รายงานวิจัยฉบับสมบูรณ์

โครงการ : การเปลี่ยนแปลงแอนติเจนในส่วนโอ-โพลีแซ็กคาไรด์ของลิโพโพลีแซ็กคาไรด์ของเชื้อเบอร์โคลเดอเรีย สูโดมาลลิไอ

Antigenic variation of O-polysaccharide moiety of *Burkholderia pseudomallei* lipopolysaccharide

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สัญญาเลขที่ TRG5580004

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จากการเฝ้าสังเกตในห้องปฏิบัติการพบว่าเชื้อ *B. pseudomallei* บาง strain เมื่อเลี้ยงบนอาหารเลี้ยงเชื้อ TSA จะแสดงลักษณะโคโลนี 2 แบบ ได้แก่ โคโลนีแบบมันเยิ้ม และโคโลนีแบบแห้ง ซึ่งลักษณะโคโลนีของเชื้อจะสัมพันธ์กับความสามารถในการจับกับโมโนโคนอลแอนติบอดี 9D5 ซึ่งเป็นแอนติบอดีที่จับกับส่วนโอ-โพลีแซ็กคาไรด์ โคโลนีแบบแห้งจะจับกับแอนติบอดี ในขณะที่โคโลนีแบบมันเยิ้มจะไม่จับ ซึ่งโคโลนีทั้ง 2 แบบในเชื้อ strain เดียวกันจะมี PFGE pattern เหมือนกัน ซึ่งยืนยันว่าโคโลนีแบบทั้ง 2 แบบเป็นเชื้อตัวเดียวกัน โคโลนีแบบทั้ง 2 แบบนี้สามารถพบได้ในเชื้อที่ขึ้นโคโลนีครั้งแรกจากตัวอย่างคนไข้ จากการศึกษาในคนไข้ 40 คน พบ 8 คนที่เชื้อแสดงลักษณะโคโลนี 2 แบบ โดยเชื้อทั้ง 8 ตัวนี้เป็นเชื้อที่แยกได้จากทางเดินหายใจทั้งหมด ส่วนเชื้อจากคนไข้ที่เหลือ 32 คนมีโคโลนีแบบแห้งแค่แบบเดียว เนื่องจากแอนติบอดีที่ใช้จับกับเชื้อเป็นแอนติบอดีที่จำเพาะกับส่วนโอ-โพลีแซ็กคาไรด์ เราจึงสร้าง mutants โดยการตัดยีนที่เกี่ยวข้องกับการสร้างและการเปลี่ยนแปลงโอ-โพลีแซ็กคาไรด์ ซึ่งยีนที่ตัดออกได้แก่ *wbiA, wbiD* และ *oacA* เมื่อตัดยีน *wbiA* ออกพบว่าเชื้อไม่จับกับแอนติบอดี 9D5 ในขณะที่ตัดยีนที่เหลืออีก 2 ยีนไม่มีผลต่อต่อการจับกับแอนติบอดี จากนั้นทดสอบว่าโคโลนีทั้ง 2 แบบเปลี่ยนกลับไปกลับมาภายใต้สภาวะทดลองหรือไม่ เราทดสอบในเชื้อ 5 strains ซึ่งมีโคโลนี 2 แบบ ผลการศึกษาพบว่ามีการเปลี่ยนจากโคโลนีมันเยิ้มไปเป็นโคโลนีแบบแห้งเมื่อมีการเปลี่ยนแปลง pH อุณหภมิ ปริมาณออกซิเจนและสารอาหาร ตรงกันข้ามกับโคโลนีแบบแห้งที่เปลี่ยนไปเป็นโคโลนีแบบมันเยิ้มก็ต่อเมื่อเลี้ยงเชื้อใน TSB เป็นเวลา 7 วัน เราจึงสรุปว่าการเปลี่ยนแปลงลักษณะโคโลนีระหว่างโคโลนีแบบแห้งและมันเยิ้มของเชื้อ *B. pseudomallei* ส่งผลต่อการเปลี่ยนแปลงแอนติเจนในส่วนโอ-โพลีแซ็กคาไรด์ซึ่งสัมพันธ์กับการหลบหลีกจากการจับกับแอนติบอดี

คำหลัก: เบอร์โคลเดอเรีย สูโดมาลลิไอ, ลิโพโพลีแซ็กคาไรด์, antigenic variation

Abstract

Project Code: TRG5580004

Project Title: Antigenic variation of O-polysaccharide moiety of *Burkholderia pseudomallei* lipopolysaccharide

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We observed some strains of *B. pseudomallei* in laboratory contain 2 colony morphotypes: mucoid and non-mucoid. Colony morphotype was correlated to reaction with 9D5 monoclonal antibody (Mab) specific to O-polysaccharide (O-PS). Non-mucoid colonies reacted with 9D5 Mab, while mucoid colonies did not. Both types of the same strain showed identical PFGE pattern which confirmed that they were originated from the same clone. Visual inspection of *B. pseudomallei* colonies growing on primary culture plates inoculated with samples, which were from 40 patients with melioidosis revealed a mixture of mucoid and non-mucoid colonies in 8 samples obtained from the respiratory samples. We found that these different colony types also reflect a difference in LPS reaction with Mab. We constructed 3 mutants defective in genes involving in O-PS synthesis and modification including *wbiA, wbiD* and *oacA* by deleted fragment mutagenesis. *WbiA* mutant showed negative reaction with 9D5 MAb indicating that 2-*O*-acetyl modification on O-PS is required for Mab reaction. We tested whether colonies could switch between the two phenotypes under a range of laboratory conditions for 5 isolates. Switching from the mucoid to non-mucoid phenotype occurred in response to changes in pH, temperature, oxygen tension, and availability of nutrients. By contrast, non-mucoid colonies were generated only in a condition of incubation in TSB for 7 days. We concluded that switching between mucoid and non-mucoid *B. pseudomallei* colonies reflects variation in modification of O-PS, and that antigenic variation is associated with the avoidance of immune recognization by antibody.

Keywords: *Burkholderia pseudomallei*, lipopolysaccharide, antigenic variation

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เนื้อหางานวิจัย

Introduction

Burkholderia pseudomallei is a Gram-negative, saprophytic and free living bacterium that is commonly found in soil and surface water such as in rice paddies. It is the causative agent of meliodosis, a common infectious disease endemic in northern Australia and Southeast Asia, especially in the northeastern part of Thailand (Chaowagul et al., 1989). The disease is acquired by inhalation of contaminated dust particles or inoculation of the organism from soil and wet environment through wound or abrasion. The clinical presentation is diverse, varying from subclinical to acute or chronic forms of localized and disseminated infection. Melioidosis is a multiple-organ involvement and lungs are the most commonly affected organs (Ip et al., 1995). The organism can remain latent, causing symptoms up to 62 years following exposure (Ngauy et al., 2005). The disease is usually associated with high mortality rate, up to approximately 50% or more (Wiersinga et al., 2006), and is a major cause of death from acute severe pneumonia and septicemia. *B. pseudomallei* is intrinsically resistant to many antibiotics including penicillin, first and second generation of cephalosporins, macrolides and aminoglycosides. Infection by this bacterium often responds poorly to antibiotic treatment. Moreover, *B. pseudomallei* can cause latent infection, commonly in association with an alteration in immune status. Recurrent infection occurs despite prolonged antibiotic treatment but 75% of recurrent infection is relapse (failure of initial eradicative treatment) and 25% is re-infection with a new strain (Limmathurotsakul et al., 2008).

Structure analysis of O-polysaccharide (O-PS) component of smooth LPS type A revealed that it expresses unbranched heteropolymer with repeating D-glucose and L-talose residues with the structure –3)-β-D-glucopyranose-(1->3)-6- deoxy-α-L-talopyranose-(1- in which 33% of this residue contains 2-*O*-methyl and 4-*O*-acetyl substitution while the remainder bears 2-*O*-acetyl modification (Knirel et al., 1992; Perry et al., 1995). A closely-related species of *B. pseudomallei, B. mallei* expresses O-PS antigen that are structurally similar to those expressed by *B. pseudomallei* and *B.thailandensis,* excepted that their L-6dTalp residues lack acetyl modifications at the *O*-4 position (Burtnick et al., 2002). By expression the different structure, Brett et al (2011) demonstrated that this phenomenon has explained the ability to bind with a monoclonal antibody Pp-PS-W, 9C1-2 and 3D11 (Brett et al., 2011). Two acetyltransferase genes in *B. pseudomallei* and *B. thailandensis* have been identified, *wbiA* located within and *oacA* (a BPSL1936 ortholog of *B. pseudomallei,*) located outside the conserved O-PS biosynthetic gene cluster. Further studies showed that *wbiA* gene is required for the 2-*O* acetylation of *B. pseudomallei* while *oacA* is required for the 4-*O* acetylation and 2-*O* methylation of *B. thailandensis* and suggested the important of these *O*-acetyl groups to the immunogenicity of *B. pseudomallei (*Brett et al., 2011). We have established a 9D5 monoclonal antibody (MAb) that reacted specifically with O-PS of *B. pseudomallei*. This antibody demonstrates the same characteristic with Pp-PS-W described by Brett and colleagues (Brett et al., 2003) in that it reacted specifically for *B. pseudomallei* and *B. thailandensis* LPSs and does not cross-react to six *B. mallei* LPS (Anuntagool and Sirisinha, 2002). The reaction of our 9D5 MAb is most likely related to the acetylation of O-PS and may be a useful tool for detection the antigenic variation in expression of acetyl group on *B. pseudomallei* LPS.

We previously reported that B*. pseudomallei* from clinical specimens on Ashdown's selective medium (Ashdown, 1979) expressed seven unique colony morphotypes. Type I is the most common (75%); the remaining 25% belonged to Types II-VII. Mixed *B. pseudomallei* morphotypes with clonally related have been observed in 8% of primary culture from clinical samples. (Chantratita et al., 2007). Colony morphology switching exhibits under adverse environmental stimuli such as starvation, iron limitation, growth at 42^{o}C, and also occur in vivo (Chantratita et al., 2007). There is a relationship between morphotype and phenotype. For example, morphotype switching is associated with bacterial length, production of extracellular enzymes, biofilm formation, motility-associated factors, bacterial-cell surface-associated factors, interaction with and replication in macrophage cell lines (Chantratita et al., 2007) In many bacterial pathogens, such phenotypic variation has long been recognized, mostly by the observation readily visible as colony variation, is often associated with virulence of the bacterial strain. Alternating between two phenotypes in a heritable and reversible manner can be classified as phase variation or antigenic variation.

Although the colony variation of *B. pseudomallei* is clearly demonstrated in primary culture on Ashdown agar, the molecular mechanism and the relationship with antigenic variation have not been elucidated. In the laboratory, we observed that the colony morphology of *B. pseudomallei* on Ashdown selective agar and on enrichment agar such as blood agar and trypticase soy agar (TSA) are different. Colonies on enrichment agar of the same isolate also varies between mucoid and non-mucoid colonies. Our pilot study focused on the variation of the LPS component of this bacteria, clearly demonstrated there is a differential expression of an immunoreactive component on LPS that can be detected using 9D5 anti O-PS monoclonal antibody between the Ashdown selective agar and other enrichment agar. Screening over 200 isolates from Ashdown agar indicated that all different colony morphologies expressed this epitope on the cell surface. In contrast, colonies on enrichment media showed a mixed result for the reaction with 9D5 MAb, the mucoid colonies were not reacted with monoclonal antibody 9D5, while non-mucoid colonies did. Given that all colony morphologies of *B. pseudomallei* contain a typical complete structure of LPS consisting of O-polysaccharide, core-oligosaccharide and lipid A observed by SDS-PAGE and silver staining, we hypothesize that *B. pseudomallei* can regulate the expression of an antigen on O-PS moiety which can be defined by the specific reaction with 9D5 monoclonal and this phenomenon is associated with colony switching. The most likely role of the switching on-off expression of LPS antigen is to provide a proportion of *B. pseudomallei* with a strategy for adapting to more than one particular environment including the changes in culture conditions and environment in host. At present, the phenomenon has not been described in *B. pseudomallei*. The significance of this observation with regard to virulence and evasion of host immune responses need to be defined.

This study will use a combination of molecular genetics and immunological approaches to identify the gene(s) responsible for the expression of an epitope on O-PS that reacts specifically to 9D5 monoclonal antibody. By immunoblot, antigenic variation of *B. pseudomallei* will be identified from primary culture of clinical specimens. A range of laboratory conditions that induce this variation as well as the possible association with the fitness advantage in environments and host invasion will be examined. This study will provide novel evidence for *B. pseudomallei* adaptability in environment and may explain persistent infection for human melioidosis.

Materials and Methods

Bacterial isolates and culture conditions

A prospective *B. pseudomallei* isolate was collected during this study, which were picked from sheep blood agar that had been inoculated with diagnostic specimens taken from 40 patients presenting to Sappasithiprasong Hospital between July 2011 and November 2012 with suspected melioidosis. The clinical specimens were sputum (n=13), tracheal secretions (n=6), pus (n=11), wound swab (n=3), synovial fluid (n=1), and blood culture (n=6). Colonies were picked after incubation for 2 days at 37°C in air. These samples were randomly selected from those that grew *B. pseudomallei* on a primary blood agar plate during routine diagnostic processing. The other laboratory strains of *B. pseudomallei* and *Escherichia coli* were used during the study. Unless otherwise stated, these were cultured on TSA or Luria-Bertani (LB) agar (BD), respectively. All isolates were stored in trypticase soy broth (TSB) with 15% glycerol at -80^{o}C.

Latex agglutination assay

A latex agglutination test was made using the IgG3 isotype monoclonal antibody (Mab), 9D5 (Anuntagool and Sirisinha, 2002). This is known to recognize *B. pseudomallei* O-PS and reacts with *B. pseudomallei* and the closely related non-virulent species *B. thailandensis*, but not with *B. mallei* (Anuntagool and Sirisinha, 2002). The Mab was purified using a Hi-trap protein A HP column (GE Healthcare), and sensitized on latex particles (Mab-latex) according to the manufacturer's instructions (Invitrogen). *B. pseudomallei* reactivity was assessed by mixing bacterial cells with 5 µl of Mab-latex suspension on a glass slide, and observing for agglutination within 2 minutes.

