type, Δ*treA* mutant or complemented mutants in M9 medium containing 0.4% glucose with agitation (A) or 0.4% trehalose with agitation (B). The ability of complemented mutants (*ΔtreA*/pBHR-nat*treA* and *ΔtreA*/pDA17-*treA*) to grow in M9 medium containing 0.4% trehalose, in a static 96 well plate format, was recorded after 48 hours (C). Values represent the mean from one experiment performed in triplicate. Error bars show standard error of the mean (SEM).

pseudomallei ΔtreA/pDA17-*treA* were standardized to 100 CFU and incubated at 4°C for 5 d before enumeration on LB agar at 37°C. On average the survival frequency was $10^{-0.8}$ (Fig. 2B). In contrast the survival frequency of the *ΔtreA* mutant was $10^{-0.3}$ ($p = 0.004$ compared to wild type). The *B. pseudomallei ΔtreA*/pBHRnat-*treA* strain had a survival frequency of approximately 10^{-1}, whereas *B. pseudomallei ΔtreA*/pDA17-*treA* had no survival.

*Δ*treA *had reduced ability to survive inside macrophages*

The intracellular survival of *B. pseudomallei* wild type, the Δ*treA* mutant or the pDA17-*treA* complemented mutant were determined by counting the number of bacteria at each time point. The overall patterns of infection by the wild type and Δ*treA* mutant were similar; the numbers of intracellular bacteria decreased between 2

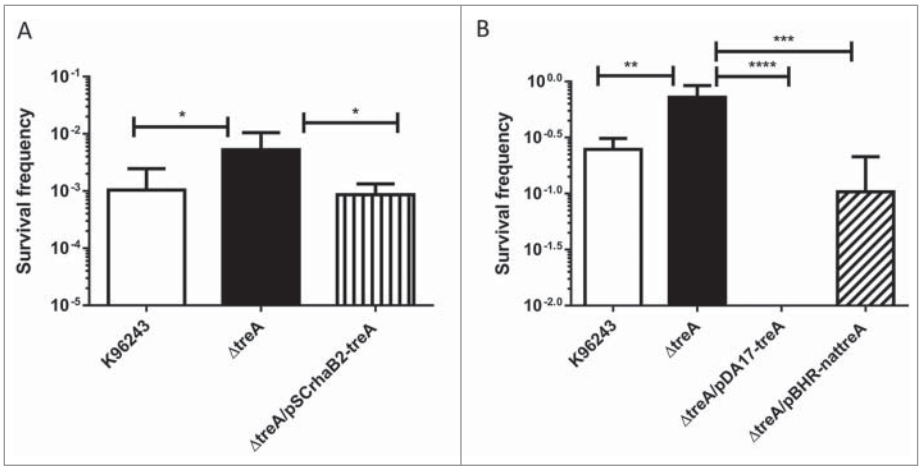

Figure 2. Survival of *B. pseudomallei* wild type Δ*treA* mutant, pSCrhaB2-*treA* complement, pDA17-*treA* complement or pBHR-nat*treA* complement after exposure to heat stress at 65°C (A) or cold stress at 4°C (B) Values represent the mean from 5 independent experiments performed in triplicate or 2 independent experiments with 9 replicates respectively. Error bars show SEM. $^{*} = p < 0.05$, $^{***} = p < 0.001$, $^{****} = p < 0.0001$ following one way Anova, Tukey post-test.

and 4 hours post infection but increased by 6 to 8 hours post infection. However, even though the J774.A.1 macrophages were infected with wild type and Δ*treA* mutant at an MOI of 10 the numbers of Δ*treA* mutant bacteria were significantly lower than the parental strain at 2, 4, 6, and 8 hours post infection ($p = 0.018$, 0.085, 0.0003, and 0.0008 respectively). This defect in intracellular survival was restored in the complemented strain (Fig. 3).

Effects of biochemical-induced stress conditions on growth

Next, stresses that *B. pseudomallei* may encounter in macrophages were tested *in vitro*. We saw no difference in survival of the mutant, compared to the wild type, after exposure to pH 4 for up to 60 min, or with 1 M hydrogen peroxide (data not shown). Compared with the wild-type, sensitivity of the Δ*treA* mutant was not significantly different either to intracellularly or to extracellularly generated superoxide using paraquat or xanthine/xanthine oxidase, respectively (data not shown). However, the mutant was more susceptible to killing by 0.04 M tert-butyl hydroperoxide (tBOOH) with $p = 0.016$ and the phenotype was restored in the pDA17-*treA* complemented strain (Fig. 4).

treA *is important in biofilm formation*

B. pseudomallei, *B. pseudomallei* Δ*treA* or *B. pseudomallei* Δ*treA*/pDA17-*treA* were inoculated into TSB and biofilms were grown on peg lids inserted into the cultures. Following 48 hours growth, pegs were stained with crystal violet and the optical density measured following an ethanol wash to quantify biofilm levels. The wild type *B. pseudomallei* and *B. pseudomallei* Δ*treA*/pDA17-*treA* strains had similar levels of staining giving standardized optical density readings (OD490 nm/OD595 nm) of 0.1203 ± 0.01 and 0.0984 ± 0.01 respectively (Fig. 5). In contrast the *B. pseudomallei* Δ*treA* mutant had significantly reduced staining and the

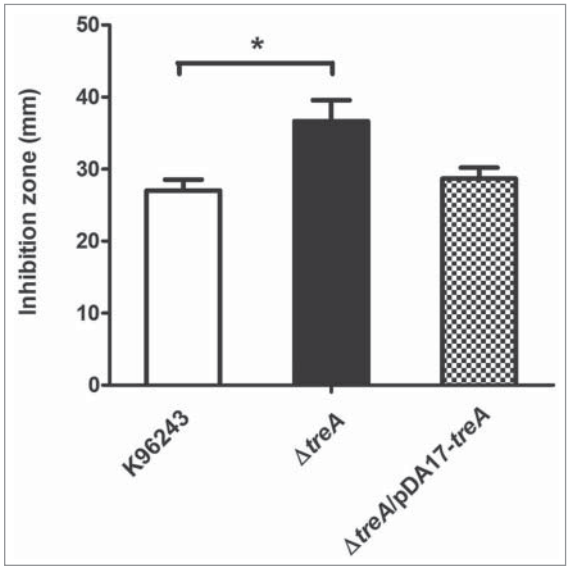

Figure 4. Response of *B. pseudomallei* wild type, Δ*treA* mutant or pDA17-*treA* complemented mutant to 0.04 M tBOOH by disc diffusion assay. Values represent the mean from 3 independent experiments performed in triplicate, with error bars showing SEM. * = $p < 0.05$ following students t-test.

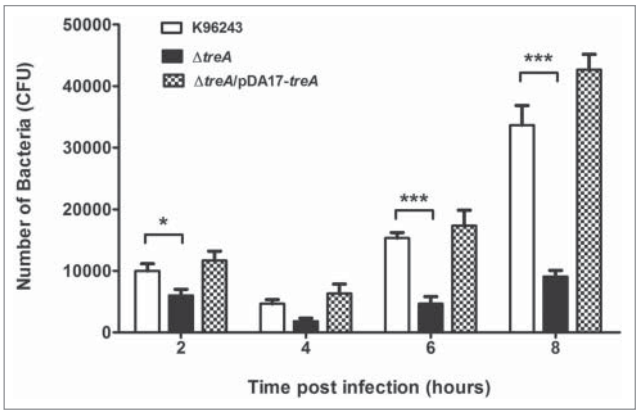

Figure 3. Intracellular survival of *B. pseudomallei* wild type, Δ*treA* mutant or pDA17-*treA* complemented mutant in J774.A.1 macrophages. Macrophages were infected with bacteria at an MOI of 10 and intracellular bacteria enumerated on LB agar. Values represent the mean from 3 independent experiments, performed in triplicate, with error bars showing the SEM s. * = $p < 0.05$, *** = $p < 0.001$ following students t-test.

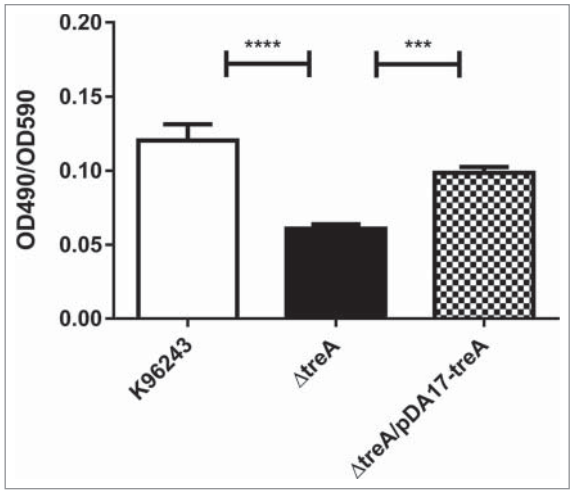

Figure 5. Relative biofilm formation by *B. pseudomallei* wild type, Δ*treA* mutant or pDA17-*treA* complement measured as crystal violet (CV) bound (OD490nm) / turbidity of bacterial culture (OD590nm). Values represent the mean from 3 independent experiments each with 12 replicates. Error bars show SEM. *** = $p < 0.001$, **** = $p < 0.0001$ following one-way Anova, Tukey post-test.

standardized optical density of the released crystal violet solution was (OD490nm/OD595nm) 0.06 ± 0.01.

To determine if the difference in biofilm formation was due to differences in capsular polysaccharide production, the bacteria were incubated with an anti-capsule antibody and binding was quantified using a GFP labeled secondary antibody and fluorescence microscopy. Labeled *B. pseudomallei, B. pseudomallei ΔtreA* or *B. pseudomallei ΔtreA*/pDA17-*treA* all had similar pixel intensities following image quantification (Fig. S1) indicating that similar amounts of capsule polysaccharide were present on the bacterial cell surface. Incubating each strain with an anti-LPS antibody and quantifying with a GFP labeled secondary antibody as a control, did not result in fluorescence (data not shown). This evidence is consistent with the presence of a CPS layer on all the strains blocking binding of the anti-LPS antibody.

*Δ*treA *is attenuated in the* G. mellonella *and mouse infection model*

After challenging *G. mellonella* with 10^3 Colony forming units (CFU), all larvae infected with wild type *B. pseudomallei* were dead at 27 hours, whereas 40% of larvae challenged with the Δ*treA* mutant were alive (Fig. 6A). We were unable to complement the wild type phenotype when challenging *G. mellonella* with *B. pseudomallei* Δ*treA*/pDA17-*treA* or *B. pseudomallei* Δ*treA*/pBHRnat-*treA* and speculate this could be due to the lack of antibiotic selection to retain the complementation plasmids during infection (Data not shown). When mice were challenged with wild type *B. pseudomallei*, death of the mice was recorded from day 4 until day 42. In contrast, all mice challenged with *B. pseudomallei* Δ*treA* were still alive at the end of the study (Fig. 6B). However, when the Δ*treA* infected mice were culled, bacteria were isolated from lungs, livers, and spleens with average CFU counts of 2.3×10^4, 4.9×10^3 and 7.5×10^2 CFU/ml respectively. Our results suggest a marked reduction in virulence of the *B. pseudomallei* Δ*treA* mutant in both *G. mellonella* and mice.