Pulsed-Field Gel Electrophoresis (PFGE)

PFGE pattern of *B. pseudomallei* was performed as previously described (Maharjan et al., 2005). An overnight cultured of individual *B. pseudomallei* colonies were suspended in TE buffer and adjusted OD_{600} to 1.2. The bacterial suspension was mixed with equal volume of molten 2% low melting point (LMP) agarose. The mixture was added into PFGE plug mold and then incubated at 4 °C for 30 min. The plug was transferred into TE buffer containing 500 µg/ml of protease K and incubated at 56 °C for overnight. The plug was washed 3 times with TE buffer for 1 h and digested overnight with *SpeI* at 37 °C. The plug was loaded into 1% agarose gel in 0.5% TBE. PFGE was performed on CHEF Mapper at 14 °C for 29 h. Gel was stained with ethidium bromide, washed in water, and photographed under UV light.

Silver staining

LPS of *B. pseudomallei* was extracted as described previously (Anuntagool et al., 1998). One loop full of the bacteria was washed once with PBS and the bacterial suspension was adjusted to $1x10^{9}$ cfu/ml based on OD_{600} to 0.8. One milliliter of the bacterial suspension was centrifuged at 10,000 rpm for 2 min. The pellet was collected and lysed with lysis buffer before heating at 100 °C for 10 min. The lysate was treated with 0.5 mg/ml protease K and then incubated at 60 °C for overnight. The purified LPS was separated in 12% SDS-PAGE. The SDS-PAGE gel was oxidizied with 0.7% periodic acid containing 40% ethanol and 5% acetic acid for 20 min. The gel was washed with deionized distilled water 3 times for 5 min and then stained

with silver for 1 h. Following washing as above, the gel was incubated in developing solution until LPS ladder appears.

Construction of mutants and complemented strains

Three *B. pseudomallei* LPS mutants defective in *wbiA* (BPSL2680), *wbiD* (BPSL2677), or *oacA* (BPSL1936) were constructed using a fragment mutagenesis protocol as described previously (Lopez et. al. 2009; Chantratita et. al., 2012). LPS mutants were constructed in *B. pseudomallei* K96243. Gene sequences for *wbiA, wbiD* and *oacA* were obtained from Genbank (accession numbers 3094050, 3093124 and 3091646 respectively). PCR primers were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) (Table 1). Mutagenesis and complementation was performed using pEXKm5-based allele replacement. Phenotypic verification of LPS expression was performed achieved by SDS-PAGE of proteinase K extracts and Western blot analysis which probing with 9D5 Mab (Anuntagool et al., 2002).

Effect of different culture conditions on *B. pseudomallei* colony appearance

The effect of a range of laboratory conditions on colony appearance was tested using 5 randomly isogenic pairs of non-mucoid or mucoid *B. pseudomallei* colonies from the same sample or patient. Colonies picked from TSA were suspended in PBS and adjusted to $1x10^8$ CFU/ml using spectrophotometry at OD_{600}. Two hundred microliters of suspension was inoculated into 2 ml of distilled water (DW) or TSB and incubated with one of these following conditions: (i) DW at 37 °C for 24 h, (ii) TSB pH 7.4 at 37 °C for 24 h, (iii) TSB pH 4.0 at 37 °C for 24 h, (iv) TSB pH 8.5 at 37 °C for 24 h, (v) TSB pH 7.4 at 42 °C for 24 h, (vi) TSB pH 7.4 + 350 mM NaCl at 37 °C for 24 h,(vii) TSB pH 7.4 + 50 mM $NaNO_2$ at 37 °C for 24 h, (viii) TSB pH 7.4 + 2 mM H_2O_2 at 37 °C for 24 h, (ix) TSB pH 7.4 at 37 °C for 7 days (all incubated in air), and (x) TSB pH 7.4 at 37 °C for 24 h in anaerobic jar (Oxoid). Thereafter, bacteria were diluted in PBS and spread plated onto TSA, and the proportion of mucoid and non-mucoid colonies in the sample was enumerated. LPS was characterized and the alteration of LPS antigen expression was tested in 5 colonies of emergent morphotype using 9D5 Mab-latex. The result was compared to those of 5 colonies of the original colony type on the same plate. The experiments were performed in duplicate for 3 independent experiments.

Stability of non-mucoid and mucoid colonies

Stability of non-mucoid and mucoid morphotypes was determined in 5 pairs which contain both morphotypes from the same sample. Bacteria from stock were cultured on TSA and incubated at 37 °C for 2 days. Ten colonies of each morphotype were suspended in PBS and the bacterial number was adjusted to $2x10^8$ CFU/ml. The bacterial suspension was diluted by 10-fold serial dilution. One hundred microliter of dilution at 10^{-5} was spread on TSA for total of 60 plates. TSA plates were incubated at 37 °C for 2 days. Total colonies were counted and observed for non-mucoid and mucoid colonies. One hundred colonies in each morphotype were reacted with 9D5 Mab-latex.

Human lung epithelial cell line (A549) infection

Human lung epithelial cell line (A549) was originally obtained from American Type Culture Collection (ATCC). A549 cells were cultured in Ham's F12 medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone) at 37 °C in 5% CO_2 incubator. A549 cells ($4.5x10^5$ cells) were seeded into 6-well plate and incubating the plate overnight at 37 °C in humidified 5% CO_2 incubator. The cell monolayer was infected with *B. pseudomallei* mucoid and non-mucoid colonies at a multiplicity of infection (MOI) of 50:1. For adherence assay, the infected cells were washed 3 times with PBS at 1 h of infection followed by lysing of the infected cells with 0.1% Triton X-100. For invasion assay, the infected cells were added with 250 μg/ml of kanamycin at 2 h of incubation and kept for further 1 h. The infected cells were lysed with 0.1% Triton X-100. The lysate was diluted and spread on TSA. Adherence and invasive bacteria were counted and calculated into CFU/ml.

Human monocyte cell line (THP-1) infection

THP-1 cells were cultured in RPMI medium (Gibco) supplemented with 10% heat inactivated FBS. THP-1 cells ($3x10^5$ cells) were seeded into each well of a 24-well plate and incubated overnight at 37 °C in 5% CO_2 incubator. The monocyres were infected with *B. pseudomallei* mucoid and non-mucoid colonies at MOI of 1:1 and then incubated at 37 °C in 5% CO_2 for 2 h. Extracellular bacteria were killed by exposure to medium containing 250 μg/ml of kanamycin for a further 2 h before replacing with the medium containing 20 μg/ml of kanamycin. At the indicated time, intracellular bacteria were quantified by lysing infected cells with 0.1% Triton X-100. The viable bacteria were counted on TSA after 1 days of incubation at 37 °C.

LPS characterization using NMR Spectroscopy

B. pseudomallei 4095a (non-mucoid) and 4095c (mucoid) were cultured on TSA, from which colonies were scraped from the plate and purified LPS and O-PS obtained as previously described (Brett et al., 2011). LPS was extracted using a modified hot aqueous phenol procedure (Perry et al., 1995). O-PS samples were deuterium exchanged by dissolving in D_2O and lyophilizing, and then dissolved in 0.27 ml D_2O containing 1 μl acetone. 1-D Proton and 2-D gradient-enhanced COSY (gCOSY), TOCSY, NOESY, gHSQC, and gHMBC spectra were obtained on a Varian Inova-600 MHz spectrometer at 50°C using standard Varian pulse sequences. The spectral width was 3.17 kHz in the ^1H dimension and 18.1 kHz in the ^{13}C dimension. The number of scans and increments was 4 and 400 for gCOSY, 8 and 200 for TOCSY and NOESY, 64 and 128 for gHSQC, and 128 and 200 for gHMBC. Acquisition times were 2 s for 1-D ^1H, 137 ms for gCOSY, TOCSY, and NOESY, 150 ms for gHSQC, and 128 ms for gHMBC. Mixing times for TOCSY and NOESY experiments were 120 and 300 ms, respectively. Proton chemical shifts were measured relative to internal acetone (δ_H=2.218 ppm, δ_C=33.0 ppm) (Wishart et al., 1995). Monosaccharide residues in O-PS samples were identified using the 2-D HSQC spectra by comparison with the assigned spectra from *B. pseudomallei* 1026b O-PS (Heiss et al., 2013). Integration of the spectra was performed using the MNova software (Mestrelab Research) after baseplane correction with a third order Bernstein polynomial.

Table 1 Primer pairs used in mutagenesis

Primers	Sequence (5'-3')	Position[§]	Product size (bp)
wbiA (BPSL2680)			
***wbiA*-F1**	CGATTCGATGCCGCCGACGT	34-53	297
***wbiA*-R1**	CAACGCATCCGCTCGCATGC	311-330	
***wbiA*-F2**	GCATGCGAGCGGATGCGTTGTGCTGCTTGGCTATCCGGCG	911-930	305
***wbiA*-R2**	TCCATCCTTGTCCGGGCCCC	1176-1190	
***wbiA*-F1**	CGATTCGATGCCGCCGACGT	34-53	1162
***wbiA*-R2**	TCCATCCTTGTCCGGGCCCC	1176-1190	
***wbiA*-Fex**	GGAATCTGCGTCTCCGGCTT	256-275	300
***wbiA*-Rex**	ATAGGGTGTCGTGTCTCGCAG	535-555	
oacA (BPSL1936)			
***oacA*-F1**	AAAGGCCGACGCATTCCGGG	(-162)-(-144)	311
***oacA*-R1**	AATGCCCGCTGGAGCGTGTC	130-149	
***oacA*-F2**	GACACGCTCCAGCGGGCATTCCCCAAGCACCATTGGCGGT	995-1014	344
***oacA*-R2**	ATGTGGGCATGGGGAAGCGC	(+114)-(+133)	
***oacA*-F1**	AAAGGCCGACGCATTCCGGG	(-162)-(-144)	1480
***oacA*-R2**	ATGTGGGCATGGGGAAGCGC	(+114)-(+133)	
wbiD (BPSL2677)			
***wbiD*-F1**	CGGTGTACAGCAATGTCGTT	41-60	206
***wbiD*-R1**	CAGACGGTGCAGGTCGATTC	227-246	
***wbiD*-F2**	GAATCGACCTGCACCGTCTGTCTACCGAAAGTGGCGTTCC	1409-1428	259
***wbiD*-R2**	CGGATGCCTGACAAAGAACC	1628-1647	
***wbiD*-F1**	CGGTGTACAGCAATGTCGTT	41-60	1607
***wbiD*-R2**	CGGATGCCTGACAAAGAACC	1628-1647	
***wbiD*-Fex**	TCGATGCAGACGAGGATTCG	170-189	318
***wbiD*-Rex**	TGTGATCGAGAAGGCCAACC	468-487	
16S			
Univ_*16S*_F	TGGCTCAGAACGAACGCTGGCGGC	21-44	336
Univ_*16S*_R	CCCACTGCTGCCTCCCGTAGGAGGAGT	327-356	
pEXKm5			
OriT_F	TCCGCTGCATAACCCTGCTTC	598-578	236
OriT_R	CAGCCTCGCAGAGCAGGATTC	368-383	

[§] Positions corresponding to the nucleotide sequence of *B. pseudomallei* K96243 chromosome 1 (NCBI Reference Sequences NC 006350 for *wbiA, wbiD* and *oacA*)

Position (-) = gene at 5'UTR of *wbiA* and position (+) = gene at 3'UTR of *wbiA*

Results

I. Identification of the expression of 9D5 reactive epitope on clinical *B. pseudomallei* isolates

Primary cultures of *B. pseudomallei* from melioidosis patients were cultured on sheep blood agar. Total 40 strains were reacted with monoclonal antibody against *B. pseudomallei* LPS (9D5 Mab). Twenty colonies in each strain were tested with 9D5 Mab. We have developed latex agglutination test for rapid screening 9D5 Mab reactive epitope on *B. pseudomallei* colonies. The latex test was specific to *B. pseudomallei* LPS. It reacted with only 9D5 reactive strains but not reacted with 9D5 non-reactive strains and other bacteria (Figure 1).

Specimen types from patients were blood (n=6), sputum (n=13), tracheal suction (n=6), pus (n=11), wound swab (n=3) and synovial fluid (n=1). Of these strains, 8 strain cultures (20%) demonstrated two colony morphotypes on the same plate (Figure 2), among which 6 strains were obtained from sputum and 2 strains were obtained from tracheal suction. The PFGE banding patterns were identical for mixed colony types for these 8 strains (figure 3). Forty (83.3%) of these isolates were typed non-mucoid, and 8 (16.7%) belonged to mucoid colonies (Table 2).

A smooth LPS serotype A was demonstrated for all isolates. Positive reaction with 9D5 MAb was observed in all 40 non-mucoid and none of 8 mucoid colonies (Table 2). The results confirmed that clinical *B. pseudomallei* isolates with smooth LPS type A pattern divided into two LPS antigenic types which were distinguishable by the reaction with Mab and it was associated with colony appearance. The colonies with mixed antigenic types represent two phenotypes of the same infecting clones and mucoid colonies were often isolated from respiratory secretions.

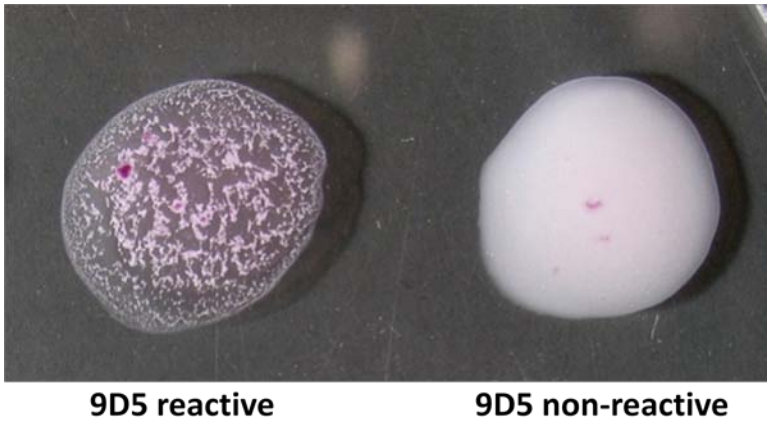

Figure 1 Latex agglutination test. Bacterial colonies were mixed with 9D5 latex reagent and agglutination was observed within 2 min. 9D5 reactive strains showed agglutinated particle while 9D5 non-reactive showed milky suspension.

Table 2 Colony type and reaction with 9D5 Mab of primary *B. pseudomallei* isolates on sheep blood agar from different specimen types

Specimen types	No. of specimens	Colony types (total colonies)		Reaction with 9D5	
		Mixed	Mucoid/ Nonmucoid	Positive	Negative
Blood	6	0	Nonmucoid (6)	6	0
Sputum	13	6	Nonmucoid (13) Mucoid (6)	13 0	0 6
Tracheal suction	6	2	Nonmucoid (6) Mucoid (2)	6 0	0 2
Pus	11	0	Nonmucoid (11)	11	0
Wound swab	3	0	Nonmucoid (3)	3	0
Synovial fluid	1	0	Nonmucoid (1)	1	0
Total	40	8	Nonmucoid (40) Mucoid (8)	40 0	0 8

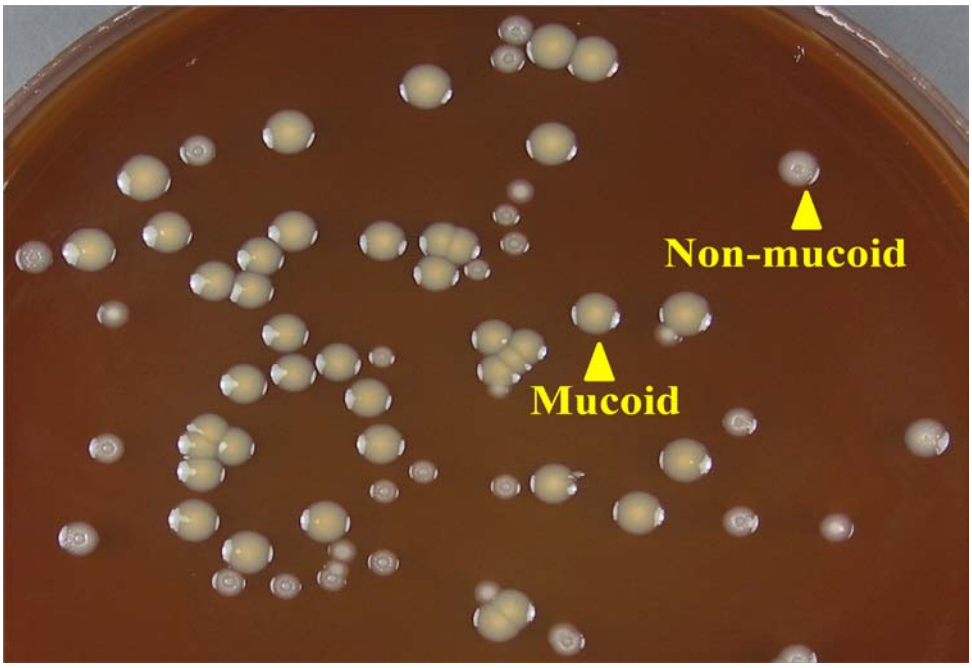

Figure 2 Mixture of colony morphotypes of *B. pseudomallei* from clinical isolates on blood agar. Overnight culture of *B. pseudomallei* on blood agar was resuspended in PBS and bacterial number was adjusted to 10^8 CFU/ml. Bacterial suspensions were spread on blood agar and the plates were incubated at 37 °C for 3 days.

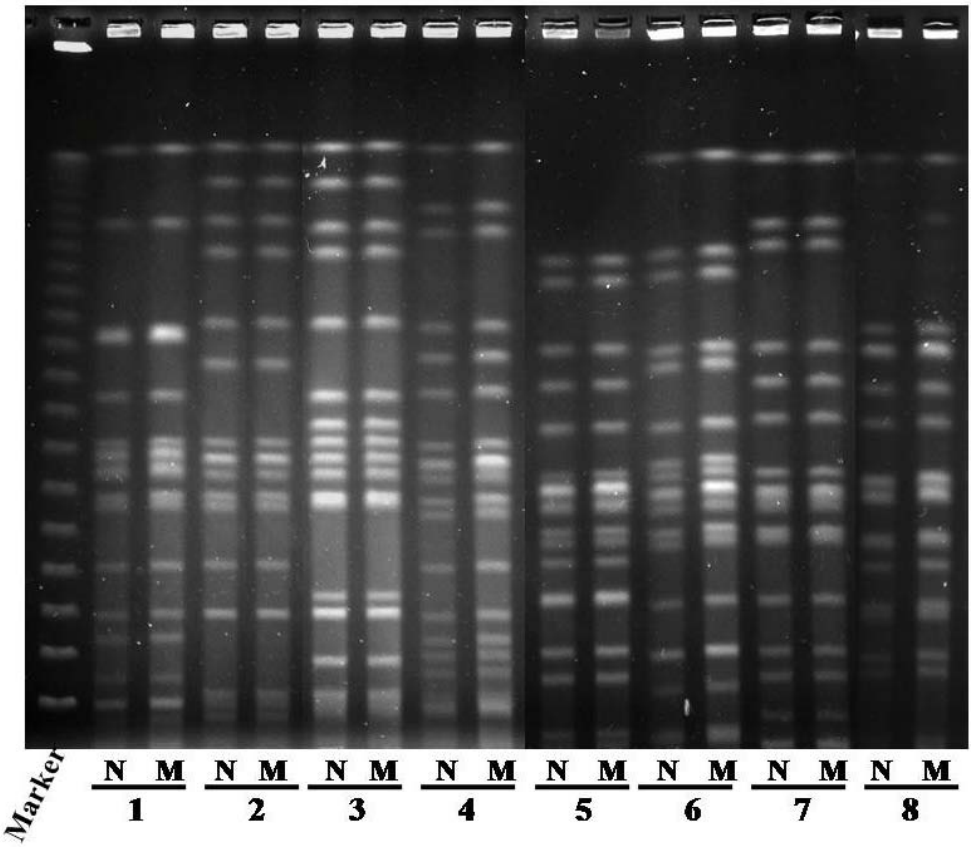

Figure 3 PFGE patterns of non-mucoid and mucoid colonies of *B. pseudomallei* from 8 clinial isolates with mixed colony morphologies. Mixed morphotypes for each of 8 strains were streaked on blood agar and incubated overnight at 37 °C. The cultures were resuspended in TE buffer and adjusted OD_{600} to 1.2. The cultures were mixed with an equal volume of 2% LMP agarose and loaded into PFGE block. Gel blocks were incubated in buffer containing protease K and then digested with *SpeI* enzymes. Gel blocks were cut and run in 1% agarose. The gel was stained with ethidium bromide and visualized under UV light. Marker: Lamda marker, N: non-mucoid colony, M: mucoid colony, No. 1-8: strain No. of *B. pseudomallei*

II. Identification of gene(s) responsible for the expression of 9D5 reactive-epitope on O-PS

Since B. *pseudomallei* with different LPS antigenic phenotype contain a typical ladder type A pattern representing complete structure of LPS consisting of O-polysaccharide, core-oligosaccharide and lipid A observed by SDS-PAGE and silver staining, we postulated that *B. pseudomallei* regulated the modification of an antigen on O-PS moiety which was defined by to the specific reaction with 9D5 Mab and this phenomenon is associated with colony morphology switching. Structure of *B. pseudomallei* O-PS contains 2-*O*-methyl and 4-*O*-acetyl substitution and 2-*O*-acetyl modification (Knirel et al., 1992; Perry et al., 1995; Brett et al., 2011). To investigate whether these modification had effect on an epitope for Mab binding, we performed knock-out deletion of two acetyltransferase genes and one methytransferase gene as follows: *wbiA* which is required for 2-*O*-acetylation, *BPSL1936* (*ocaA*) which is required for 4-*O*-aceylation and 2-*O*-methylation and *wbiD* which is required for 2-*O*-methylation (Brett et al., 2011).

We generated mutants according to mutagenesis protocol described by LÓpez and colleagues (LÓpez et al., 2009). Fragment mutagenesis, where a linear DNA fragment containing the mutation was assembled in vitro by PCR, was constructed for *wbiA, wbiD* and *oacA* genes. Regions of deleted mutations were cloned into a non-replicative plasmid, pEXKm5. The mutant alleles were transferred to the host *B. pseudomallei* strain K96243 by conjugation using RHO3.

1. Primer design

WbiA, wbiD and *oacA* sequences were obtained from the genome sequence of *B. pseudomallei* K96243. PCR primers corresponding to 5′ and 3′ fragments of these genes were designed by using Primer-BLAST from NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast). The forward primer for amplifying the 3' fragment was designed to have oligonucleotide tail homologous to the 3' end of the 5' fragment. The primer sequences, positions and product sizes are shown in Table 1.

2. Construction of *B. pseudomallei* mutants

2.1 The DNA of *B. pseudomallei* was extracted using a High pure PCR template preparation kit (Roche). PCR were performed using 5′ and 3′ primers under optimized conditions. Primers of fragment 1 (5′ fragment) for *wbiA, wbiD* and *oacA* genes are *wbiA*-F1/R1, *wbiD*-F1/R1 and *oacA*-F1/R1 respectively. Primers of fragment 2 (3′ fragment) for *wbiA, wbiD* and *oacA* genes are *wbiA*-F21/R2, *wbiD*-F2/R2 and *oacA*-F2/R2 respectively. Fragment 1 and 2 were connected by PCR which was facilitated by tail on 3′ forward primer to give new PCR product. This connection was performed using primer F1/R2. The new PCR products contained deleted fragment of each gene. The size of new PCR product of *wbiA, wbiD* and *oacA* gene was 580 bp, 465 bp and 635 bp respectively.

2.2 The connected products were cloned into pGEM®-T -Easy and transformed into *E. coli* DH5α. The white colonies were selected from LB agar supplemented with X-Gal and Ampicillin. The deleted fragments were confirmed by PCR. The positive clones containing pGEMT-*wbiA,* pGEMT-*wbiD* or pGEMT-*oacA* were cultured for overnight. The plasmids were purified and then cut with *EcoR*I before running in agarose gel. *WbiA, wbiD* and *oacA* genes after *EcoR*I cut were observed as small product (Figure 4). All 3 genes were

purified and ligated into *EcoR*I digested pEXKm5, which is a non-replicating plasmid that used for allelic exchange for introducing the mutation into *B. pseudomallei* K96243.

2.3 The pEXKM5-*wbiA,* pEXKM5-*wbiD* and pEXKM5-*oacA* were transformed into *E. coli* DH5α. The clones were selected using LB agar containing X-Gal and kanamycin. The white colonies were picked to PCR amplification to check products for *wbiA, wbiD* and *oacA*, respectively. The plasmids were extracted and transformed into *E. coli* RHO3. The clones were selected using kanamycin plates. The kanamycin resistance colonies were selected and the gene fragments in each gene were detected by PCR. *E. coli* RHO3 containing *wbiA, wbiD* and *oacA* were conjugated with *B. pseudomallei* K96243. The mutant clones were selected on LB agar supplemented with X-Gluc and 1,000 μg/ml kanamycin. The mutants were checked by PCR. The wild type and mutant strains were distinguished by PCR using primer sets flanking the mutant deletion allele primers. The product sizes of gene mutant were 580 bp in *wbiA,* 465 bp in *wbiD* and 635 bp in *oacA*, whereas wild type retained 1,162 bp, 1,607 bp and 1,480 bp of full length *wbiA, wbiD* and *oacA* gene respectively (Figure 5).

2.4 The antigenic phenotype O-PS of mutants was compared to wild type by immunoblot against 9D5 Mab. LPS from *B. pseudomallei* wild type and mutants was purified by protease K digestion and probed with 9D5 Mab. *WbiA* and *wbiD* mutants did not react with 9D5 Mab (Figure 6A) while *oacA* mutant reacted with the Mab similar to wild type (Figure 6A). To ensure that *wbiA* and *wbiD* mutants have either LPS epitope modification or incomplete LPS structure, LPS from wild type and mutants was stained with silver stain. Both *wbiA* and *oacA* mutants exhibited a ladder of O-PS moieties of LPS as smooth type A similar to that in the wild type (Figure 6B). However, *wbiD* exhibited rough LPS type showing no ladder. (Figure 6A, B). It is confirmed 9D5 Mab reacted with an epitope on O-PS which was acetylated by *wbiA.*

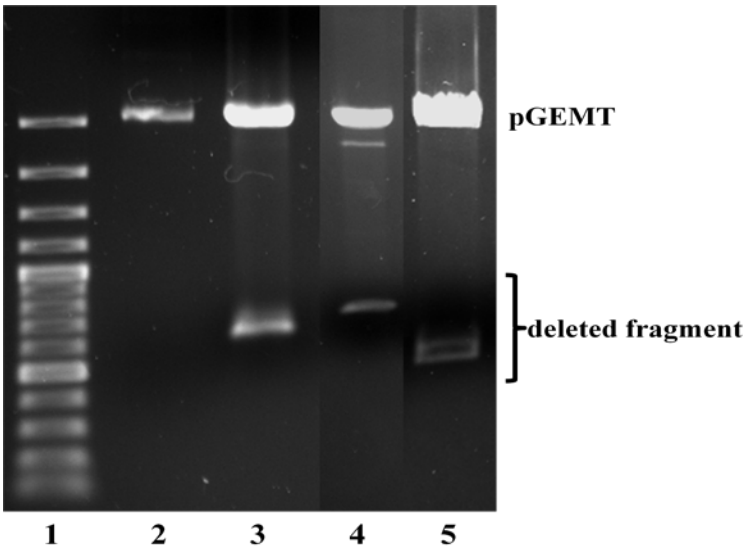

Figure 4 Agarose gel electrophoresis of the products after *EcoR*I cut. Lane 1: 100-3,000 bp markers, lane 2: pGEMT, lane 3: pGEMT-*wbiA*, lane 4: pGEMT-*wbiD* and lane 5: pGEMT-*oacA*.