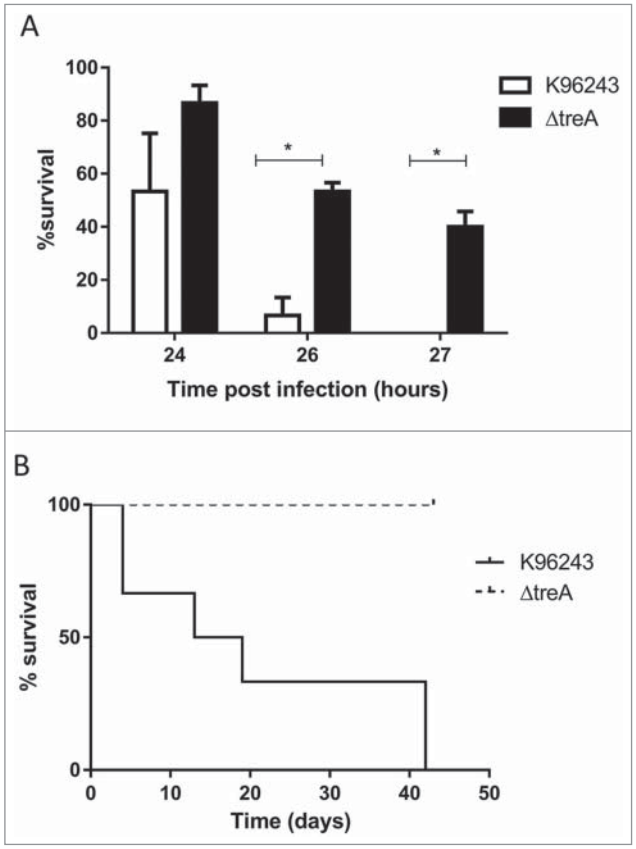

Figure 6. Virulence of *B. pseudomallei* wild type or Δ*treA* mutant in *G. mellonella* larvae or in mice. (A) Groups of 10 *G. mellonella* larvae were challenged with 10^3 CFU of *B. pseudomallei* K96243 or the *ΔtreA* mutant. Values represent the mean from 3 independent experiments. Error bars show SEM. * = $p < 0.05$ following 2 way Anova, Sidak's multiple comparisons test. (B) Groups of 6 BALB/c mice challenged via i.p. route with 3.4×10^4 CFU of *B. pseudomallei* K96243 (solid line) or 6.7×10^4 CFU of the *ΔtreA* mutant (dotted line).

Discussion

Trehalose plays a number of distinct functions in plants, invertebrate and microbial cells from serving as an energy storage carbohydrate, to protecting membranes and proteins from stress-induced damage and to a regulator of the glycolytic path and signaling pathways.[19] In this study we have identified a single trehalase encoding gene in *B. pseudomallei*. The protein was similar to the *E. coli* TreA trehalase, which is located in the periplasmic space of the bacterium. *E. coli* also possesses a second trehalase (TreC) which is cytoplasmic, and a TreB transporter which is located in the inner membrane and is responsible for import of trehalose into the cell. Neither TreB nor TreC were identified in the genome sequences of *B. pseudomallei*. However at high osmolarity the normal pathway for the uptake and utilization of trehalose is blocked.[20] *E. coli* can also utilize periplasmic trehalose by splitting it into glucose molecules, which are subsequently taken up by the phosphotransferase- mediated uptake system.[20] This may be the same mechanism that *B. pseudomallei* uses to utilize trehalose without having a trehalose transporter. *B. pseudomallei* also has no apparent cytoplasmic trehalase within its genome. However, it does contain a putative trehalose synthase (*treS*) gene. TreS catalyzses the reversible interconversion of trehalose and maltose within the cytoplasm.[21] Like many bacteria *B. pseudomallei* possess an *otsBA* operon, which encodes trehalose sythetase/phosphatase. Therefore, it appears that *B. pseudomallei* may both produce and

metabilize trehalose within the bacterial cell. The biosynthetic pathway is broadly paralleled in fungi. In fungi, acid and neutral trehalases are involved in trehalose degradation. The former are found in vacuoles within the fungal cell, while neutral trehalases are cytoplasmically located.[22]

In this study we have shown that the *treA* gene plays a role in mitigating stress tolerance and in virulence of *B. pseudomallei* in murine and insect infection models. Whole genome transcriptome profiling of *B. pseudomallei*, has previously shown that *treA* is expressed in many different conditions but importantly appears to be upregulated in conditions of nutrient deprivation aswell as in normal human serum.[23] The ability of trehalose to protect cells against stress is well documented in invertebrates, plants and fungi.[6] The protective properties of trehalose are attributed to different mechanisms ranging from the formation of an inert "glass" around macromolecules to the ability of the molecule to replace or exclude water molecules.[24] As a consequence of the protective properties of trehalose the oxidation and degradation of macromolecules is minimized.[24] Deletion of the *treA* gene increased the ability of the bacterium to survive exposure to both heat and cold stress. Deletion of the *Listeria monocytogenes* trehalase (*treA*) has previously been shown to increase heat tolerance [18] and was associated with increased intracellular trehalose. Like *E. coli, treA* in *L. monocytogenes* is predicted to encode a cytoplasmic protein. Fungal trehalase mutants have also been reported to show enhanced thermal stress.[25] There is less evidence linking intracellular trehalose levels to cold stress, but trehalose is believed to act as a cryoprotectant in insects [26] and the addition of trehalose to media during prolonged cold storage has been shown to increase the long term survival of *Salmonella enterica*.[27] Our finding that the constitutive expression of *treA* (in the *B. pseudomallei* Δ*treA*/pDA17-*treA* strain) abolished the ability of the bacterium to withstand cold shock, is consistent with increased levels of trehalase and consequently reduced levels of trehalose in the bacterial cell. However, quantification of trehalose levels would be needed to validate this. Furthermore, we can't rule out the possibility that deletion of trehalase is also having an effect upon the regulation and biosynthesis of trehalose via the genes *otsBA*. It's possible that the Δ*treA* mutant is instead upregulated for trehalose production. Including a *B. psedomallei* Δ*treA*Δ*otsBA* mutant in our assays would be needed to exclude this possibility.

Further investigations into the role of *treA* might reveal whether our findings are linked to the observations that *B. pseudomallei* is only found in soils in tropical or sub-tropical regions of the world.

We also showed the *B. pseudomallei ΔtreA* mutant had decreased biofilm formation and our results are similar to the findings with a T6P hydrolase *(treC)* mutant of *Klebsiella pneumonia*.[28] The reduction in biofilm by the *K. pneumoniae ΔtreC* mutant was associated with reduced CPS production. Biofilm and CPS production were restored by the addition of glucose to the growth media. Previous studies have shown that adding glucose to *B. pseudomallei* growth media have also shown increased biofilm formation.[29] However our studies failed to reveal a difference in capsular polysaccharide (CPS) production by *B. pseudomallei, B. pseudomallei* Δ*treA* or the complemented strains. Additionally we were unable to complement for the loss of biofilm with the addition of glucose in the growth media. Overall our findings indicate that different mechanisms link trehalose metabolism and biofilm production in *B. pseudomallei* and *K. pneumonia*.

While trehalose metabolism has previously been linked to the virulence of *Burkholderia glumae* and *Pseudomonas aeruginosa* in plants, [9,30] it has not been linked to virulence of bacterial pathogens in mammals. The *P. aeruginosa* Δ*treYZ*Δ*treS* trehalose biosynthesis mutant, which was attenuated in plants, retained full virulence in mice and insects.[9] Our results indicate a marked reduction in virulence of the *B. pseudomallei* Δ*treA* mutant in *G. mellonella* and in mice (although complementation would ideally be needed to provide further evidence) and this was reflected in the reduced ability of the Δ*treA* mutant to grow in murine macrophages.The ability to resist intracellular killing by oxidative stress is not related to trehalase because we found no difference in H_2O_2 response between *B pseudomallei* wild-type and the Δ*treA* mutant. Our data did show a difference in resistance to tert-butyl hydroperoxide (tBOOH). tBOOH is an organic hydroperoxide that decomposes to alkoxyl and peroxyl radicals that can further react to metal ions to generate reactive oxygen species (ROS) such as H2O2.[31] [32] tBOOH is more stable than H202 and additionally decomposition of tBOOH accelerates lipid peroxidation and depletes cell glutathione, so has additional effects to just hydrogen peroxide.[33] Furthermore, the proteins that respond to each stressor are different. H2O2 requires catalase, but tBOOH requires Ohr or AhpC.[34] This may explain the different outcome of both experiments.

While trehalase has not previously been linked to bacterial virulence of mammals or insects, there are reports of trehalase playing a role in fungal virulence.[25,35,36] In the study by Sanchez-Fresneda et al, the virulence potential of *Candida parapsilosis* trehalase mutants were also

reduced in a murine model, as determined by fewer CFU numbers isolated from kidney tissue compared to wild type and complemented strains.[25] Attenuation of the *B. pseudomallei ΔtreA* mutant in mice may also be due to lower cell numbers. This could be due to slower growth or increased bacterial clearance by the host immune system. Alternatively it may be linked to reduced ability to form biofilm, since*B. pseudomallei* that produce low levels of biofilm have previously been shown to have reduced virulence in a mouse infection model.[37]

Proteins involved in trehalose biosynthesis have been proposed as novel drug targets. This pathway is absent in mammalian cells and the enzymes involved in this pathway are highly specific.[19] Our finding that trehalase plays a role in virulence of *B. pseudomallei* suggests that that this pathway merits further investigation, as a drug target for the treatment of melioidosis.

Materials and methods

Bacterial stains, macrophage cell line, culture conditions, and reagents

E. coli strains DH5α, S17–1 *λ pir*, and *B. pseudomallei* K96243 were grown at 37°C in Luria Bertani broth (LB). Where indicated *B. pseudomallei* was gown in M9 minimal medium (pH 7.4) containing 0.4% w/v glucose or trehalose. Chloramphenicol (30 μg/ml for *E. coli* and 50 μg/ml for *B. pseudomallei*) and kanamycin (50 μg/ml for *E. coli* and 400 μg/ml for *B. pseudomallei*) were added as required. LB agar without sodium chloride but containing 10% (w/v) sucrose was used in the final step of mutant selection. Bacterial growth was determined by measuring the optical density at 600 nm in triplicate. The mouse macrophage cell line J774A.1 was obtained from the American Type Culture Collection (ATCC, Manasssas, Va.) and cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (HYCLONE)), 1% L-glutamine (250 mM) (Hyclone) and 1% penicillin / streptomycin (Hyclone) at 37°C with 5% CO_2. For infection with *B. pseudomallei*, macrophages were cultured in Gibco L-15 media (Invitrogen) at 37°C without CO_2. Reagents used in this study were obtained from Sigma-Aldrich unless stated otherwise.

Construction of treA mutant

A DNA fragment that included 540-bp upstream and 440-bp downstream regions of the *B. pseudomallei* K96243 *treA* coding region and flanked by *Bgl*II and *Nhe*I restriction sites was synthesized by GENEART. The DNA fragment was ligated into suitably digested pMo130 [17] and electroporated into *E. coli* DH5α. Recombinant plasmids were selected on LB agar containing 400μg/ml kanamycin. The recombinant plasmid was isolated and electroporated into *E. coli* S17-1 λpir, then conjugated with *B. pseudomallei* K96243. The conjugation and *sacB* counter selection was carried out as previously described.[38] Transconjugants were selected on LB agar containing 50 μg/ml chloramphenicol. Colonies were selected and sub cultured into LB broth and an overnight culture was diluted 10^4 fold and plated onto LB agar without salt containing 10% sucrose. After incubation at 24°C for 2–3 d colonies were picked and the *treA* deletion verified by PCR using primers F1 (5′- GTGCGAT GAAAGCGTAGAGG-3′ binds 700 bp upstream of treA ORF) and R2 (5′-AATACGGACCGCTCCATGC 3′ binds 550 bp downstream of the treA ORF) or F2 (5′- CGCTCAAGCTGCTCGATCTG-3′ which binds within the last 30 bp at the 3′ end of treA) and R2.

Genome sequencing of the ΔtreA mutant

B pseudomallei genomic DNA was harvested by the method described previously.[39] Rehydrated DNA was checked for quality by running on an agarose gel and running on a bioanalyser. The DNA was sequenced by the University of Exeter sequencing service and output files were aligned and analyzed against the published K96243 genome using artemis genome browser and annotation tool.

Construction of complementation plasmids

The *treA* gene was PCR amplified using Failsafe polymerase and the primers treA_fwd 5′- TTATGGATCCATGGT-CACGCCGCGGCATCGCCCGCTTCAT-3′ and treA_rv 5′- GCTAAAGCTTTCAGCCGCCGTACAGATCGAG-CAGCTTGAG-3′. The purified PCR product was then cloned into the *Bam*HI and *Hind*III sites of pSCrhaB2 [40] or pDA17.[41] For cloning *treA* and the predicted promoter region into pBHR4, the *treA* open reading frame and a region 300-bp upstream and 200-bp downstream was PCR amplified using primers treA_FWD_Nat 5′-TTAA GAGCTCCACGGTCATTTGCGCGAAGTGTGGGAAG-3′ and treA_RV_Nat 5′-ATTAGGATCCCGGTTTCC GACGGCGCATC-3′. The purified product was cloned into the *Sac*I and *Bam*HI sites of pBHR[42] removing the GroES promoter.

Biofilm assay

Bacterial cultures were grown on tryptic soy agar (TSA) plates at 37°C for 16 hour and then inoculated into PBS

standardizing to OD_{590}nm 1.0. Cultures were then diluted 1:2000 in fresh tryptic soy broth (TSB). 150μl aliquots were added to a 96-well peg lidded plate (12 wells for each culture) and incubated at 37°C. After 24 hours the peg lid was transferred into fresh TSB media for a further 24 hours before measuring the optical density of the plate at 590 nm. The peg lids were then washed in PBS before baking at 65°C for 30 minutes. Biofilms were stained with crystal violet for 30 minutes and then washed 3x with PBS. Crystal violet stain was released with ethanol treatment for 20 minutes and measured at OD490 nm. Relative biofilm levels were quantified by comparing OD490nm/OD590nm.

CPS staining

Overnight cultures of *B. pseudomallei* were diluted to OD_{590}nm 0.1 in 1 ml of PBS containing 4 % Paraformaldehyde (PFA) and incubated for 15 minutes on the bench. Cultures were then centrifuged at 13000 × *g* for 7 minutes and cells resuspended in 500 μl 5 % Bovine serum albumin (BSA) / phosphate buffer saline (PBS) and incubated for 1 hour. The IgG 2b isotype anti-CPS I monoclonal antibodyMab 4VIH12 [43] was then added to each strain at a final concentration of 25 μg/ml and incubated for 1 hour at 37°C. The cells were then harvested by centrifugation and washed twice in 500 μl PBS. The cells were then resuspended in 500 μl 5 % BSA/PBS containing anti- goat anti-rabbit GFP tagged secondary antibody (Licor) at a 1:400 dilution and incubated at 1 hour at 37°C. The cells were then harvested by centrifugation and washed twice in PBS. Finally the cells were suspended in 100 μl PBS. Ten microlitre μl aliquots were spotted onto a microscope slide, air-dried and overlaid with vector shield. Slides were visualized under the GFP filter on a fluorescence microscope (100 × objective) and imaged. Quantification of pixel intensity was carried out using image J software. The fluorescence intensity of all bacteria in at least 3 fields of view per strain was calculated.

Heat stress

Overnight cultures were standardized to OD_{590}nm 0.1 in LB broth and heated at 65°C for 2 hours. Cells were serial diluted and enumerated on LB agar. Survival frequency was calculated by the number of bacteria post heat treatment compared to pre-treatment.

Cold stress

Overnight cultures were standardized and serial diluted in LB before spread plating 100 bacteria onto LB agar plates. Half the plates were incubated at 37°C overnight (non-treated) and half incubated at 4°C for 5 d. After 5 d these plates were then incubated at 37°C for 1 day to enumerate survivors. Percentage survival was calculated by the number of CFU following cold stress compared to the number of CFU for non-treated samples.

Hydrogen peroxide and tBOOH stress

Overnight cultures were standardized to OD_{590}nm 0.1 in LB broth and 100 μl of culture was spread plated onto LB agar plates. Five millimeter diameter paper discs were then placed onto the plates and 5 μl of 2 M H_2O_2 (Sigma-Aldrich) or 6 μl of 0.04 M tert-butyl hydroperoxide (t-BOOH, Sigma-Aldrich) was applied to each disc. Plates were then incubated at 37°C for 24 hours before measuring the area of growth inhibition around the discs.

Superoxide stress

To investigate the effect of intracellular superoxide on viability, a paraquat assay was used. The optical density (OD_{590}nm) of an overnight bacterial culture was adjusted to 0.1 and 130 μl of the culture was spread onto LB agar. Paraquat solutions (100, 50, 10 and 5 mM) were pipetted onto either the surface of the inoculated agar or onto 6 mm sterile filter discs on the plates. After incubation at 37°C for 1 day or 20°C for 3 d the zones of inhibition were measured.

To determine sensitivity to extracellular superoxide, a xanthine/xanthine oxidase assay was used. Overnight cultures were diluted to OD_{590}nm of 0.1 ($\sim 10^8$ CFU/mL) and 1 ml of the culture was incubated with xanthine/xanthine oxidase (250 mM/0.14 U final concentrations, respectively). Catalase (100 U) was added to protect cells from H_2O_2 generated as a superoxide degradation product. After 30, 60, 90 or 120 minutes, bacteria were enumerated on LB agar. Colonies were counted after 1 day incubation at 37°C.

Acid stress assay

Bacterial overnight cultures were washed with PBS and adjusted to OD_{590}nm 0.1 in LB at pH 4, (acidified with 1 M Hydrochloric acid) and incubated at 37°C for 0, 10, 20, 30 or 60 minutes. Following incubation, bacteria were washed in PBS and enumerated. The percentage of survival was calculated by comparing against the number of bacteria at T0.

Galleria mellonella killing assay

Bacterial virulence toward *G. mellonella* (waxmoth) larvae was assessed by killing assays as described

previously.[42] Briefly, 10^3 CFU in a volume of 10 μl were injected into the hemocoel of 10 larvae per bacterial strain. Larvae were incubated at 37°C and the number of dead larvae scored after 24, 26, and 27 hours.

Survival in macrophages

Prior to infection, J774.A.1 macrophages were cultured in DMEM medium. Overnight cultures of *B. pseudomallei* were diluted to 1×10^6 CFU/ml in DMEM medium, then added to wells seeded with 1×10^5 macrophages (MOI of 10). After incubation at 37°C for 2 h extracellular bacteria were killed by replacing growth medium with DMEM medium containing 250 μg/ml kanamycin for 2 h, followed by maintenance in DMEM containing 10 μg/ml kanamycin. At 2, 4, 6 or 8 h post infection, cells were washed with pre-warmed PBS to remove the antibiotic. Viable intracellular bacteria were released from the infected cells by adding 0.1% (v/v) Triton X-100. The cell lysates were serially diluted with sterile distilled water and appropriate dilutions plated on LB agar. The numbers of colony forming unit (CFU) were counted after incubation at 37°C for 24 to 36 hours.

Virulence studies in mice

Female BALB/c age-matched mice, approximately 6 weeks old, were used in this study. The mice were grouped together in cages of 5 with free access to food and water and subjected to a 12 hour light/dark cycle. For challenge the animals were handled under bio-safety level III containment conditions. All investigations involving animals were carried out according to the requirements of the Animal (Scientific Procedures) Act 1986. In two separate experiments, groups of 6 mice were challenged with wild type *B. pseudomallei* strain K96243 or Δ*treA* by the intraperitoneal (i.p.) route and the infection monitored for 5 weeks. Humane endpoints were strictly observed and animals deemed incapable of survival were humanely killed by cervical dislocation.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Analysis of the Prevalence, Secretion and Function of a Cell Cycle-Inhibiting Factor in the Melioidosis Pathogen *Burkholderia pseudomallei*

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Abstract

Enteropathogenic and enterohaemorrhagic *Escherichia coli* express a cell cycle-inhibiting factor (Cif), that is injected into host cells via a Type III secretion system (T3SS) leading to arrest of cell division, delayed apoptosis and cytoskeletal rearrangements. A homologue of Cif has been identified in *Burkholderia pseudomallei* (CHBP; Cif homologue in *B. pseudomallei*; BPSS1385), which shares catalytic activity, but its prevalence, secretion and function are ill-defined. Among 43 available *B. pseudomallei* genome sequences, 33 genomes (76.7%) harbor the gene encoding CHBP. Western blot analysis using antiserum raised to a synthetic CHBP peptide detected CHBP in 46.6% (7/15) of clinical *B. pseudomallei* isolates from the endemic area. Secretion of CHBP into bacterial culture supernatant could not be detected under conditions where a known effector (BopE) was secreted in a manner dependent on the Bsa T3SS. In contrast, CHBP could be detected in U937 cells infected with *B. pseudomallei* by immunofluorescence microscopy and Western blotting in a manner dependent on *bsaQ*. Unlike *E. coli* Cif, CHBP was localized within the cytoplasm of *B. pseudomallei*-infected cells. A *B. pseudomallei chbP* insertion mutant showed a significant reduction in cytotoxicity and plaque formation compared to the wild-type strain that could be restored by plasmid-mediated *trans*-complementation. However, there was no defect in actin-based motility or multinucleated giant cell formation by the *chbP* mutant. The data suggest that the level or timing of CHBP secretion differs from a known Bsa-secreted effector and that CHBP is required for selected virulence-associated phenotypes *in vitro*.