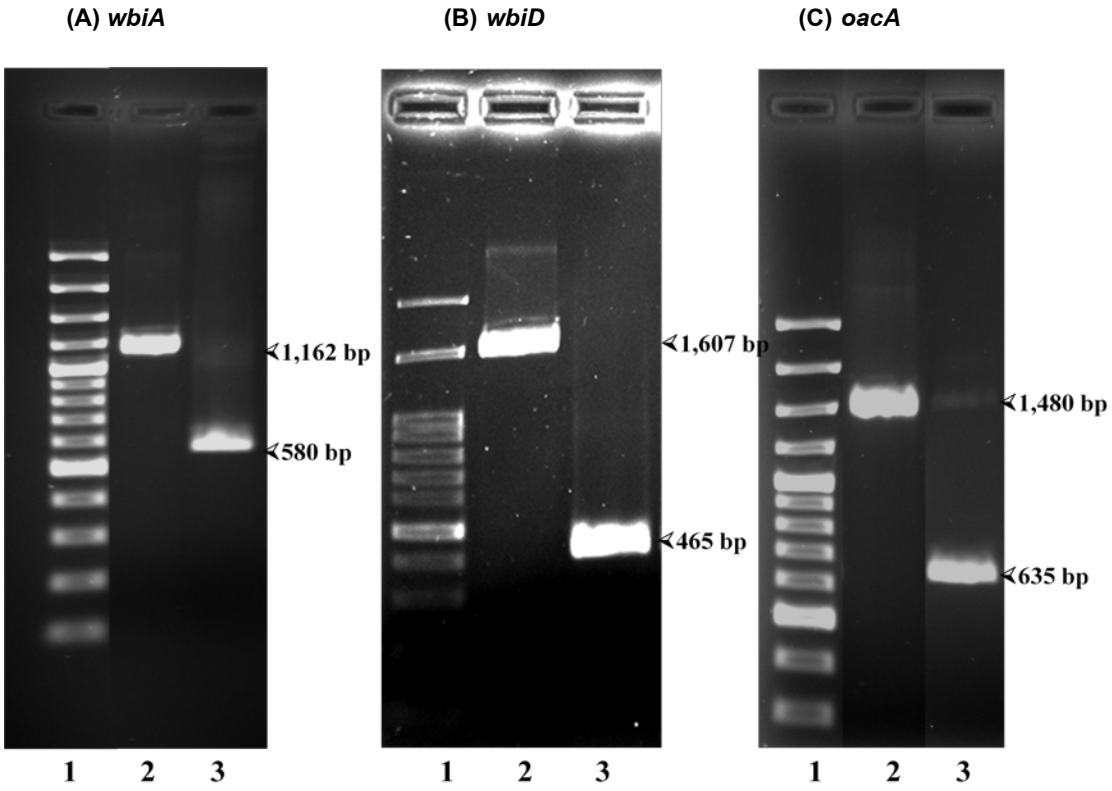

Figure 5 Construction of *wbiA, wbiD* and *oacA* defective mutants. PCR products of *wbiA* (A), *wbiD* (B) and *oacA* genes (C) were separated in 1.5% agarose. Lanes 1: 100-3,000 bp markers, lane 2: *B. pseudomallei* K96243 wild type and lane 3: mutants

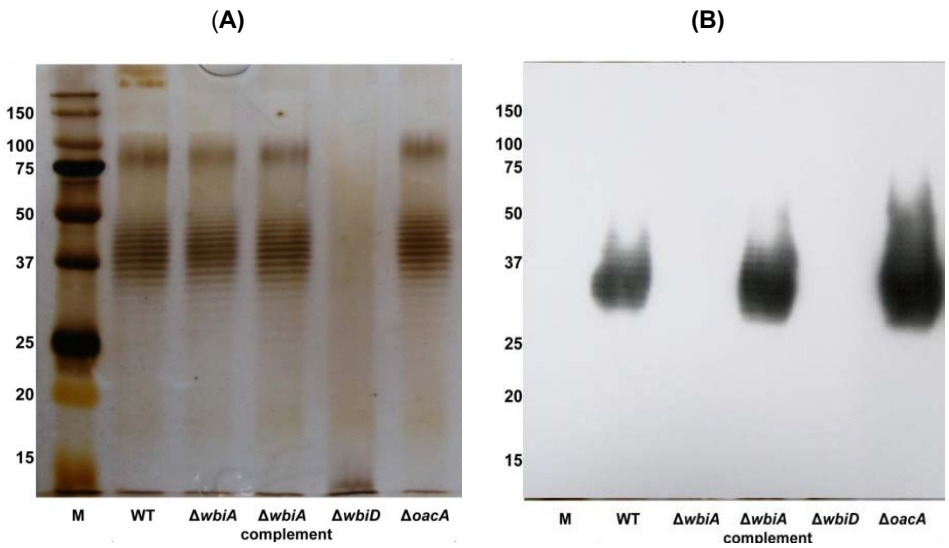

Figure 6 Immunoblot (A) and silver stain (B) of LPS of *B. pseudomallei* wild type and mutants. LPS of all strains was prepared by protease K digestion, separated on 12% SDS-PAGE gel electrophoresis.

III. Identification of laboratory conditions that associated with the O-PS modification of *B. pseudomallei*

1. **Colony morphology switching of *B. pseudomallei* under various stress conditions**

B. pseudomallei 5 isolates (5 mucoid and 5 non-mucoid) were cultured for 1 day in 9 stress conditions including control (TSB pH 7.4), acid (TSB pH 4.0), alkaline (TSB pH 8.5), temperature (42 °C), oxygen deficiency, starvation (DW), salt (350 mM NaCl), reactive nitrogen intermediate (RNI; 50 mM $NaNO_2$), reactive oxygen species (ROS; 2 mM H_2O_2), and for 7 days in TBS. The colony morphology of emergent morphotype that were different from original type was determined. Ten colonies of emergent morphotype from each strain were randomly picked to test for the reaction with Mab-latex and compared with the original type. The experiments were performed in duplicate for 3 independent experiments.

The result in Figure 7 suggested that the pattern and rate of switching between mucoid and non-mucoid colonies in all conditions was specific to isolates. Starting from a mixture of 5 non-mucoid colonies at $1x10^7$ CFU/ml for each strain, only three (4095a, 10457A and 11017A) of five strains demonstrated colony morphology switching to mucoid (Figure 7A). The mucoid colonies emerged in two stress conditions. The 7-day incubation in TSB generated 72% and 60% mucoid colonies of total colony count for strains 4095a and 11017A respectively and condition of 42^oC incubation in TSB generated 30% mucoid colonies of total colony count. We did not observe switching to mucoid colonies on the plates for strains 10971B and MSHR295 in any conditions tested.

In contrast, several conditions affected colony switching from mucoid to non-mucoid and the rate of switching varied between isolates (Figure 7B). Non-mucoid emerged from parental mucoid in all conditions for strain 10457A and in most conditions for 10971B and 11017A. Starting from a mixture of 5 mucoid colonies at $1x10^7$ CFU/ml for each strain, the percentage of non-mucoid colonies was observed highest from culture of DW (78 % for 10457A and 78% for 10971B) and 7 days in TSB (64% for 11017A). Switching from mucoid to non-mucoid of 4095c and MHSR295S was observed in DW only.

2. Stability of mucoid and non-mucoid colonies in non-stress condition

The emergent colonies were tested for the stability of morphotype after stress removal. Five colonies of each emergent strain were streaked on TSA and the plates were incubated overnight. The colony morphology was examined. The subculture was performed ten times. We observe the switch of 10457A (M) and 10971B (M) to non-mucoid after subculturing for 2 and 3 times respectively (Figure 8B). The reversible rate was increased by times starting from 4.4% on second passage to 17.8% on the 8^{th} passage for strain 10457A (M). For 10971B (M), the rate increased from 3% of 3^{rd} passage to 8.2% on 6^{th} passage (36R). We did not observe the switching of other 3 mucoid colonies (4095c, 11017A and MSHR295) nor all non-mucoid to alternate types after 10 continuous subculturing (Figure 8A). The result suggested that the non-mucoid colony variants were stable than mucoid colonies in the absence of continued stress condition.

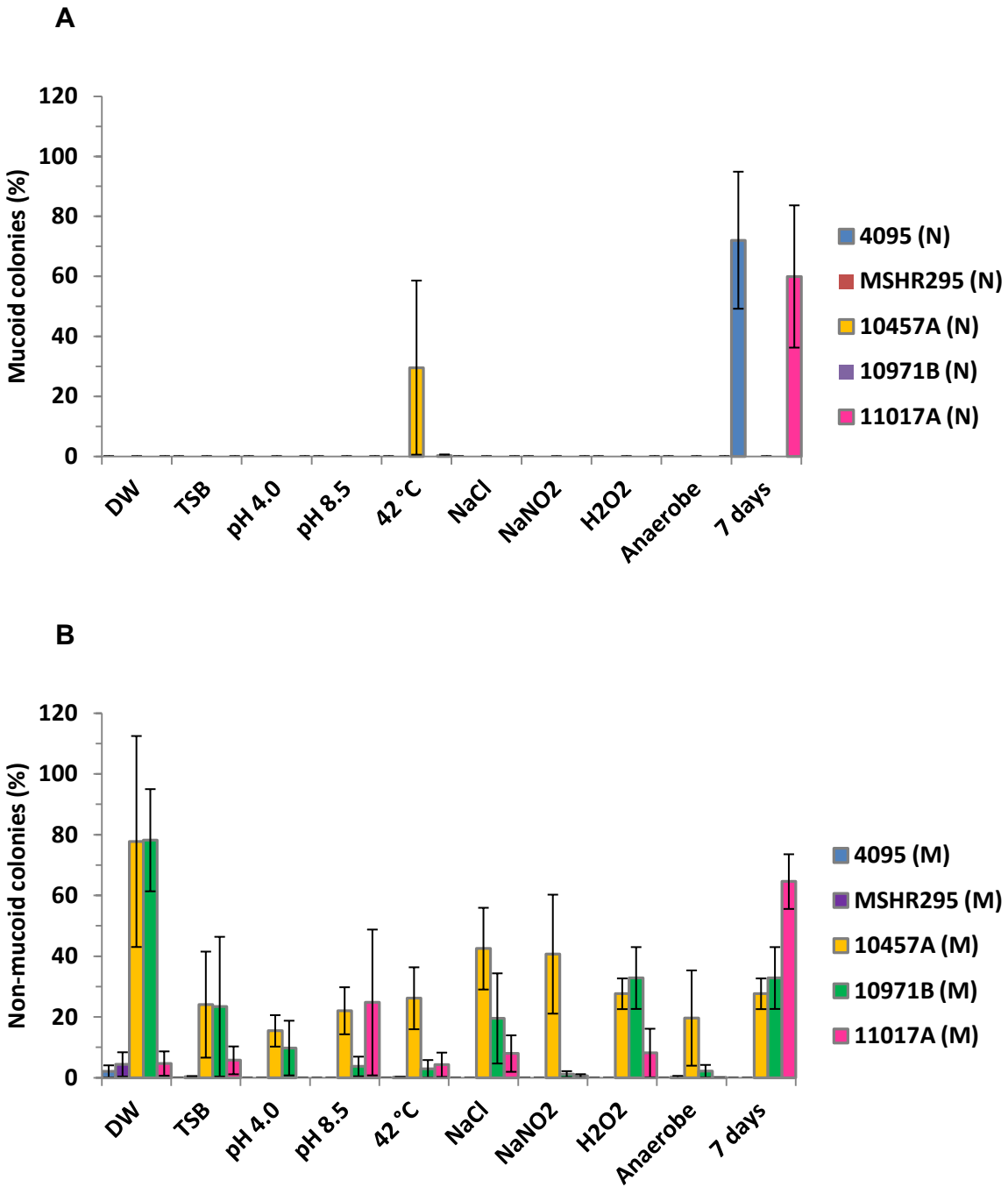

Figure 7 Effect of laboratory stress conditions on colony morphology switching**.** *B. pseudomallei* 10 isolates ($1x10^7$ CFU/ml of 5 mucoid and 5 non-mucoid) were cultured for 1 day in 9 stress conditions including control (TSB pH 7.4), acid (TSB pH 4.0), alkaline (TSB pH 8.5), temperature (42 °C), oxygen deficiency, starvation (DW), salt (350 mM NaCl), reactive nitrogen intermediate (RNI; 50 mM $NaNO_2$), reactive oxygen species (ROS; 2 mM H_2O_2), and for 7 days in TSB. Colony morphology and 9D5 reaction were observed after 1 or 7 days of incubation. N=non-mucoid colonies, M=mucoid colonies

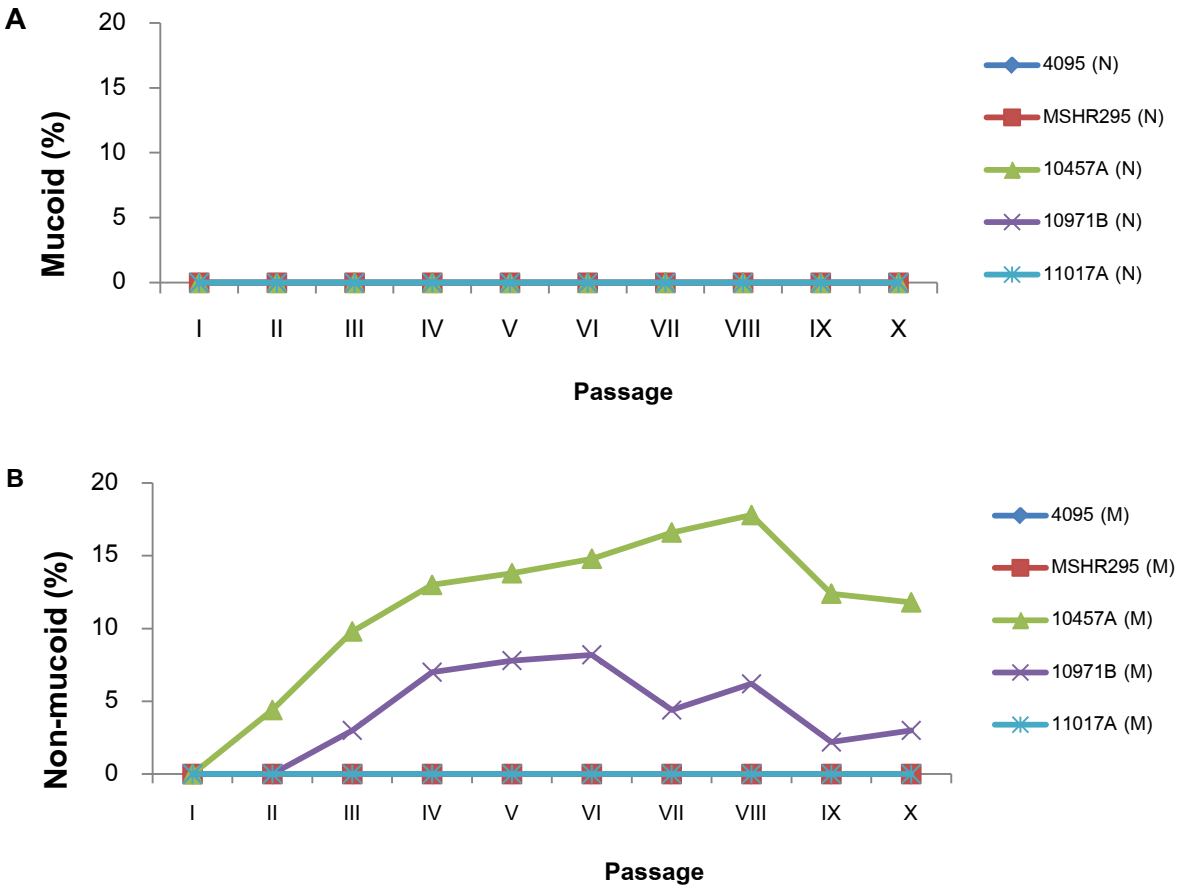

Figure 8 Stability of mucoid and non-mucoid colonies in non-stress condition. *B. pseudomallei* were streaked on TSA for 10 times. (A) Percent switching of non-mucoid (NM) to mucoid (M) colonies and (B) Percent switching of mucoid to non-mucoid colonies.

IV. Investigation the effect of O-PS modification of *B. pseudomallei* for survival in host invasion

1. Adherence and invasion of *B. pseudomallei* to human epithelial cell line (A549)

Adherence and invasion of *B. pseudomallei* 10 isolates (5 mucoid and 5 non-mucoid colonies) were investigated in non-phagotytic human epithelial cell line (A549). Approximately $4.5x10^5$ epithelial cells were seeded into 6-well plate and cultured overnight. The cell monolayer was infected with *B. pseudomallei* 5 reactive and 5 non-reactive strains at an MOI of 50:1. The infected cells were incubated at 37 °C in humidified 5% CO_2 incubator. For adherence assay, at 1 h of infection, the infected cells were washed 3 times with PBS to remove non-adherence bacteria. Thereafter, 1 ml of 0.1% Triton X-100 was added to each well and the numbers of adherence bacteria were determined by plating onto TSA. For invasion assay, the cells were infected with *B. pseudomallei* for 2 h. The extracellular bacteria were killed by incubation of the infected cells in the medium containing 250 μg/ml of kanamycin for 1 h.

The result of adherence was showed in Figure 9A. At 1 h, there was no statistically significance of adherence in epithelial cell between mucoid and non-mucoid colonies. There was no consistency difference between mucoid and non-mucoid colonies for invasion. Mucoid colonies of strains 4095 and MHSR295 had better invasion but strains 10971B and 11017A had lower adherence into A549 cells (Figure 9B). Strains 10457A lost ability to invade into epithelial cells.

2. Internalization and intracellular survival of *B. pseudomallei* to human monocyte cell line (THP-1)

To determine the internalization and intracellular survival of *B. pseudomallei* in phagocytic cell, internalization and survival of 5 pairs of *B. pseudomallei* (5 mucoid and 5 non-mucoid colonies) were investigated in phagotytic human monocyte cell line (THP-1). Approximately $3x10^5$ THP-1 cells were seeded into 24-well plate and cultured overnight. The cells were infected with *B. pseudomallei* 5 non-mucoid and 5 mucoid strains at an MOI of 1:1. The infected cells were incubated at 37 °C in humidified 5% CO_2 incubator. At 2 h of infection, the infected cells were added with 250 μg/ml of kanamycin and incubated further for 2 h before replace with 20 μg/ml of kanamycin. At the indicated time, the infected cells were washed 3 times with PBS and 1 ml of 0.1% Triton X-100 were added to each well. The numbers of intracellular bacteria were determined by plating onto TSA.

The result of internalization (4 h of infection) was showed in Figure 10A. All of 5 mucoid colonies are able to enter into human monocyte cells better than non-mucoid colonies. However, the intracellular survival of both mucoid and non-mucoid colonies was not significant different (Figure 10B). Both mucoid and non-mucoid colonies can multiply inside the cells after internalization.

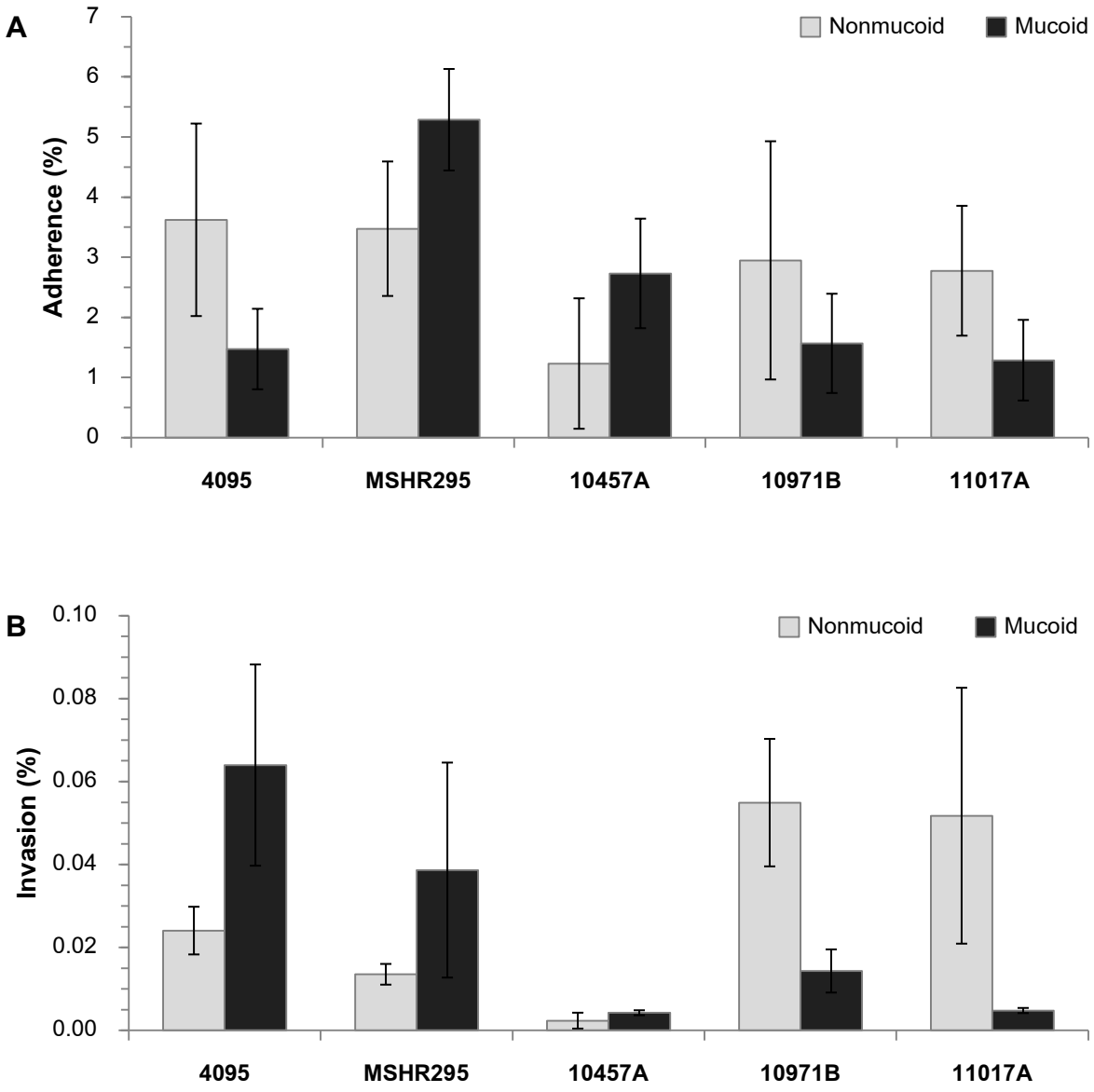

Figure 9 Adherence and invasion of *B. pseudomallei* to human epithelial cell line (A549). A549 cells were infected with *B. pseudomallei* for 1 h (A: adherence) and 3 h (B: invasion). The results represent mean± SD of 3 independent experiments.

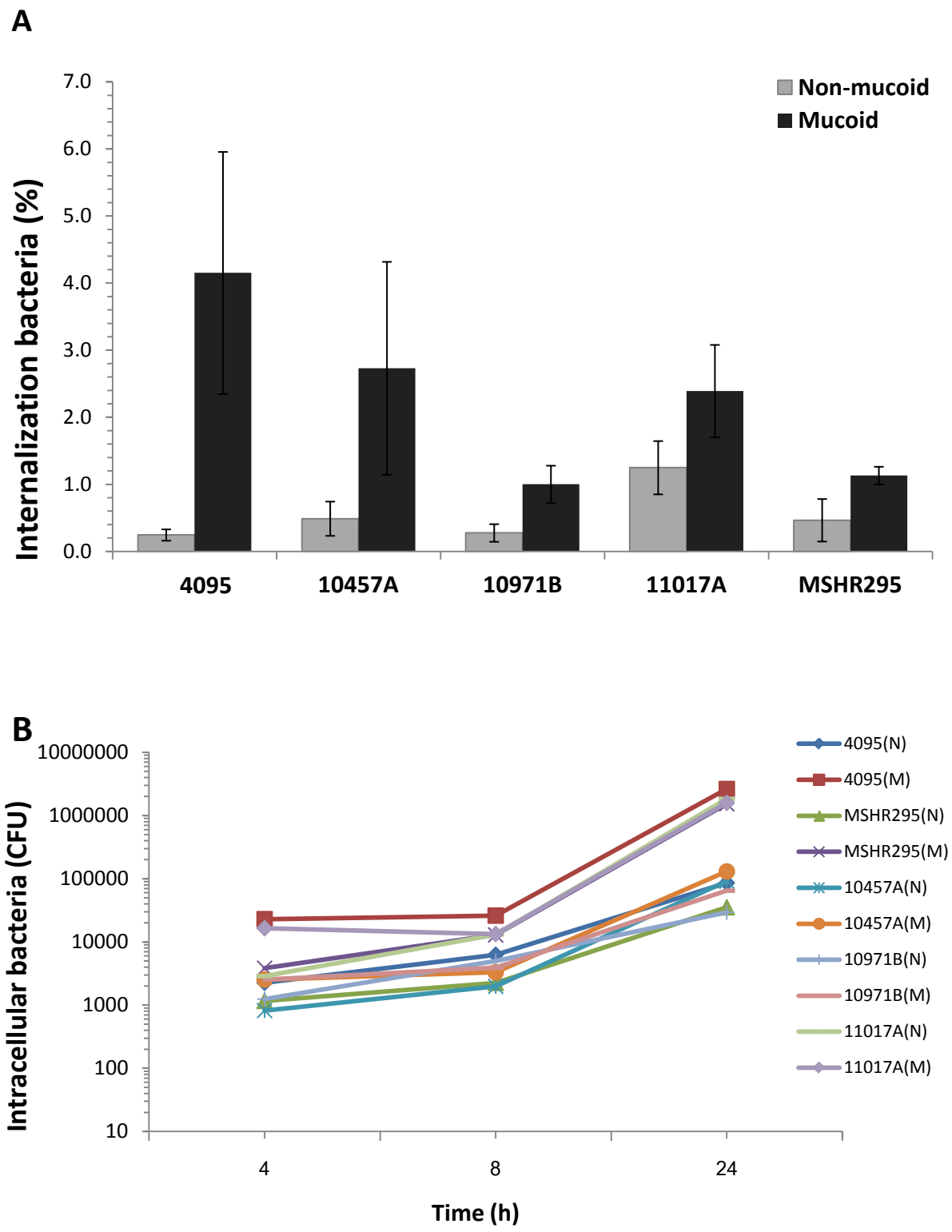

Figure 10 Internalization (A) and intracellular survival (B) of *B. pseudomallei* mucoid and non-mucoid colonies into human monocyte cell line (THP-1). THP-1 cells were infected with *B. pseudomallei* for 2 h before adding with 250 μg/ml of kanamycin for 2 h to kill extracellular bacteria and then the concentration of kanamycin was keep at 20 μg/ml until end of experiment. The results represent mean± SD of 3 independent experiments. N=non-mucoid colonies, M=mucoid colonies

V. NMR analysis of OPS structure

To further investigate the structural characteristics of the O-PS antigens expressed by *B. pseudomallei* 4095a (non-mucoid) and 4095c (mucoid), O-PS was isolated from purified *B. pseudomallei* LPS by mild-acid hydrolysis and gel-permeation chromatography. Utilizing a combination of 1-D and 2-D ^{1}H and ^{13}C NMR spectroscopy experiments, results indicated that the 4095a and 4095c O-PS antigens exhibited 2-*O*-methyl and 4-*O*-acetyl substitution patterns that differed from one other as well as from the predominant O-PS serotype expressed by *B. pseudomallei* 1026b (Figure 11). More specifically, the 6dTal residues of the *B. pseudomallei* 4095a sample were found to be devoid of any 4-*O*-acetyl modifications (except at the non-reducing end residue) while the 6dTal residues of the *B. pseudomallei* 4095c sample were shown to be lacking both 4-*O*-acetyl (except at the non-reducing end residue) and 2-*O*-methyl modifications. The data indicate that the epitope recognized by the 9D5 mAb requires 2-*O*-acetyl and 2-*O*-methyl substitution of 6dTal residues. *B. pseudomallei* OPS is subjected to antigenic variation within a host.

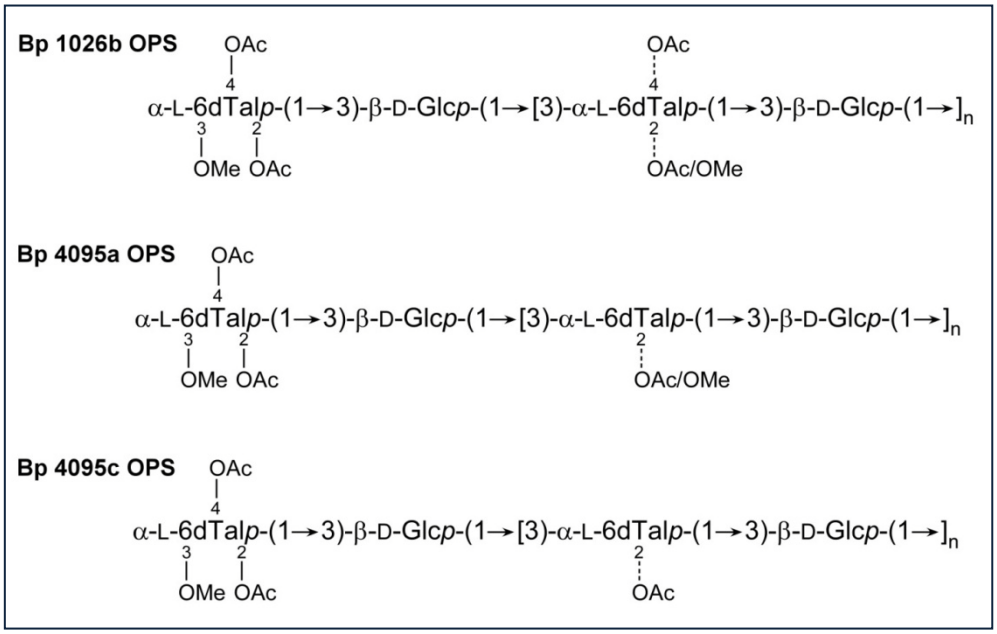

Figure 11 Chemical structure of O-polysaccharide expressed by non-mucoid and mucoid *B. pseudomallei* strain 4095a (non-mucoid) and 4095c (mucoid), and a previous described O-PS structure of strain1026b.