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Introduction

Burkholderia pseudomallei is a facultative intracellular pathogen that causes melioidosis, a severe invasive disease of humans that may involve subacute and latent phases. The basis of entry and persistence of *B. pseudomallei* in host cells is ill-defined, but the *bsa*-encoded Inv/Mxi-Spa-like Type III secretion system (T3SS-3) has been identified as a key virulence factor [1,2]. T3SSs are nano-machines that inject bacterial effector proteins directly into host cells in order to subvert host cellular processes [3]. Only a small number of effectors have been confirmed to be substrates of the Bsa T3SS in *B. pseudomallei*, including BopC [4] and the guanine nucleotide exchange factor BopE [5]. A further candidate effector (BopA) was demonstrated to be Type III secreted in a surrogate bacterial host [6] and to interfere with LC3-associated phagocytosis [7]. A homologue of an *E. coli* Type III secreted effector termed Cif (cycle-inhibiting factor) was identified in *B. pseudomallei* and exhibits 21% amino acid identity and 40% similarity [8], but no evidence has yet been presented that it is secreted via the Bsa apparatus or that it influences pathogenesis during melioidosis.

In a subset of enteropathogenic and enterohaemorrhagic *Escherichia coli* (EPEC and EHEC), Cif is an effector of the locus of enterocyte effacement (LEE)-encoded T3SS [8,9] and belongs to the cyclomodulin family of proteins that interfere with the eukaryotic cell cycle [10]. Upon contact with epithelial cells, the bacteria inject this protein into the host cell where it induces cell enlargement, arrests the cell cycle G1/S and G2/M transitions, disrupts the actin network, delays cell death and triggers macrophage-specific apoptosis [8,11–13]. Recently, Cif was reported to act by deamidation of ubiquitin or the ubiquitin-like protein NEDD8 that regulates Cullin-RING-ubiquitin ligase (CRL) complexes [14–18]. The homologues of *E. coli* Cif in other bacterial pathogens of invertebrates and mammals have been described, including *B. pseudomallei* [15,17,19,20], *Yersinia pseudotuberculosis* [14,19], *Photorhabdus luminescens* [19–21] and *Photorhabdus asymbiotica* [19].

Jubilin et al [19] demonstrated that treatment of HeLa cells with the purified Cif homologue in *B. pseudomallei* (CHBP) mixed with BioPORTER reagent induced cell enlargement, cell cycle arrest at G2 phase and stress fiber formation in an identical manner to that of *E. coli* Cif. Analysis of the crystal structures of CHBP revealed that it possesses a papain-like fold with a Cys-His-Gln catalytic triad similar to *E. coli* Cif [20,22]. In addition, a recent study showed that CHBP is recognized by melioidosis patient sera [23] indicating that it is expressed *in vivo* and may play a role in pathogenesis.

In this study, we investigated the prevalence of CHBP in *B. pseudomallei* strains by genome sequence analysis and by using an antibody raised against a CHBP synthetic peptide to detect the protein in clinical isolates of *B. pseudomallei*. Whilst it is assumed that Cif family members are Type III secreted, no evidence has yet been presented that CHBP is secreted through the Bsa apparatus. We therefore explored whether CHBP is secreted via the Bsa T3SS and evaluated phenotypes of a *B. pseudomallei chbP* mutant and *trans*-complemented strains in a variety of cell culture infection assays.

Materials and Methods

Bacterial Strains, Cell Lines and Culture Conditions

The prototype genome-sequenced *B. pseudomallei* strain K96243, *bsaQ* mutant [24] and 14 clinical isolates [25] were routinely maintained in Luria-Bertani (LB) broth or agar (Hardy Diagnostic, USA) containing 40 μg/ml chloramphenicol where needed (*bsaQ*). All cultures were grown at 37°C. Cell lines used in this study including HeLa (human cervical carcinoma), J774A.1 (murine macrophage-like cell) and U937 (human monocyte cell) were obtained from the American Type Culture Collection (ATCC, Manasssas, VA). HeLa, J774.1 and U937 cell lines were routinely maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS). All cells were cultured in a 5% CO_2 atmosphere at 37°C in a humidified incubator.

Bioinformatic Analysis of CHBP

The 43 full or partial *B. pseudomallei* genome sequences available at the time of writing were interrogated using the K96243 CHBP protein sequence (accession number NC_006351.1) using a Basic Local Sequence Alignment Tool (tBLASTn) to determine prevalence and sequence conservation. All CHBP amino acid sequences were aligned using Clustal W to identify regions of homology or divergence.

Construction of *B. pseudomallei chbP* Insertion Mutant and *trans*-complemented Strains

A *B. pseudomallei chbP (bpss1385)* mutant was created by insertion of a plasmid with a conditional origin of replication and chloramphenicol resistance gene into the *chbP* gene on chromosome 2 of strain K92643. A 316 bp internal fragment of *B. pseudomallei chbP* (corresponding to nucleotide positions 183–498) was amplified using primers Cif-f (5′-CTCGGA TCCGAGTTT-GAAGATGTTGTTG-3′) and Cif-r (5′-CACTCTA-GAAACTGGCG AAAATCCTATG-3′) and the product was cloned into the suicide vector pKNOCK-Cm [26]. The recombinant plasmid pKNOCK-*chbP* was transformed into *E. coli* S17–1λ*pir* [27] and mobilized into *B. pseudomallei* K96243 by conjugation and recipients selected by plating on agar supplemented with 40 μg/ml chloramphenicol and 30 μg/ml kanamycin. The resulting *B. pseudomallei chbP*::pKNOCK mutant was verified by polymerase chain reaction (PCR) using the primer pairs KNOCK1/Cif-R (5′-CACTTAACGGCTG ACATGG-3′/5′-CCGACTAGTACATCTGCTGCGGTCTCAC-3′; product size of 935 bp) and KNOCK2/Cif-F (5′-GTAGCACCAGGCGTT-TAA-3′/5′-CCGCT CGAGATGCATCATCATCATCAT-CATCTACTATTGTTGGAGCACG-3′; product size of 1,584 bp), and by Southern blot analysis using genomic DNA double digested with *Apa*I/*Sma*I enzymes and a *chbP*-specific probe amplified by the Cif-f and Cif-r primers.

For complementation studies, the *chbP* open-reading frame was amplified from *B. pseudomallei* K96243 genomic DNA using primers BpsCifTEM (5′-ATATATGAGCTCCAGA-CAATCTGTGTGGG-3′) and BpsCif6His (5′-ATATATA-GATCTCTAGTGGTGGTGGTGGTGGTGGCCAAG GCCGACGACGTATTG-3′). The amplified DNA fragment was cloned into the IPTG-inducible broad host range vector pME6032 [28], generating pCHBP. This plasmid was delivered into the *B. pseudomallei chbP* mutant by electroporation to produce the *B. pseudomallei chbP*/pCHBP strain, which was confirmed by plasmid DNA extraction and sequencing.

Generation of a CHBP-specific Antibody and Western Blot Analysis

A polyclonal rabbit antiserum against CHBP was generated by Cambridge Research Biochemicals (Cleveland, UK) by immunization with the synthetic peptide ASHEYDFRQFQRNAQ. Specificity of the purified IgG was confirmed by Western blotting of lysates prepared from wild-type *B. pseudomallei* and *chbP* insertion mutant strains. To detect secretion of CHBP in culture supernatants, overnight cultures of *B. pseudomallei* strains were sub-cultured into LB broth or serum-free DMEM with or without induction with 10 mM IPTG where appropriate and incubated at 37°C for 6 h. After centrifugation, *B. pseudomallei* cell pellets were lysed with B-PER II Reagent (Pierce, Rockford, USA) to release intracellular proteins whereas bacterial cell culture supernatant was filtered through 0.22 μM low protein-binding membranes before protein precipitation using a final concentration of 50% (v/v) ethanol. Whole bacterial cell lysates and precipitated secreted proteins were resolved by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins were transferred to nitrocellulose membranes (Pierce). The blotted proteins were probed with rabbit BopE-specific [5] or CHBP-specific antibodies at a dilution of 1:500 for 3 h. Horseradish peroxidase (HRP)-conjugated mouse anti-rabbit IgG (DAKO, USA) at the dilution of 1:3000 and a chromogenic substrate-3, 3′-diaminobenzidine (DAB; Sigma Chemical Co., USA) were added to detect bound antibodies.

For detection of CHBP protein in infected host cells, U937 cells were activated with 20 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma Chemical Co.) for 48 h in DMEM supplemented with 10% (v/v) FBS then inoculated with *B. pseudomallei* strains at a multiplicity of infection (MOI) of 2 or 100. To induce the expression of CHBP from the pCHBP plasmid in the *trans*-complemented strain, IPTG was added to a final concentration of 10 mM to the culture medium. Two hours after addition of bacterial strains, infected cells were washed with phosphate-buffered saline (PBS) and maintained in media containing 250 μg/ml kanamycin to kill extracellular bacteria for a further 2 h. Media was replaced at 4 h post-infection with fresh medium containing 20 μg/ml kanamycin until 6 h post-infection. Thereafter, the infected cells were washed and lysed with 0.1% (v/v) Triton X-100 in PBS. Protein lysates of infected U937 cells were centrifuged for 1 min at 13,000×g to separate the bacteria and insoluble cytoskeleton of the cells from the cytosolic cell supernatant. Then, the supernatants were resolved by SDS-PAGE, transferred onto

nitrocellulose membranes and independently probed with anti-CHBP and anti-BopE antibodies as above. Bound primary antibodies were detected with HRP-conjugated mouse anti-rabbit IgG (DAKO, USA) at a 1:3000 dilution using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific Pierce, USA).

Confocal analysis of CHBP Following Infection

PMA-activated U937 macrophage cells were seeded on 22×22 mm square glass coverslips (Menzel-Glaser, Germany) in 6-well plates (Costar, USA) and incubated at 37°C in a humidified 5% CO_2 atmosphere. Overnight cultures of *B. pseudomallei* K96243, *chbP* mutant or the *trans*-complemented strain were used to infect U937 cells at a MOI of 2 for 2 h. A 10 mM IPTG (final concentration) was added to the culture medium for induction of CHBP expression from pCHBP. The duration of incubation and procedures for killing of extracellular bacteria were as described above for detection of CHBP in infected cells by Western blotting. At 6 h post-infection cells were washed and fixed with 4% (v/v) paraformaldehyde in PBS. The fixed cells were washed with PBS and permeabilized with 0.5% (v/v) Triton X-100 in PBS for 30 min. Then, 1% (w/v) bovine serum albumin (BSA) in PBS was added and incubated for 30 min at room temperature. Subsequently, the infected cells were stained with 1:500 of rabbit CHBP-specific antibody at 37°C for 1 h, followed by washing with PBS and bound antibodies were detected with a 1:1000 goat anti-rabbit antibody-Alexa Fluor^{488} (Molecular Probes, USA) in 1% (w/v) BSA. The staining was observed by confocal laser scanning microscope using a Zeiss LSM 510 META instrument (Carl Zeiss, Germany) and analyzed by DP Manager (version 3.1.1) equipped with LSM (release 3.2) software. Where necessary coverslips were stained for actin filaments using Alexa Fluor^{568}-conjugated phalloidin (Molecular Probes) and DNA stained using 4′, 6′ diamidine-2′-phenylindole dihydrochloride (DAPI, Molecular Probes). Bacteria were stained using mouse monoclonal anti-*B. pseudomallei* lipopolysaccharide antibody (Camlab, Cambridge, United Kingdom) detected with Alexa Fluor^{488}-conjugated anti-mouse Immunoglobulin (Molecular Probes).

Cell Infection Assays

To assay net intracellular replication**,** PMA-activated U937 cells were seeded and infected with *B. pseudomallei* strains at an MOI of 2. After 2 h infection at 37°C, cells were washed with PBS, media was replaced with medium containing 250 μg/ml of kanamycin to kill extracellular bacteria, and incubated for another 2 h. Thereafter, the infected cells were incubated with medium containing 20 μg/ml kanamycin. At 3, 6, 9 and 12 h post-infection, the infected host cells were washed with PBS and lysed with 0.1% (v/v) Triton X-100 in PBS. Viable intracellular bacteria were quantitated by plating serial ten-fold dilutions of lysates on trypticase soy agar and counting colonies after 24–36 h of incubation at 37°C.

Plaque-forming efficiency was evaluated as previously described [29] with some modifications. HeLa cells were infected with *B. pseudomallei* at an MOI of 20 and incubated at 37°C with 5% CO_2 for 2 h. After 2 h incubation, the infected cell monolayers were washed and replaced with a medium containing kanamycin (250 μg/ml). The plates were incubated at 37°C in a humidified 5% CO_2 atmosphere for at least a further 16 h. Plaques were stained with 1% (w/v) crystal violet in 20% (v/v) methanol and counted by microscopy. Plaque-forming efficiency was calculated by the following equation: number of plaques/CFU of bacterial added per well.

The efficiency of multinucleated giant cell (MNGC) formation [29] and cell cytotoxicity [30] in monolayers infected with wild-type, the *chbP* mutant and the *trans*-complemented strains of *B. pseudomallei* were assessed as described by Suparak et al [29] and Korbsrisate et al [30].

Statistical Analysis

All experiments were independently performed a minimum of three times. The significance of differences between groups was assessed using the unpaired *t*-test using GraphPad Prism 6 software (STATCON). P values ≤ 0.05 were taken to be significant.

Results

Prevalence and Sequence Diversity of CHBP in *B. pseudomallei*

B. pseudomallei K96243 chromosome 2 harbors *bpss1385*, the gene encoding the Cif homologue CHBP, a hypothetical 328 amino acid protein with a predicted molecular weight of 35.8 kDa. To examine the conservation of CHBP among sequenced *B. pseudomallei* strains, 43 available complete or draft *B. pseudomallei* genome sequences were searched for homologues to the CHBP protein of K96243 using tBLASTn and homologous sequences aligned using the ClustalW multiple sequence alignment tool. Of the 43 available genomes, 33 (76.7%) *B. pseudomallei* strains harbored CHBP with >99% amino acid sequence identity to CHBP of *B. pseudomallei* strain K96243. Apart from amino acid differences detected at E32G, T88M, G157R, G223E, G237E and T278M in a small number of strains, the amino acid sequences were remarkably highly conserved, with complete conservation of the predicted catalytic Cys-His-Gln triad [20] (Figure S1). A 1.5 kb deletion of *chbP* (*bpss1385*) between the predicted transposase genes *bpss1384* and *bpss1385a* was detected in the draft genome sequence of the virulent strain 10276 used to identify the *bsa* locus, and was confirmed by PCR with flanking primers (data not shown). The same deletion boundaries were present in all the deposited genome sequences that lack *chbP*, indicating that the gene is likely to be absent in these strains rather than *chbP* sequence reads being absent or not aligned to the scaffold. It is noteworthy that *chbP* homologues were lacking in the related but avirulent species *B. thailandensis* (6 genomes) and the glanders pathogen *B. mallei* (10 genomes). In addition, there was no evidence of any truncations in the *chbP* sequences that may ablate function as described previously from analysis of *E. coli* Cif sequences [8].

Additionally, a selection of *B. pseudomallei* clinical isolates from the endemic area [25] were studied by Western blotting of bacterial cell lysates for CHBP expression using rabbit polyclonal antiserum raised against a CHBP synthetic peptide. Of 15 *B. pseudomallei* isolates, a protein of the expected size of CHBP was detected in 7 (46.6%) samples, whereas 8 samples including the 10276 strain from Bangladesh were negative (data not shown), consistent with the deletion of *chbP* detected in the draft genome sequence and PCR with *chbP*-flanking primers of 10276 genomic DNA.

Analysis of CHBP Secretion by *B. pseudomallei*

To confirm the specificity of the anti-CHBP antibody and determine if CHBP is secreted, *B. pseudomallei* strains in which *chbP* was inactivated by insertion of the pKNOCK suicide replicon via homologous recombination (*chbP*::pKNOCK) or restored by inducible expression of *chbP* from a plasmid (*chbP*/pCHBP) were constructed and validated by sequencing. Western blot analysis of whole cell extracts of such strains with anti-CHBP detected a

protein of the expected size in wild-type K96243, but not the *chbP* insertion mutant, and this was restored by introduction of pCHBP into the mutant (Figure 1). No significant difference in growth of the bacterial strains under the conditions used was detected (data not shown). *B. pseudomallei* Bsa-secreted proteins such as BopE and BipD can be detected in the supernatant of late logarithmic phase LB-grown cultures of wild-type, but not *bsa*-deficient strains [5,24]. Interestingly, under these conditions, CHBP could not be detected in the supernatant of the cultures that yielded CHBP in the whole cell extract (Figure 1), even though we were able to confirm that the supernatants contained the known Bsa effector BopE by Western blotting using anti-BopE antibody (Figure 1). These data suggest that secretion of CHBP may be regulated in a manner distinct from BopE, though we cannot preclude the possibility that failure to detect CHBP in culture supernatant may reflect low abundance, low antibody affinity or avidity or the insensitivity of the detection system.

CHBP can be Detected in *B. pseudomallei*-infected Cells

Since CHBP could not be detected in *B. pseudomallei* culture supernatants under conditions where BopE was detected, we investigated whether host cell contact may trigger CHBP secretion. U937 macrophage-like cells were separately infected with *B. pseudomallei* K96243, the *chbP* mutant and the *trans*-complemented strain. After 6 h, cells were fixed and stained with rabbit anti-CHBP antibody followed by anti-rabbit Alexa Fluor^{488} conjugate. Confocal micrographs revealed diffuse punctate staining in the cytoplasm of *B. pseudomallei* K96243-infected cells; but with the same conditions for excitation and capture of confocal images, such staining was absent in cells infected with the *chbP* mutant (Figure 2). No differences in intracellular survival of the *B. pseudomallei* K96243 and *chbP* mutant strains were detected over the duration of the assay (Figure S2). The intensity of staining was restored by induction of CHBP expression from a plasmid in the *chbP* mutant (Figure 2). These results indicate that *B. pseudomallei* K96243 is able to secrete CHBP in infected host cells, and that unlike BopE its secretion may require host cell contact.

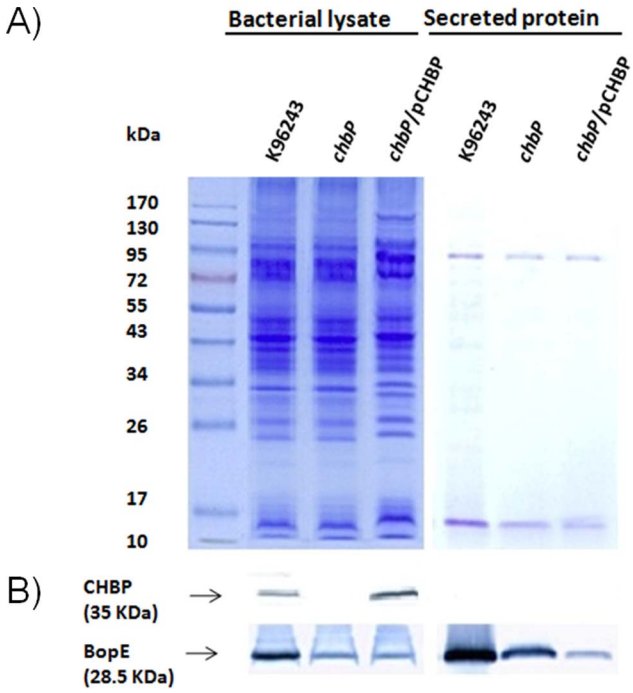

Figure 1. SDS-PAGE and Western blot analysis of CHBP in *B. pseudomallei* K96243 wild-type, *chbP* mutant and *trans*-complemented strains. A) SDS-PAGE. Bacterial lysates and secreted proteins of *B. pseudomallei* K96243, *chbP* mutant or *chbP*/pCHBP strain cultured in LB broth for 6 h were separated by 12% polyacrylamide gel electrophoresis. B) Western blot analysis. The blotted proteins from A) were separately probed with anti-CHBP and anti-BopE antibodies. Molecular mass markers are shown on the left. Lanes 1–3 are bacterial cell lysates and lanes 4–6 are secreted proteins precipitated from culture supernatants.
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To exclude the possibility that CHBP secretion might be influenced by eukaryotic cell culture medium, bacterial lysates and secreted proteins of *B. pseudomallei* strains cultured in serum-free DMEM were prepared and Western blot analysis of CHBP secretion was performed. CHBP and BopE could not be detected in the supernatants of cultures of the *B. pseudomallei* strains, even though both effector proteins were identified in the bacterial lysates (except from *B. pseudomallei chbP* mutant which does not produce CHBP protein) (Figure S3). This implies that CHBP is secreted in response to host cell infection rather than cues from the culture medium.