Discussion and Conclusion

The predominant colonies of *B. pseudomallei* on solid agar are typically dry, rough colonies. In this study, we observed mucoid colonies from clinical and environment isolates, indicating the occurrence of mucoid colonies in various environment. Identification of mucoid colonies from primary culture of melioidosis patients confirmed the occurrence of colony variation in human infection. Conversion from mucoid to non-mucoid colonies or vice versa of *B. pseudomallei* may be a mechanism in adaptation. Mucoid colonies are less stable than non-mucoid colonies. Starvation in DW and in TSB for 7 days is more relevant to conversion from mucoid to non-mucoid colonies. It is unclear whether mucoid or non-mucoid colonies are the parental colony. Whole genome sequencing of mucoid and non-mucoid colonies is needed to solve this problem. However, mucoid colonies seem to be an origin colony since mucoid colonies have more mutation rate when non-mucoid colonies have not. Colony morphology variation of *B. pseudomallei* had been reported when culture on selective medium Ashdown's agar, however different morphotypes were not related to antibody recognition (Chantratita et. al. 2007). The difference on colony appearance of *B. pseudomallei* is related to alteration on expression of the surface determinant. This study showed correlation between colony morphology of *B. pseudomallei* and 9D5 Mab specific to O-PS. It also indicates the influence of LPS structure on colony morphology.

Alteration on O-PS modification of *B. pseudomallei* avoids 9D5 Mab recognition. We have demonstrated a novel O-PS expressing by *B. pseudomallei* that does not react with 9D5 Mab. A comparison of O-PS structure of non-mucoid and mucoid colonies by NMR suggested that mucoid colonies loss of 2-*O*-methyl group at *O*-2 position of the L-6dTal*p* residues. The *wbiA* mutant defective in 2-*O*-acetylation showed LPS smooth type A but lose of 9D5 reaction. It is now obviously seen that 9D5 MAb reacts only with polymers of 3)-β-D-glucopyranose-(1 $\rightarrow$ 3)-6-deoxy-α-L-talopyranose-(1 in which the L-6dTal*p* residues are coordinately acetylated and methylated at *O*-2 position. The 9D5 MAb need to bind with conformational structure of *O*-methyl and *O*-acetyl groups on O-PS antigen. De-acetylation or de-methylation affects the three-dimensional structure of O-antigen by destroying a series of conformational antigenic determinant. O-antigen modification might be a mechanism of bacteria to adapt in particular niches. Escape from antibody recognition of *B. pseudomallei* can block the initiation of alternative pathway of complement activation. This mechanism increase survival of bacteria in the host during infection.

Change in colony morphology is associated with survival adaptation in stress conditions and particularly environment. Mucoid colonies which lack of 2-*O*-methyl group on O-PS are not stable and try to convert to the common non-mucoid colonies. This is the reason why mucoid colonies are observed as a minor population of *B. pseudomallei*. There are many gene clusters involved in LPS biosynthesis of *B. pseudomallei* (DeShazer et. al., 1998). *WbiA* gene seem to be involved in *O*-acetylation on O-PS of *B. pseudomallei* but it is not involved in generating of mucoid colonies since *wbiA* mutant exhibit non-mucoid colonies. The further study needs to identify gene involved in regulation of LPS modification and colony morphology variation.

De-acetylation or de-methylation on O-PS moieties of *B. pseudomallei* mucoid colonies increased internalization human monocytes. The LPS impart a strongly negative charge to surface of gram negative bacteria. Alteration on O-PS structure might affect surface charge of bacteria that increase phagocytosis of *B.*

pseudomallei into the human monocytes. These results are consistence with the previous report that substitution of O-antigen of *E. coli* and *S. enterica* serovar Typhimurium is correlated with pathogenesis by increased activation of alternative complement pathway, leading to increase phagocytosis (Reeves, 1995). O-PS of *B. pseudomallei* LPS is required for serum resistance and virulence (DeShazer et. al., 1998). We found de-acetylation or de-methylation on O-PS moieties did not affect serum susceptibility. O-PS of 9D5 non-reactive strains or mucoid colonies was still retained in LPS structure. The length of O-PS with O-PS alteration is long enough to prevent access of the C5b-C9 complement membrane attack complex (MAC) to outer membrane so modification on O-PS structure of *B. pseudomallei* is not relevant to serum resistance. However, we still not know why primary isolate of strain 10457A which contained smooth type A LPS was susceptible to serum killing and had less ability to enter into primary human monocytes.

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Outputs

1. Publication

A manuscript is preparing and is not complete. A manuscript entitled “Colony morphology variation of *Burkholderia pseudomallei* is associated with O-polysaccharide modification” will be submitted to Infection and Immunity (Appendix).

Presentations

A part of this study was presented in international meeting.

1) “7th World melioidosis congress 2013” in Bangkok, Thailand on 18-20 September 2013. The title of poster is “*Burkholderia pseudomallei* modifies O-polysaccharide to modulate immune recognition”
2) “The 12th FIMSA Advance Training Course” in Chiang Mai, Thailand on 15-28 October 2013. The title of poster is “Modification on O-polysaccharide moieties of *Burkholderia pseudomallei* LPS effect on immune recognition and response”

2. Advantage of the research

The results from this research is basic knowledge for understanding of the pathogenesis and virulent of *Burkholderia pseudomallei.* It may lead to the discovery of relapse mechanism. The techniques and methods in this research are used as reference for other studies.

Appendix

COLONY MORPHOLOGY VARIATION OF *BURKHOLDERIA PSEUDOMALLEI* IS ASSOCIATED WITH O-POLYSACCHARIDE MODIFICATION

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Running title: Modification of *Burkholderia pseudomallei* lipopolysaccharide

INTRODUCTION

Burkholderia pseudomallei is an environmental saprophyte that causes a fatal disease mostly endemic in tropical countries. It is classified as a CDC Tier 1 select agent based on potential hazard to public health (1)[Butler D Nature 2012]. *B. pseudomallei* infection is acquired by inoculation, inhalation and ingestion. Once infection is established, bacteria may disseminate via the bloodstream and affect numerous organs, with common clinical manifestations including pneumonia and multiple abscesses in the liver and/or spleen. The mortality rate is 40% (2)[Limmathurotsakul D et al Am J Trop Med Hyg 2010], and eradication of *B. pseudomallei* is difficult to achieve, leading to the need for prolonged antimicrobial treatment and relapse in up to 10% of cases. *B. pseudomallei* is a facultative intracellular organism and can survive in a range of host cell types including phagocytes (3, 4)[Wiersinga NEJM 2012; Charoensap 2009]. Potential mechanisms that contribute to bacterial persistence are poorly understood, although *B. pseudomallei* has been reported to be highly adaptability and able to survive under extreme conditions (5, 6)[Pumpuang et al 2011; Thanhavanand S BMC 2010].

Lipopolysaccharide is an important *B. pseudomallei* virulence factor, a major stimulator of the host immune response, and has been considered to be a vaccine target (7)[Burtnick et al Frontiers 2012]. Three different LPS types based on SDS-PAGE pattern have been described for *B. pseudomallei* which have distinct serological reactivity: ladder type A, ladder type B and rough LPS (8)[Anuntagool 2006]. Type A and Type B LPSs are composed of three covalently linked domains (lipid A, core-oligosaccharide and O-polysaccharide (OPS), while rough LPS lacks OPS (8)[Anuntagool 2006]. Type A is the predominant LPS type expressed by isolates from Thailand (97%) and Australia (80%) (8)[Anuntagool 2006]. The structure of OPS type A is an unbranched heteropolymer with repeating D-glucose and L-talose residues with the structure -3)-β-D-glucopyranose-(1->3)-6-deoxy-α-L-talopyranose-(1- in which 33% of this residue contains 2-*O*-methly and 4-*O*-acetyl substitution while the remainder has 2-*O*-acetyl modification (9-11)[Knirel et al, 1992; Perry et al., 1995; Brett et al 2011]. It is believed that there is only one serotype for type A LPS.

Bacterial switching between colony morphotypes often reflects a change in expression of one or more phenotypic determinants. This may be a reversible process and results from genetic or epigenetic alterations at specific genetic loci [Ref]. In several Gram-negative bacteria, LPS variation has been shown to occur and generates diversity that is associated with a survival fitness in different environments. For example, different states may be associated

with enhanced invasion, reduced virulence, or avoidance of host immune recognition and persistence (12)[Ref P aeruginosa paper, Chung JW et al]. Our previous study has shown that *B. pseudomallei* have seven colony morphotypes which undergo switching under stress conditions. The different colonies that were observed on Ashdown agar are related to phenotypic variation, including the ability to produce extracellular enzymes, flagella production, motility and biofilm formation (13)[Chantratita et al, 2007]. Type I morphotype represents the predominant type (88% of 241 isolates), but this can switch to other types in vivo and in vitro. The relationship between this and LPS variation is unidentified (13)[Chantratita et al, 2007]. We have subsequently noted the presence of two distinct colony types in a proportion of clinical samples plated onto non-selective agar such as trypticase soy agar (TSA) and blood agar (BA). These two types of colonies are described as a mucoid and non-mucoid but the related phenotypes on these media are unknown.

We hypothesized that different colony types of *B. pseudomalle* was associated with LPS variation. We developed a latex agglutination test based on a LPS specific monoclonal antibody (Mab) to screen the LPS antigenic variation in *B. pseudomallei* mucoid and non-mucoid colonies, verified the distinct OPS types by Western blot analysis. We also identified the different antigenic molecules on OPS using site-directed mutagenesis and NMR spectroscopy.

MATERIALS AND METHODS

Bacterial isolates and culture conditions

Three retrospective *B. pseudomallei* isolate collections were used, as follows. (1) Two hundred isolates, one from each of 200 patients presenting to Sappasithiprasong Hospital, northeast Thailand with their first episode of melioidosis between 1986-2004 who did not develop relapse during follow up to July 2005 (14)[Limmathurotsakul et al, CTD 2006]. (2) One hundred and sixty-six isolates from 78 melioidosis patients from the same cohort who did develop relapse during follow up to July 2005 (14)[Limmathurotsakul et al, CTD 2006]. Of these, 69 patients had two isolates and the remainder had 3 isolates (24 patients) or 4 isolates (1 patient). (3) Fifty-two isolates from 38 patients, 10 animals, 3 soil samples and 1 water sample from northern Australia, provided by Prof. Bart Currie, Charles Darwin University in 2002 (8)[Anuntagool N. AJTMH 2006]. A fourth prospective collection was assembled during this study, in which colonies were picked from primary culture plates

(sheep blood agar) from 40 patients presenting to Sappasithiprasong Hospital between July 2011 and November 2012 with suspected melioidosis. The clinical specimens were sputum (n=13), tracheal secretions (n=6), pus (n=11), wound swab (n=3), synovial fluid (n=1), and blood culture (n=6). Colonies were picked after incubation for 2 days at 37°C in air. These samples were randomly selected from those that grew *B. pseudomallei* on a primary blood agar plate during routine diagnostic processing. The laboratory strains of *B. pseudomallei* and *Escherichia coli* used during the study are described in Table 1. Unless otherwise stated, these were cultured on trypticase soy agar (TSA) or Luria-Bertani (LB) agar (BD), respectively. All isolates were stored in trypticase soy broth (TSB) with 15% glycerol at -80^{o}C.

Latex agglutination assay

A latex agglutination test was performed as described previously (15)[Anuntagool and Sirisinha, 2002]. This is based on a monoclonal antibody (Mab) termed 9D5, which recognizes *B. pseudomallei* OPS and is positive for *B. pseudomallei* and the closely related non-virulent *B. thailandensis*, but negative for *B. mallei* (15)[Anuntagool and Sirisinha, 2002]. The Mab was purified using a Hi-trap protein A HP column (GE Healthcare), and sensitized onto latex particles (Mab-latex) according to the manufacturer's instructions (Invitrogen). *B. pseudomallei* reactivity was assessed by mixing bacterial cells with 10 µl of Mab-latex suspension on a glass slide, and observing for agglutination within 2 minutes.

Isolation and characterization of mucoid and non-mucoid colonies

B. pseudomallei from the three retrospective collections were streaked from the freezer vial onto TSA, incubated at 37°C in air for 2 days, and the plates observed for the presence of non-mucoid and mucoid colonies. Non-mucoid colonies were defined as white, opaque and matt with an umbilicated or umbonated surface, and mucoid colonies were defined as white or yellow, and shiny with a smooth surface. Five colonies of each of the two types from the same sample (or all colonies if less than 5) were tested with the Mab-latex test. For the prospective study of clinical samples, *B. pseudomallei* colonies on blood agar were observed for mucoid and non-mucoid colonies after incubation at 37°C in air for 2 days. Ten colonies of each of the two types from the same sample (or all colonies if less than 10) were tested with the Mab-latex assay. Separate freezer vials were prepared for all colony picks, and stored in TSB with 15% glycerol at -80°C. Each colony was tested for the following: (i) LPS

type using 12% gel SDS-PAGE and silver stain, and (ii) expression of the Mab 9D5 specific antigen using Western blot (8)[Anuntagool et. al., 2006]. LPS was extracted using proteinase K (Invitrogen) digestion. LPS types were defined as ladder type A, type B or rough type (no ladder) (8)[Anuntagool et. al., 2006]. In the event that a single sample contained both mucoid and non-mucoid colonies, one colony of each was compared by pulsed-field gel electrophoresis, as previously described (13)[Chantratita et al., 2007].