The timing of expression and localization of CHBP in infected cells was also followed over time by confocal microscopy. In U937 cells infected with *B. pseudomallei* K96243, staining was consistently detected in the cytoplasm at intervals from 3 to 12 h post-infection (Figure 3), with no obvious concentration in the nucleus as previously reported for *E. coli* Cif over the time intervals tested [19]. Staining could not be detected in the cytosol of U937 cells infected with the *B. pseudomallei chbP* mutant over the same 12 h time course.

The immunofluorescence microscopy data were verified by detection of CHBP protein in infected cells by Western blotting. When using the same MOI and duration of incubation as used for immunofluorescence microscopy CHBP could be detected in lysates of U937 cells infected with the wild-type and *trans*-complemented strains, but not the *chbP* mutant (Figure 4A). BopE could be detected in cells infected with each of the strains, with the exception of the *bsaQ* mutant, and the intensity of signals were increased when an MOI of 100 was used (Figure 4B). The absence of BopE in the lysates of *bsaQ*-infected cells indicates that the signals obtained did not arise from the lysis of bacteria in the samples.

Secretion of CHBP in Host Cells is Bsa-dependent

As it has been reported that CHBP can be injected by the *E. coli* T3SS in an identical manner to *E. coli* Cif [19], we speculated that CHBP can be secreted via the virulence-associated Bsa T3SS. To investigate this possibility, cells were infected with *B. pseudomallei* wild-type or an isogenic *bsaQ* mutant [24]. The *B. pseudomallei bsaQ* mutant lacks a structural component of T3SS and exhibits a defect in secretion of the known Bsa-secreted proteins BopE and BipD and delayed escape from endosomes [24]. During the 12 h infection time course, the *bsaQ* mutant exhibited comparable intracellular net replication in U937 cells to the *B. pseudomallei* wild-type K96243 strain (Figure S4). Confocal microscopy indicated that the *bsaQ* mutant could not secrete CHBP into the cell cytoplasm even at 12 h post-infection, despite the ability of the *bsaQ* mutant to express the protein as shown in Figure S4. In addition, Western blotting for CHBP in U937 cells infected with the *bsaQ* mutant failed to detect CHBP in cell lysates either at an MOI of 2 (Figure 4A) or 100 (Figure 4B). The data indicate that CHBP secretion in host cells is Bsa-dependent, though further studies are required to determine if this reflects the direct requirement for Bsa to secrete CHBP or the requirement for

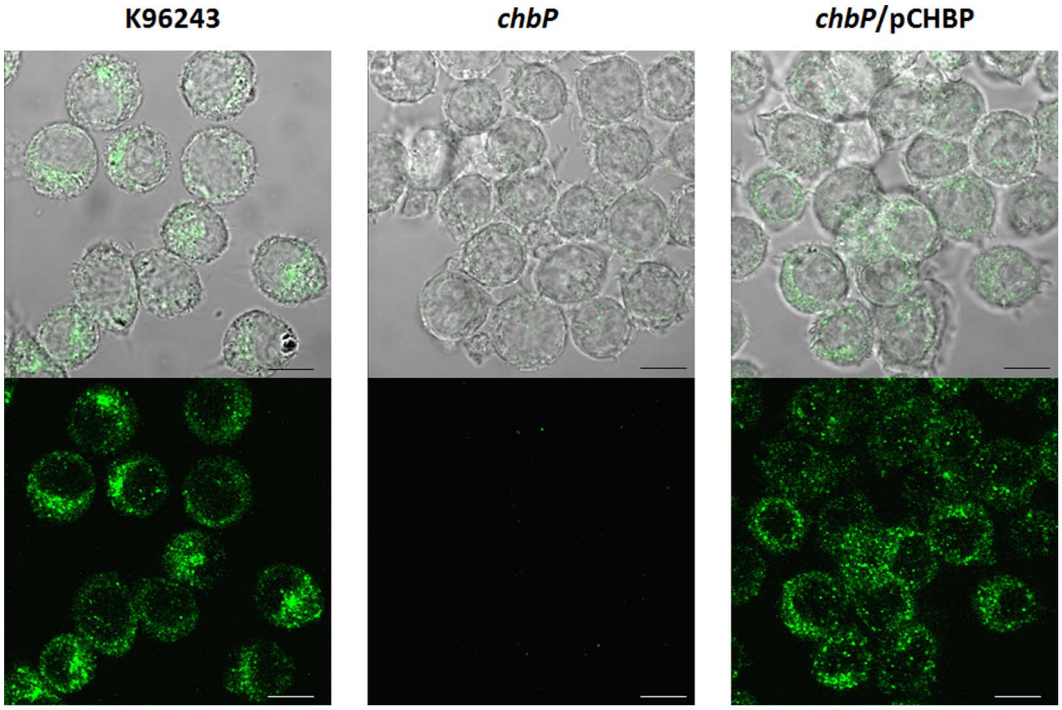

Figure 2. Confocal micrographs of CHBP expression and localization in U937 cells infected with *B. pseudomallei*. PMA-activated U937 cells were separately infected with three strains of *B. pseudomallei* (K96243, *chbP* mutant or *chbP*/pCHBP strain). After 6 h, infected cells were fixed, permeabilized andstained using purified rabbit anti-CHBP antibody detected with anti-rabbit Ig-Alexa Fluor^{488} (Molecular Probes). The bottom panel shows the localization of CHBP and the top panel merges this signal with differential interference contrast (DIC) images showing the position of infected cells. Scale bars, 20 µm.
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Bsa-dependent bacterial escape to the cytosol where CHBP may then be secreted.

B. pseudomallei CHBP Influences Virulence-associated Interactions with Host Cells

During EPEC and EHEC infections, Cif was initially reported to induce a progressive cytopathic effect involving stress fibre formation, as well as arrest of the cell cycle as detected by a change in DNA content [19]. These phenotypes took several days to fully develop, and it was possible to sterilise the cell cultures of bacteria after a period of T3SS-mediated injection of Cif by antibiotic treatment. We repeatedly attempted to sterilise cell cultures infected with *B. pseudomallei* wild-type and *chbP* mutant strains to investigate effects on the cytoskeleton and cell cycle, but were impeded by the high intrinsic resistance of *B. pseudomallei* to diverse antibiotics and loss of viability of infected host cells at the intervals where phenotypes had previously been detected (data not shown). We were nevertheless able to examine whether CHBP influenced interactions between *B. pseudomallei* and host cells that have been linked to virulence.

The capacity for cell-to-cell spread is an important characteristic of *B. pseudomallei* pathogenesis [31]. The ability of *B. pseudomallei* K96243 and the *chbP* mutant to disseminate from cell-to-cell was evaluated by infection of non-phagocytic HeLa cells. We found that plaque-forming efficiency of *B. pseudomallei chbP* mutant ($7.6\pm3.7\times10^{-4}$ pfu/bacteria) was significantly reduced compared to the wild-type strain ($28.7\pm4.4\times10^{-4}$ pfu/bacteria) (Figure 5A). Moreover, the *B. pseudomallei chbP* mutant consistently produced smaller plaques when compared to the wild-type strain (Figure 5B). Cell-to-cell spreading of the *chbP* mutant was restored by introduction of pCHBP and infection of cells in the presence of inducer.

Additionally, we compared the level of host cell damage (cytotoxicity) induced by *B. pseudomallei* wild-type and *chbP* mutant by measuring the LDH release of infected HeLa cells and U937 cells at 6 h post-infection. The *B. pseudomallei chbP* mutant caused a significantly lower level of cytotoxicity compared to the wild-type strain upon infection of HeLa cells (at the MOI of 25, 50 and 100; Figure 6A) and U937 cells (at the MOI of 25 and 50; Figure 6B).

Discussion

Cif is a bacterial cyclomodulin that arrests the cell cycle and modulates multiple cellular processes, as first described during EPEC and EHEC infection of cultured cells [8]. Proteins homologous to *E. coli* Cif have been identified in diverse bacterial pathogens including *Y. pseudotuberculosis*, *P. luminescens*, *P. asymbiotica* and *B. pseudomallei* [19]. Though the subject of intense study at the molecular level; the prevalence, secretion and role in infection of Cif homologues has received little attention. Recently, it has been reported that CHBP is recognized by melioidosis patient sera [23], however its function during interactions between *B. pseudomallei* and host cells is ill-defined.

Analysis of draft or complete *B. pseudomallei* genome sequences available at the time of writing indicated that *chbP* is present in 33 of 43 strains (76.7%), with minimal variation in predicted amino acid sequences and full conservation of the catalytic triad in CHBP-positive strains. A survey of 15 clinical *B. pseudomallei* isolates from the endemic area indicated that approximately half produced CHBP, indicating that CHBP is not an absolute requirement for *B. pseudomallei* to cause melioidosis (or *B. mallei*

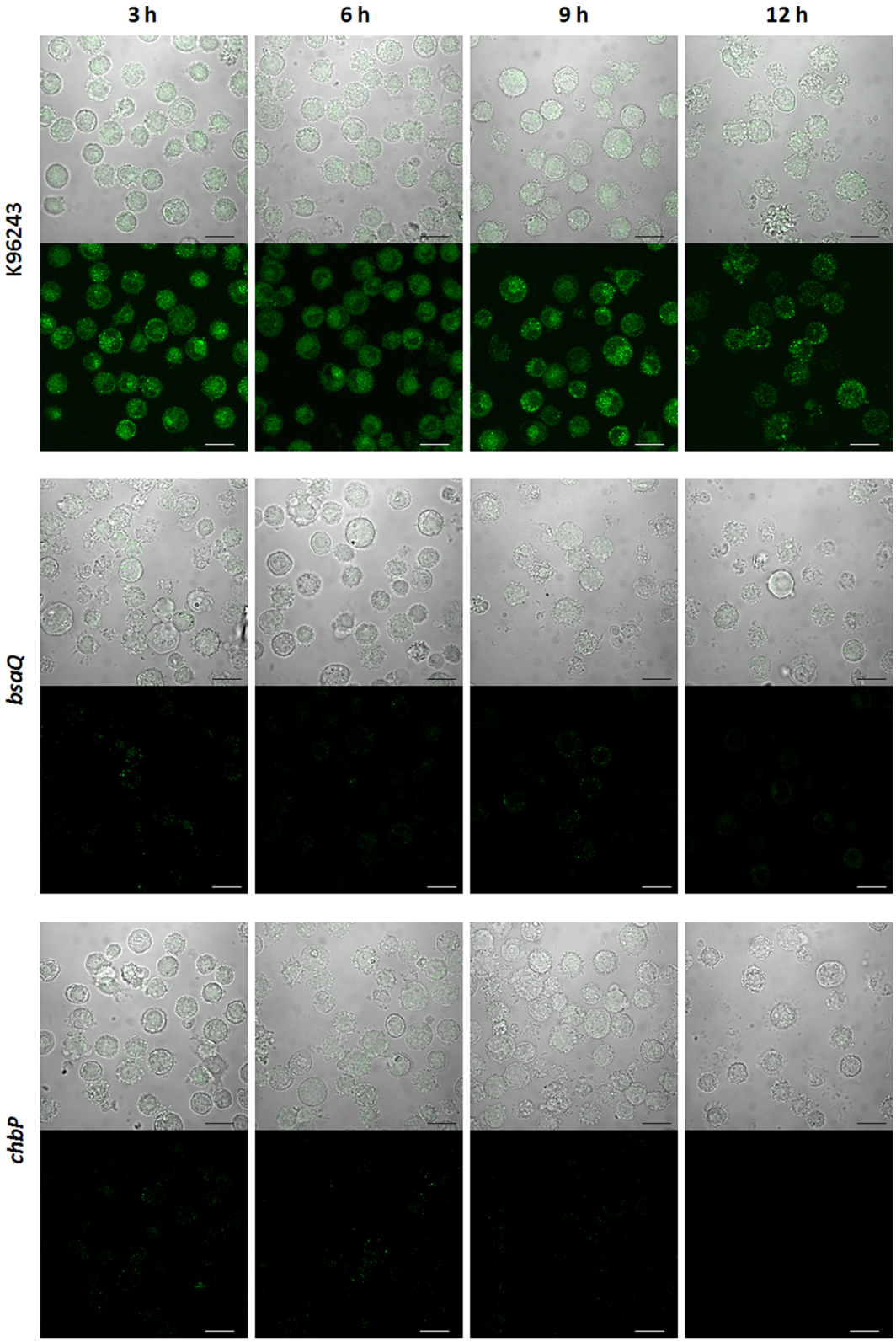

Figure 3. Confocal micrographs indicating *bsaQ*-dependent secretion of CHBP in U937 cells infected with *B. pseudomallei*. PMA-activated U937 cells were separately infected with *B. pseudomallei* (K96243, *bsaQ* or *chbP* mutant strain). At different time points of infection (3, 6, 9 and 12 h), infected cells were stained using purified rabbit anti-CHBP antibody detected with anti-rabbit Ig-Alexa Fluor^{488} (Molecular Probes). The bottom panel shows the localization of CHBP and the top panel merges this signal with DIC images showing the position of infected cells. Scale bars, 10 μm.
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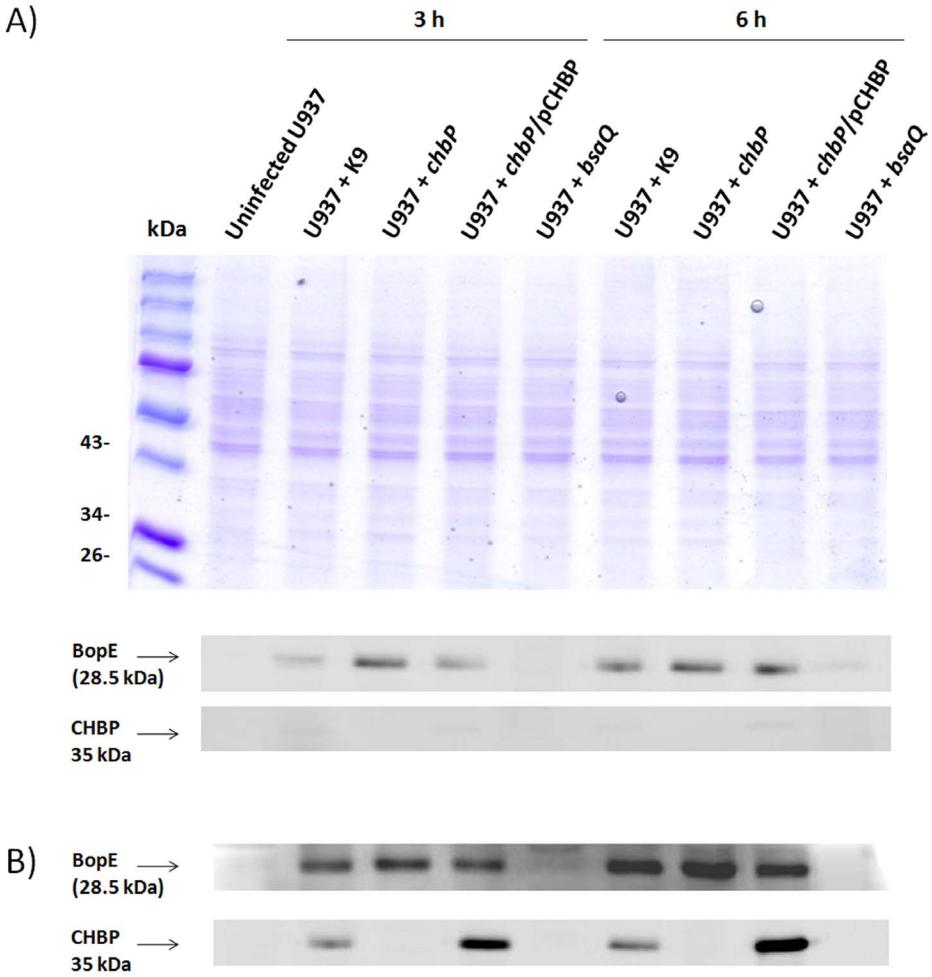

Figure 4. SDS-PAGE and Western blot analysis of CHBP in *B. pseudomallei*-infected U937 cells. A) Protein from lysates of U937 cells infected at an MOI of 2 with *B. pseudomallei* K96243, *chbP* mutant, *chbP*/pCHBP strain or *bsaQ* mutant for 3 or 6 h were separated by 12% polyacrylamide gel electrophoresis and blotted with anti-CHBP and anti-BopE antibodies. Molecular mass markers are shown on the left. Panel B shows data from an identical experiment, except using an MOI of 100.
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to cause glanders). Indeed, the *B. pseudomallei* 10276 strain isolated from a human melioidosis patient in Bangladesh is known to be virulent in murine models of melioidosis [32] despite the proven deletion in the *bpss1384-bpss1385a* region and absence of CHBP protein by Western blotting with specific antibody. Similarly, Marchès et al [8] reported that *cif* is not universally present in pathogenic EPEC and EHEC, and that some strains encode a truncated variant that is inactive. Variation in the repertoire of Type III secreted effectors is well known and it is possible that CHBP is non-essential for virulence, that functional redundancy may exist, or that presence or absence of Cif is related to subtle differences in virulence. Other cyclomodulins are known in *E. coli* (e.g. cytolethal-distending toxin and cytotoxic necrotizing factor) and it will be of interest to determine if other toxins of this kind exist in pathogenic *Burkholderia*.

B. pseudomallei is predicted to encode three Type III protein secretion systems and it has yet to be demonstrated that CHBP is secreted by the virulence-associated Bsa apparatus. An antibody raised against a synthetic peptide of CHBP reacted specifically with a 35 kDa protein in whole cell lysates of *B. pseudomallei* K96243 consistent with predictions, but no protein was detected at this position in a lysate of an isogenic *chbP* insertion mutant. Reactivity was restored when cloned *chbP* was introduced into the mutant on an inducible plasmid. In contrast to BopE, which was readily detected in the supernatant of LB-grown *B. pseudomallei* as before [5], we were unable to detect CHBP despite evidence that the protein was present in the whole-cell fraction. Interestingly, we were able to detect CHBP-specific staining in the cytosol of U937 human macrophage cells after infection with *B. pseudomallei* K96243 or the *trans*-complemented strain, but not the *chbP* mutant, suggesting that secretion of CHBP may be activated on host cell contact or induced by an intracellular signal. It is noteworthy that the *P. luminescens* Cif homologue CHPL is secreted into the culture supernatant at a time when the well characterized Type III secreted effector LopT is not [21], even though it has been proposed to be an effector of the T3SS.

Despite the absence of CHBP in the secreted fraction when BopE was detected, appearance of cytosolic CHBP in infected cells was dependent on a functional Bsa system, as it was absent in a *bsaQ* mutant previously reported to be deficient in Type III secretion [4]. Though it is tempting to speculate that the failure of CHBP to appear in lysates of U937 cells infected with the *bsaQ* mutant is evidence that CHBP is secreted via the Bsa apparatus, it should be noted that Bsa is required for the bacteria to escape