Effect of different culture conditions on *B. pseudomallei* colony appearance

The effect of a range of laboratory conditions on colony appearance was tested on the same isogenic pairs. Ten colonies of mucoid or non-mucoid types per strain scrapeded from TSA were suspended in PBS and adjusted to $1x10^8$ CFU/ml using spectrophotometry at OD_{600}. Two hundred microliters of suspension of each type was inoculated into 2 ml of distilled water (DW) or TSB and incubated under one of the following conditions: (i) DW at 37^{o}C for 24 h, (ii) TSB pH 7.4 at 37^{o}C for 24 h, (iii) TSB pH 4.0 at 37^{o}C for 24 h, (iv) TSB pH 8.5 at 37^{o}C for 24 h, (v) TSB pH 7.4 at 42^{o}C for 24 h, (vi) TSB pH 7.4 + 350 mM NaCl at 37^{o}C for 24 h, (vii) TSB pH 7.4 + 50 mM $NaNO_2$ at 37^{o}C for 24 h, (viii) TSB pH 7.4 + 2 mM H_2O_2 at 37^{o}C for 24 h, (ix) TSB pH 7.4 at 37^{o}C for 7 days (all incubated in air), and (x) TSB pH 7.4 at 37^{o}C for 24 h in anaerobic jar (Oxoid). Thereafter, bacteria were diluted in PBS and spread plated onto TSA, and the proportion of mucoid and non-mucoid colonies in the sample enumerated. Three separate experiments were performed in duplicate. Five colonies of mucoid and non-mucoid colonies (where present) were tested for each plate using the Mab-latex, and one colony of each mucoid and non-mucoid colonies tested for stability of colony appearance by serial plating onto TSA on ten occasions.

Bacterial internalization by human monocytes

Human monocytic cell line THP1 (ATCC# TIB-202) were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS. All cultures were maintained at 37°C in a humidified 5% CO_2 incubator. 2 x 10^5 monocytes were seeded into each well of a 96-well plate and incubated overnight at 37 °C in 5% CO_2. Cells were infected with *B. pseudomallei* at a multiplicity of infection (MOI) of 1:1 at 37 °C in 5% CO_2 for 2 h. Extracellular bacteria were killed by exposure to medium containing 250 μg/ml kanamycin for a further 2 h. Intracellular bacteria were quantified by lysing infected monocytes with 0.1% Triton X-100 and plating followed by colony counts on TSA.

Construction of mutants and complemented strains

Five *B. pseudomallei* mutants (three LPS mutants defective in *wbiA* (BPSL2680), *wbiD* (BPSL2677), or *oacA* (BPSL1936), and two capsule mutants defective in *wcbB* (BPSL2808)) were constructed using a fragment mutagenesis method, as described previously (16, 17)[Lopez et. al. 2009; Chantratita et. al., 2012]. LPS mutants were constructed in *B. pseudomallei* K96243, and capsule mutants were constructed in *B. pseudomallei* 4095a (non-mucoid) and 4095c (mucoid), which are isogenic pairs from the same patient (Table 1). Gene sequences for *wbiA, wbiD, oacA* and *wcbB* were obtained from Genbank (accession numbers 3094050, 3093124, 3091646 and 3093047, respectively). PCR primers were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) (Table 2). Mutagenesis and complementation was performed using pEXKm5-based allele replacement, as described previously (16, 17)[Lopez et. al. 2009; Chantratita et. al., 2012]. Phenotypic evaluation of LPS and capsule expression was achieved by SDS-PAGE of proteinase K extracts and Western blot analysis, respectively (15)[Anuntagool et al 2002]. LPS expression was demonstrated using the 9D5 Mab, while capsule expression was demonstrated using the capsule-specific Mab, 4B11 (15)[Anuntagool et al 2002].

DNA sequencing and reverse transcriptase PCR of *wbiA*

Genomic DNA was extracted from *B. pseudomallei* using the QIAamp DNA mini kit (Qiagen). *B. pseudomallei wbiA* was amplified using two overlapping primer pairs ((*wbiA*-seqA and *wbiA*-seqB, and *wbiA*-seqC and *wbiA*-seqD) shown in Table 2. PCR products were cleaned using ExoSAP-IT (Affymetrix) and sequenced by Macrogen (Korea). One-step reverse transcriptase (RT-PCR) was used to compare *wbiA* expression between non-mucoid and mucoid colony types. Individual colonies were harvested from TSA and RNA extracted using Trizol reagent (Invitrogen). RT-PCR was performed using 1 μg RNA and a Superscript III One-step RT-PCR system (Invitrogen) with forward primer *wbiA*-Fex and reverse primer *wbiA*-Rex (Table 2). The conditions used were: cDNA synthesis at 45 °C for 30 min; initial denaturation at 95 °C for 2 min; 35 cycles of denaturation at 94 °C for 15 s, annealing at 58 °C for 15 s, and extension at 72 °C for 15 s; and a final elongation step at 72 °C for 5 min. The positive control was RT-PCR for 16S rDNA amplification using primers Univ_16S_F and Univ_16S_R (Table 2). The negative control was *wbiA* reaction without reverse transcriptase enzyme.

LPS characterization using NMR Spectroscopy

B. pseudomallei 4095aΔ*wcbB* (CPS deficient derivative of 4095a) and 4095cΔ*wcbB* (CPS deficient derivative of 4095c) were cultured on TSA, from which colonies were scraped from the plate and purified LPS and OPS obtained as previously described (11)[Brett et al. Infect Immun 2011]. LPS was extracted using a modified hot aqueous phenol procedure (10)[Perry et. al., 1995]. OPS samples were deuterium exchanged by dissolving in D_2O and lyophilizing, and then dissolved in 0.27 ml D_2O containing 1 µl acetone. 1-D Proton and 2-D gradient-enhanced COSY (gCOSY), TOCSY, NOESY, gHSQC, and gHMBC spectra were obtained on a Varian Inova-600 MHz spectrometer at 50°C using standard Varian pulse sequences. The spectral width was 3.17 kHz in the ^{1}H dimension and 18.1 kHz in the ^{13}C dimension. The number of scans and increments was 4 and 400 for gCOSY, 8 and 200 for TOCSY and NOESY, 64 and 128 for gHSQC, and 128 and 200 for gHMBC. Acquisition times were 2 s for 1-D ^{1}H, 137 ms for gCOSY, TOCSY, and NOESY, 150 ms for gHSQC, and 128 ms for gHMBC. Mixing times for TOCSY and NOESY experiments were 120 and 300 ms, respectively. Proton chemical shifts were measured relative to internal acetone (δ_H=2.218 ppm, δ_C=33.0 ppm) (18)[Wishart et al. J Biomol NMR 1995]. Monosaccharide residues in OPS samples were identified using the 2-D HSQC spectra by comparison with the assigned spectra from *B. pseudomallei* 1026b OPS (19)[Heiss et al Carbohydr. Res 2013]. Integration of the spectra was performed using the MNova software (Mestrelab Research) after baseplane correction with a third order Bernstein polynomial [add ref].

HEK 293 cell transfection and LPS stimulation

HEK 293 cells (CRL-1573, ATCC) were maintained in complete Dulbecco's Modification of Eagle's Medium (DMEM) without sodium pyruvate, supplemented with L-glutamine, 4.5 g/L glucose (MediatechCellgro) and 10% fetal bovine serum (FBS) (Hyclone). 5×10^4 cells were seeded into each well of a 96-well plate (BD) and transfected with 10 ng of pNiFty-Luc (Invivogen), 1 ng of pRL-TK (Promega), 1 ng of hMD2, 1 ng of hCD14, and 1 ng of hTLR4 using Polyfect (Qiagen, Germany) (20)[West et al BMC]. Cells were stimulated with 0, 1, 10 or 100 ng/ml of LPS purified from *B. pseudomallei* 4095a or 4095c (21)[Chantratita et al. LPS 2014], and maintained for 6 or 18 h before harvesting. Cells were lysed and luciferase activity determined with the Dual-Luciferase Reporter Assay on a GloMax Multi Detection System (Promega). Three separate experiments were performed in triplicate. *E. coli* K12 LPS (Invivogen) stimulation was used as a control.

Susceptibility of mucoid and non-mucoid *B. pseudomallei* colonies to serum killing

Susceptibility of *B. pseudomallei* to normal human serum killing (NHS) was performed as described previously (22)[DeShazer et al. 1998]. In brief, *B. pseudomallei* was harvested from TSA, adjusted to 1 x 10^6 CFU/ml, then incubated in 30% NHS for 2 h after which viable count (CFU/ml) was defined by plating on TSA. *E. coli* HB101 was used as a control. Three independent experiments were performed.

Statistical analysis

Statistical analysis was performed using the statistical program STATA version 11.2 (College Station, TX). Nested repeated measures ANOVA was used to test internalization and monocyte cytokine responses between mucoid and non-mucoid for each isogenic pair. The t-test was used to test NF-kB activation and susceptibility to 30% NHS killing between mucoid and non-mucoid colonies.

Results

Frequency of mucoid *B. pseudomallei* colonies

A previous study described 7 different colony types for *B. pseudomallei* grown on Ashdown agar (13)[Chantratita et al, 2007]. Using TSA and blood agar in this study, we observed only two colony types, which we described as mucoid (M) and non-mucoid (NM) (Fig. 1). The frequency of these two colony types was determined in two retrospective Thai collections representing isolates from 200 cases of primary infection that was not complicated by relapse, and 166 isolates from 78 patients who had at least one episode of relapse. The majority of cultures contained non-mucoid colonies alone, with 10% or less of cultures containing mucoid colonies either alone or mixed with non-mucoid colonies (Table 3). The frequency of mucoid colonies was not significantly different in the two collections (add p value), and mucoid colonies were observed in both primary (n=9) and relapse cultures (n=9). This indicates a lack of association with clinical relapse. A third Australian collection (n=52) was also examined since there is a marked phylogenetic distinction between isolates from Thailand and Australia. As before, the majority of cultures (n=40, 77%) contained non-mucoid colonies alone, while the remaining cultures originating from human (n=10), animal (n=1) and soil (n=1) contained a mixture of non-mucoid and mucoid colonies.

Defining the presence and frequency of the two colony types from frozen stocks has several flaws, including the fact that the colonies picked for storage at the time of isolation are usually restricted and therefore subject to selection bias. To address this, we undertook a prospective evaluation and determined the frequency of non-mucoid and mucoid colony types in fresh, primary cultures of clinical specimens from 40 patients with melioidosis. Of these, 32 samples grew only non-mucoid colonies, and the remaining 8 samples contained a mixture of non-mucoid and mucoid colonies, the sample types for which were sputum (n=6) and tracheal secretions (n=2). The mucoid type represented 5% of total *B. pseudomallei* colonies on the mixed colony plates.

Taken together, these results indicate that mucoid colonies are in the minority but appear to be widely distributed in the population and are represented in clinical samples. We then considered whether the mixed colony types were isogenic and belonged to the same clone, or whether different colony morphologies could represent the presence of more than one *B. pseudomallei* strain. To address this we selected the 8 prospectively collected clinical samples, and genotyped 5 non-mucoid and 5 mucoid colonies from each sample using PFGE. The banding pattern was identical for each of the colonies within a given sample, indicating that the different colony appearance occurred in the same genetic background and could not be explained by mixed infection with two different strains.

Five pairs of mucoid and non-mucoid *B. pseudomallei* colonies were selected for further detailed phenotype and genetic analysis in the remainder of the study. These pairs were as follows: 10457A (NM) and 10457A (M), 10971B (NM) and 10971B (M), 11017A (NM) and 11017 (M) picked from primary clinical cultures containing mixed colony types; MHSR295 (NM) and MHSR295 (M) picked from a culture of *B. pseudomallei* containing mixed colony types and originating from soil in Australia; and *B. pseudomallei* 4095a (NM) and 4095c (M) isolated from different samples taken 22 days apart from a Thai patient. PFGE demonstrated that mucoid and non-mucoid colonies from each sample had an identical PGFE banding pattern, and were considered to be isogenic. Full details of these isolates are shown in Table 1.

Stability of mucoid and non-mucoid colonies on sub-culture

Stability of colony morphology was tested by performing 10 serial subcultures on TSA. One colony of each mucoid and non-mucoid colonies picked from a starting non-mucoid colony

culture was an infrequent event (4/100), only occurred under two conditions, and was only observed to occur with 4095, 10457A and 11017A (Figure 2A). By contrast, the appearance of non-mucoid colonies from a starting mucoid culture was a more frequent event (33/100) and occurred in all conditions tested although incubation in distilled water led to the largest proportional change in the population (Figure 2B). All mucoid starting cultures were observed to contain non-mucoid colonies in at least one condition, but this was limited to DW only for 4095c and MHSR295.

Internalisation of mucoid and non-mucoid colonies to human monocytes

We hypothesized that mucoid and non-mucoid colony types may have different interactions with host cells. We evaluated whether the LPS modification associated with mucoid colonies was associated with altered internalisation using five isogenic mucoid and non-mucoid *B. pseudomallei* pairs (4095, 10457A, 10971B, 11017A and MSHR295). Bacterial internalisation by human monocytes THP1 cells following 4h incubation with live bacteria at a MOI of 1:1 varied markedly between bacterial strains (Figure 3). A consistent finding, however, was higher number of internalised bacteria for mucoid colony types compared with non-mucoid colony types (*P* = ?, respectively).

Susceptibility of mucoid and non-mucoid colonies to serum killing

The O-PS moiety of LPS is responsible for serum resistance by *B. pseudomallei* (22)[DeShazer D et al., 1998]. We evaluated whether the LPS modification associated with mucoid colonies was associated with altered serum resistance using five isogenic mucoid and non-mucoid *B. pseudomallei* pairs (4095, 10457A, 10971B, 11017A and MSHR295). Isolates were suspended in a starting inoculum of 1 x 10^6 CFU/ml (verified by plating and colony counts), and viability determined after incubation in 30% NHS at 37^{o}C for 2 h by plating and colony counts. The K96243 negative control remained viable in 30% NHS (inoculum 1.10 x 10^6 CFU/ml, post-experiment 1.04 x 10^6 CFU/ml), and the *E. coli* positive control was killed (inoculum 1.14 x 10^6, viability count 0 CFU/ml). Comparison between mucoid and non-mucoid colony pairs for strains 4095, 10971B, 11017A and MSHR295 indicated that the two colony types had comparable serum resistance between pairs (P> 0.05 for all pairs, t-test), and with the positive control. Unexpectedly, both mucoid and non-mucoid colonies of strain 10457 were susceptible to serum killing (mucoid: inoculum 1.52 x 10^6 CFU/ml/viability count 1.77 x 10^2 CFU/ml; non-mucoid: inoculum 1.03 x 106 CFU/ml/viability count 4.55 x 10^2 CFU/ml).