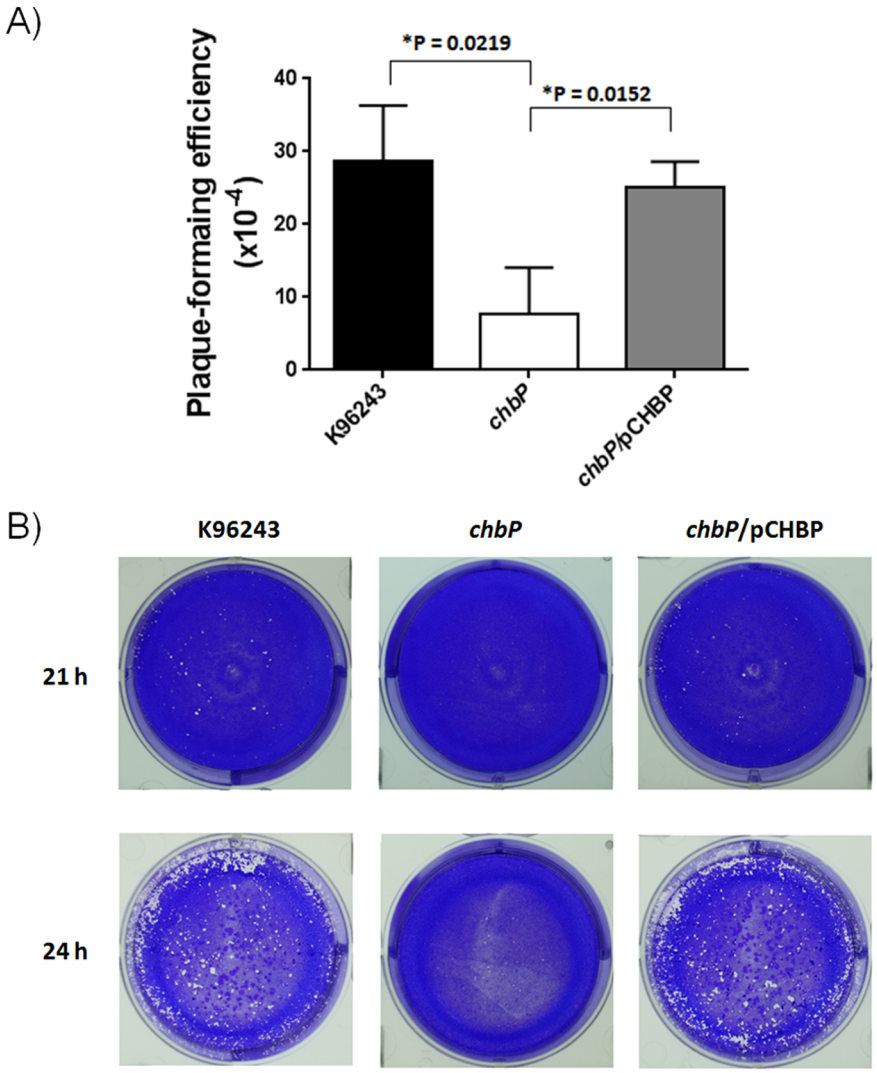

Figure 5. Effect of *chbP* mutation on *B. pseudomallei* plaque formation. A) Plaque-forming efficiency. HeLa cells were infected with *B. pseudomallei* (K96243, *chbP* mutant or *chbP*/pCHBP strain) at an MOI of 20. Plaque-forming efficiency was established following staining of the infected cells with crystal violet. Plaque-forming efficiency at 21 h was calculated by the following equation: number of plaques/CFU of bacteria added per well. Asterisks indicate significant differences (P value <0.05, *t*-test) between groups. Error bars represent standard errors of the means for experiments performed in triplicate. B) Photographs of plaques. Representative images of the infected cell monolayers after infection with *B. pseudomallei* K96243, *chbP* mutant or *chbP*/pCHBP strains for 21 and 24 h. Note the reduced number of plaques and reduced plaque size of the *chbP* mutant.
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endosomes. It remains a possibility that CHBP is secreted only once *B. pseudomallei* enters the cytosol in a Bsa-dependent way. To separate these possibilities we repeatedly attempted to detect the Bsa-dependent appearance of CHBP in cells infected with *B. pseudomallei* wild-type and mutant strains in the presence of cytochalasin D to prevent bacterial uptake. By Western blotting we were unable to detect injection of CHBP into cells where *B. pseudomallei* was prevented from uptake (data not shown), though this may reflect low levels of injection or the sensitivity of the detection method,

The cytosolic staining obtained with a CHBP-specific antibody is in contrast to observations with *E. coli* Cif, where ectopic expression leads to accumulation of the protein in the nucleus [16]. CHBP is predicted to act on nuclear targets, but we cannot preclude the possibility that it enters the nucleus at lower levels, or that it may be enriched in the nucleus at time intervals beyond those studied here.

E. coli Cif induces the accumulation of p21 and p27 that inhibit CDK1-CyclinB and CDK2-CyclinA/E, leading to cell cycle arrest at the G2/M and G1/S transitions [16]. Cui et al [17] demonstrated that this and other activities of Cif require glutamine deamidation of ubiquitin or the ubiquitin-like protein NEDD8 that regulates Cullin-RING ubiquitin ligases. We repeatedly attempted to detect CHBP-dependent inhibition of the cell cycle during *B. pseudomallei* infection as previously demonstrated by *E. coli* Cif by flow cytometric analysis of propidium iodide-stained cells, but were hindered by our inability to completely remove *B. pseudomallei* from the culture system owing to its intrinsic high level of resistance to antibiotics and induction of cell death 24–48 h post-inoculation.

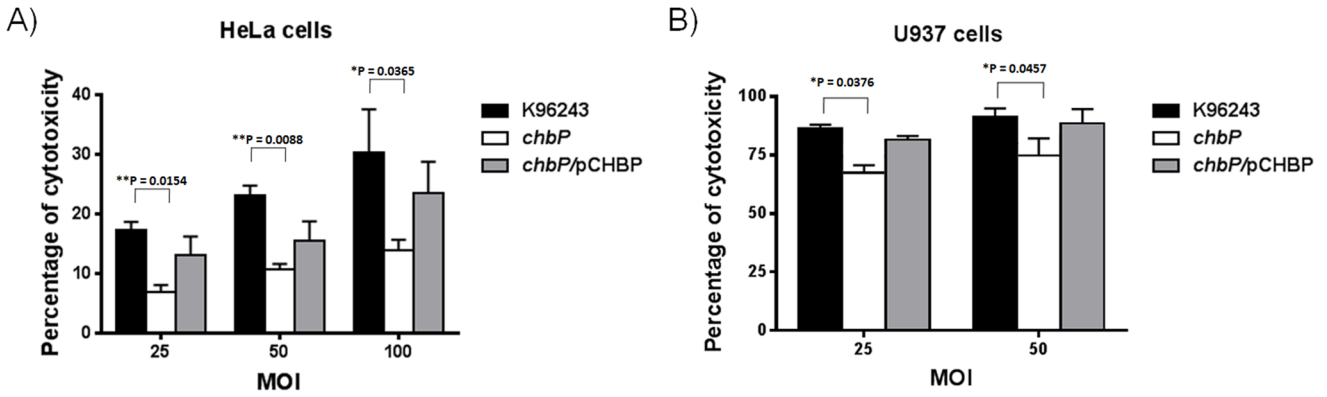

Figure 6. Effect of *chbP* mutation on *B. pseudomallei*-induced cytotoxicity. A) HeLa cells and B) U937 cells were infected with *B. pseudomallei* (K96243, *chbP* mutant or *chbP*/pCHBP strain) with a range of MOIs. After 6 h, cytotoxicity was assessed using the CytoTox96 lactate dehydrogenase (LDH)-release kit (Promega). Asterisks indicate significant differences (P value <0.05, *t*-test) between groups. Error bars represent standard errors of the means for experiments performed in triplicate.
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It has been reported that EPEC Cif induces cell damage and apoptosis of IEC-6 intestinal cells in a manner associated with LDH release and caspase-3 activation after infection [12]. Similarly, Cif homologue in *P. luminescens* triggers apoptosis in insect cells, albeit this activity is not associated with virulence in an insect model [21]. Consistent with these findings, the *B. pseudomallei chbP* mutant caused the release of lower levels of LDH in infected HeLa cells compared to the wild-type and complemented strain, despite intracellular net replication occurring at comparable levels (data not shown). *B. pseudomallei* has recently been reported to induce expression of apoptosis-related genes including caspase-3, caspase -8, caspase -9, Bax, and Bcl-2 in macrophages [33], and the role of CHBP in modulation of apoptosis during *B. pseudomallei* infection merits future study, ideally in murine models.

A significant reduction in plaque formation was detected with the *chbP* mutant that could be restored by plasmid-mediated *trans*-complementation. Plaque formation reflects the outcome of multiple processes, including uptake, endosome escape, net intracellular replication and spread to adjacent cells via actin-based motility or cell fusion. While we did not detect a defect in the net intracellular replication (Figure S2), actin tail formation or multinucleated giant cell formation (Figure S5) by the *chbP* mutant over short duration cell-based assays, it is possible that subtle phenotypes are amplified over the longer duration and multiple cycles of infection required to form a plaque. It is noteworthy that despite marked cell-based phenotypes, Cif homologue in *P. luminescens* is not required for full virulence in an insect model [21] and studies in murine melioidosis models are required before the relevance of the activities attributed to CHBP to date can be stated. Nevertheless, our study indicates a requirement for the Bsa apparatus for secretion of CHBP in host cells and indicates that distinct signals may regulate the expression or secretion of Bsa effectors.

Supporting Information

Figure S1 Sequence diversity of CHBP in sequenced *B. pseudomallei* genomes. Prototypic *B. pseudomallei* CHBP sequences were aligned using ClustalW. Note the minor differences in amino acid composition between the proteins (presented in red) and conservation of the predicted catalytic Cys-His-Gln triad (highlighted in yellow) proposed by Crow et al [20].
(TIF)

Figure S2 SDS-PAGE and Western blot analysis of CHBP in *B. pseudomallei* K96243, the *chbP* mutant and the complemented strains grown in DMEM or LB media. A) *B. pseudomallei* lysates and B) secreted proteins from K96243 wild-type, *chbP* mutant or *chbP*/pCHBP strains cultured in serum-free DMEM medium for 6 h were separated by 12% SDS-PAGE. The blotted proteins were separately probed with anti-CHBP and anti-BopE antibodies. Molecular mass markers are shown on the left of the gel. Bacterial lysate and secreted protein prepared from *B. pseudomallei* K96243 cultured in LB broth were used as the positive controls.
(TIF)

Figure S3 *B. pseudomallei* intracellular survival in U937 cells. PMA-activated U937 cells were infected with *B. pseudomallei* K96243 wild-type, *bsaQ* or *chbP* mutant strains at an MOI of 2. After 3, 6, 9 and 12 h of infection, infected cells were lysed and the numbers of viable bacteria were enumerated after plating on TSA and incubation at 37°C for 36–48 h.
(TIF)

Figure S4 SDS-PAGE and Western blot analysis of CHBP expression and secretion in *B. pseudomallei* K96243 and an isogenic *bsaQ* mutant. A) SDS-PAGE. Bacterial lysates and secreted proteins of *B. pseudomallei* K96243 or *bsaQ* mutant strain cultured in LB broth for 6 h were separated by 12% SDS-PAGE. B) Western blot analysis. The blotted proteins from A) were probed with anti-CHBP antibody. Molecular mass markers are shown on the left.
(TIF)

Figure S5 Effect of *chbP* mutation on *B. pseudomallei* intracellular movement and intercellular spreading. A) Actin tail formation. PMA-activated U937 cells were infected with two strains of *B. pseudomallei* (K96243 or *chbP* mutant strain) at an MOI of 2. After 6 h of infection, infected cells were fixed using 4% paraformaldehyde and actin filaments stained with phalloidin^{568} (red) and bacteria stained with mouse monoclonal anti-*B. pseudomallei* lipopolysaccharide antibody detected with anti-mouse Ig-Alexa Fluor^{488} (green). Bar, 10 μm. B) Multinucleated giant cell formation. MNGC formation in J774A.1 murine macrophage cells infected at an MOI of 2 with *B. pseudomallei* (K96243 or *chbP* mutant strain) was studied 6 h post-infection by Giemsa staining of the cell monolayers. The stained cells were examined under a light microscope (OLYMPUS) at a magnification of 20X.

(TIF)

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Author Contributions

Conceived and designed the experiments: PP SK MPS JMS. Performed the experiments: PP NJ CVB VM. Analyzed the data: PP JMS CVB. Contributed reagents/materials/analysis tools: SK PK KP. Wrote the paper: PP SK MPS JMS.

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