Association between mucoid colonies and LPS antigenic variation

We next considered the phenotypic basis for the difference in colony appearance between non-mucoid and mucoid colonies. LPS is a major surface-expressed determinant of *B. pseudomallei*, and we hypothesized that variation in LPS structure was responsible for the variable colony appearance. We tested all isolates from two retrospective Thai collections (200 isolates, no relapse and 166 isolates, relapse), Australian collections (n=52) and prospective isolates (n=40) (Table 3). SDS-PAGE and silver staining of LPS extracts of one non-mucoid and one mucoid colonies of each isolate demonstrated that *B. pseudomallei* colonies tested had 3 LPS types: ladder A, ladder B and rough LPS as described previously (8)[Anuntagool 2006] (table 3). The same type A and B ladder patterns, which reflects the complete structure of LPS consisting of OPS, core-oligosaccharide and lipid A were found in both mucoid and non-mucoid colonies. Rough LPS was found in only non-mucoid colonies (Table 3). Ten colonies per isolate (5 mucoid and 5 non-mucoid) were screened using the Mab-latex agglutination test. This Mab used in this assay is known to recognize *B. pseudomallei* OPS type A not type B or rough LPS by Western blot (15)[Anuntagool and Sirisinha 2002]. The latex agglutination result demonstrated that only non-mucoid colonies of all isolates with LPS type A were positive while the remainders were negative. The negative result in mucoid colonies of isolates with LPS type A suggests the presence of a modification of OPS which can be distinguished by the Mab. This result was verified using western blot, in which the Mab was observed to react with LPS extracted from non-mucoid colonies showing reactions on the type A ladder components, but did not react with LPS from mucoid colonies (Figure 4). We proposed that the difference in colony morphology variation was due to antigenic variation of LPS.

Molecular identification of an OPS molecule that reacts with 9D5 MAb

We investigated the nature of the OPS modification associated with variable colony morphology and latex agglutination reaction. *B. pseudomallei* OPS contains 2-*O*-methyl and 4-*O*-acetyl substitution and 2-*O*-acetyl modification (9-11)[Knirel et al., 1992; Perry et al., 1995; Brett et al., 2011]. We thus generated a range of allelic exchange mutants which contained unmarked deletions of *wbiA, wbiD* and *oacA* genes involved in the modifications and the LPS biosynthesis on *B. pseudomallei* K96243. *wbiA* is required for 2-*O* acetylation of LPS O-antigen, *wbiD* involves in OPS biosynthesis and *oacA* is required for 4-*O* acetylation and 2-*O* methylation of OPS (11, 22)[DeShazer MolecMicobiol 1998; Brett et al., 2011].

Analysis of PCR products showed the absence of a 236 bp *oriT* fragment of pEXKm5 backbone in all mutants. The mutants defective in *wbiA* (K96243Δ*wbiA*) indicated the desired mutant alleles of the product at 580 bp, while the wild type K96243 yielded products of 1,162 bp. The mutants defective in *wbiD* (K96243Δ*wbiD*) indicated the desired mutant alleles of the product at 465 bp, while the wild type K96243 yielded products of 1,607 bp. The mutants defective in *oacA* (K96243Δ*oacA*) indicated the desired mutant alleles of the product at 635 bp, while the wild type K96243 yielded products of 1,480 bp (Figure 5A).

SDS-PAGE and silver staining of proteinase K extracts revealed type A ladder pattern for K96243Δ*wbiA* and K96243Δ*oacA* which is the same as that of wild type, but the OPS ladder was absent for K96243Δ*wbiD* based on silver staining (Figure 5B). This suggests that the K96243Δ*wbiA* and K96243Δ*oacA* mutants but not K96243Δ*wbiD* have an OPS backbone. Utilizing the OPS specific Mab 9D5, Western blot analysis demonstrated that LPS of K96243Δ*oacA* and wild type reacted with Mab showing ladder components of LPS but no reaction was observed for K96243Δ*wbiA* and K96243Δ*wbiD* mutants (Figure 5C).

A complemented strain for the K96243Δ*wbiA* was constructed. The PCR product size using primers flanking deleted alleles demonstrated that the complemented *wbiA* mutant strains had the 1,162 bp PCR fragments. The result indicated that deletion alleles was replaced by wild type sequence, which was the same as observed with the parental strain (Figure 5A). Western blot analysis demonstrated that the complemented strain restored the expression of specific antigen as demonstrated by the ladder reaction with Mab (Figure 5C). Taken together, the result suggested that 1) LPS specific Mab, 9D5 reacted with OPS moiety of LPS, and 2) 2-*O*-acetyl modification which is regulated by *wbiA* of *B. pseudomallei* OPS, is required for the reaction with the Mab.

Comparison of the presence of *wbiA* gene and analysis of *wbiA* sequence between mucoid and non-mucoid colonies

Thirty-four *B. pseudomallei* cultures containing mucoid and non-mucoid colonies were randomly chosen from Thailand collections, and a colony of each type tested by PCR for the presence of *wbiA* encoding for the 2-*O* acetylation of OPS. All colonies were positive, indicating the presence of *wbiA*. Mutation in *wbiA* of mucoid (4095c) and non-mucoid (4095a) isogenic *B. pseudomallei* isolates was assessed based on full-length sequencing of

wbiA gene. Results indicated no mutation in mucoid isolates as all mucoid and non-mucoid pair isolates showed the identical sequences of *wbiA* gene.

Comparison of *wbiA* expression by mucoid and non-mucoid colonies

Given that the *wbiA* sequence analysis demonstrated 100% identity between mucoid and non-mucoid pair. We further investigated the functional expression of this gene. Using RT-PCR analysis to examine two *B. pseudomallei* isogenic pair isolates (4095a (NM), 4095c (M), MSHR295 (NM) and MSHR295 (M)), we observed the same levels of *wbiA* gene expression yielded a 300 bp product in mucoid versus non-mucoid isolates.The *wbiA* expression was abolished in K96243Δ*wbiA* mutant negative control. The 16S rRNA positive control samples all yielded a 336 bp product. No amplification was detected in the no-RT negative controls (data not shown). The result suggested that *wbiA* were able to function in mucoid isolates.

Construction of mutant defective in capsule and LPS purification

Having functional expression of *wbiA* gene which is responsible for the presence of 2-*O*-acetyl group of OPS, the mucoid colony may lack of additional determinant important for 9D5 Mab binding. To identify these molecules, we investigate the OPS structural difference between mucoid (4095c) and non-mucoid (4095a) isogenic isolates. To avoid the capsular polysaccharide contamination during LPS purification, *B. pseudomallei* mutants defective in *wcbB* was initially constructed on strains 4095a and 4095c. *wcbB* encoded for polysaccharide glycosyltransferase biosynthesis protein that is required for capsular synthesis (23)[Reckseidler-Zenteno et al 2005]. Using a pEXKm5-based allele replacement strategy, the PCR verification of mutants 4095aΔ*wcbB* and 4095c Δ*wcbB* demonstrated the presence of a 383 bp DNA fragment (Figure 5A) and the absence of a 236 bp *oriT* fragment indicated the desired mutant allele, while the wild type had a *wcbB* product of 1,009 bp. SDS-PAGE and silver staining of proteinase K extracts revealed ladder pattern for 4095aΔ*wcbB* and 4095cΔ*wcbB* similar to wild types suggesting that the mutagenesis for capsule did not alter the OPS synthesis (Figure 5B). Western blot analysis were used to also examined for the reaction with LPS specific Mab, 9D5 and a capsule specific Mab, 4B11. The results demonstrated that the capsule antigen at 200 KD were absent in these two capsule mutants (Figure 5D). Western blot probed with LPS Mab 9D5 showed reaction with 4095a wild type and 4095aΔ*wcbB* capsule mutant but no reaction appeared with 4095c wild type and 4095aΔ*wcbB* mutant (Figure 5C). The deletion Δ*wcbB* did not affect the LPS synthesis; we

therefore used these 4095aΔ*wcbB* and 4095cΔ*wcbB* to prepare purified LPS for NMR chemical structure characterization.

NMR analysis of OPS structure

To further investigate the structural characteristics of the OPS antigen expressed by *B. pseudomallei* 4095aΔ*wcbB* and 4095cΔ*wcbB*, OPS was isolated from LPS by mild acid hydrolysis and gel-permeation chromatography. A combination of 1-D and 2-D ^{1}H and ^{13}C NMR spectroscopy indicated that 4095a (non-mucoid) and 4095c (non-mucoid) OPS antigens exhibited 2-*O*-methyl and 4-*O*-acetyl substitution patterns that differed from one other, and from the predominant OPS serotype expressed by *B. pseudomallei* 1026b (Figure 6 and Table 4). Specifically, the 6dTal residues of *B. pseudomallei* 4095a lacked 4-*O*-acetyl modification (except at the non-reducing end residue) while the 6dTal residues of *B. pseudomallei* 4095c lacked both 4-*O*-acetyl (except at the non-reducing end residue) and 2-*O*-methyl modification. These data indicate that the epitope recognized by the 9D5 mAb requires 2-*O*-acetyl and 2-*O*-methyl substitution of 6dTal residues.

TLR4-dependent NF-κB activation by different OPS in HEK cells

B. pseudomallei LPS can activate immune cells via the TLR4 dependent pathway in the presence of MD2 and CD14 (20)[West et al.BMC 2011]. We investigated whether the LPS modification associated with mucoid colonies was associated with altered TLR4-dependent NF-κB activation. We compared NF-κB activation induced by *B. pseudomallei* LPS purified from capsule mutants of *B. pseudomallei* 4095aΔ*wcbB* and 4095cΔ*wcbB*, and LPS from *E.coli* strain K12 stimulations of transfected HEK 293 cells. LPS used at a concentration of 10 ng/ml extracted from 4095cΔ*wcbB* mucoid colonies led to a significantly lower NF-κB activation at 6 and 18 h compared with 4095aΔ*wcbB* non-mucoid colonies (P = 0.001 and ?, t-test), and the effect is dependent on stimulation time and LPS concentration (Figure 7). However, the NF-κB activation at 18 h showed no difference between two *B. pseudomallei* LPS at 100 ng/LPS (P = 0.380; t-test) (Figure 7). These data suggest that the LPS modification leads to altered innate immune activation through TLR4 signalling pathway.

DISCUSSION

The predominant type of *B. pseudomallei* LPS is a smooth type A which also found in *B. thailandensis* and *B. mallei* [Anuntagool et. al., 2006]. Our 9D5 Mab has ability to react with LPS type A of *B. pseudomallei* and *B. thailandensis* but did not react with LPS of *B. mallei* [Anantagool and Sirisinha, 2002]. LPS mutant defective in O-PS synthesis but contained lipid A and core was unable to react with 9D5 Mab [Wikraiphat et. al., 2009]. This demonstrates that the epitope for 9D5 Mab is exhibit on O-PS residue. O-PS structure of *B. pseudomallei* is identical to those of *B. thailandensis* but differ from O-PS of *B. mallei* by the presence of 4-*O*-methylation on the L-6dTal*p* residues [Brett et. al., 2003]. We have demonstrated a novel O-PS expressing by *B. pseudomallei* that does not react with 9D5 Mab. A comparison of O-PS structure of 9D5 reactive strains and 9D5 non-reactive strains by NMR suggested that 9D5 non-reactive strains loss of 2-*O*-methyl group at *O*-2 position of the L-6dTal*p* residues. The *wbiA* gene is required for 2-*O*-acetylation on *B. pseudomallei* LPS [Brett et. al., 2003]. The *wbiA* mutant defective in 2-*O*-acetylation showed LPS smooth type A but lose of 9D5 reaction. It is now obviously seen that 9D5 MAb reacts only with polymers of 3)-β-D-glucopyranose-(1 → 3)-6-deoxy-α-L-talopyranose-(1 in which the L-6dTal*p* residues are coordinately acetylated and methylated at *O*-2 position. The 9D5 MAb need to bind with conformational structure of *O*-methyl and *O*-acetyl groups on O-PS antigen. The immunological reaction with 9D5 MAb was restored in *wbiA* complement strain. However, a comparison of *wbiA* gene between 9D5 reactive strain (4095a) and 9D5 non-reactive strain (4095c) showed identical sequences and there are no different in *wbiA* gene expression between these 2 strains. We demonstrated that *wbiD* mutant shown rough LPS suggesting that *wbiD* gene encoding for O-antigen methyltransferase was essential for biosynthesis of O-antigen in *B. pseudomallei.* This result indicated that there are other genes involved in regulation of O-PS modification.

In the present study, we demonstrated that some strains of *B. pseudomallei* undergoes a conversion from a mucoid to a non-mucoid colony that correlates with increased NF-kB activation in HEK cell transfected with human TLR4 and co-receptors MD2 and CD14, increased IL-1β production and increased internalization in primary human monocytes. LPS is recognized by immune cells through TLR4. *B. pseudomallei* activate immune cells through TLR2 and TLR4 [Wiersinga et. al., 2007]. West and colleagues demonstrated that, in the presence of co-receptor MD2, TLR4-dependent signaling is essential for response to *B. pseudomallei* LPS and lipid A [West et. al., 2008]. Lipid A is part of LPS that activate TLR4

ligand. Modification on O-PS not on lipid A of *B. pseudomallei* LPS alter NF-kB activation in TLR4/MD2/CD14 transfected HEK cells. There may be conformational alteration of LPS after O-PS modification in mucoid colony that increases TLR4 recognition. LPS mutant of *B. pseudomallei* that lack of O-PS moieties was more susceptible to macrophage killing [Arjcharoen et. al., 2007; Wikraiphat et. al., 2009] but resistance to Histatin Dhvar5 and lactoferrin [Wikraiphat et. al., 2009].

O-PS of *B. pseudomallei* LPS is required for serum resistance and virulence [DeShazer et. al., 1998]. *B. pseudomallei* defective in O-PS production showed rough type of LPS and was susceptible to killing by 30% NHS [DeShazer et. al., 1998]. We found de-acetylation or de-methylation on O-PS moieties did not affect serum susceptibility. O-PS of 9D5 non-reactive strains was still retained in LPS structure. DeShazer proposed the mechanism of OPS in serum resistance is that by directly prevented the integration of C5b-C9 complement membrane attack complex (MAC) into the bacterial outer membrane [DeShazer et. al., 1998]. The length of O-PS with O-PS alteration is long enough to prevent access of MAC to outer membrane so modification on O-PS structure of *B. pseudomallei* is not relevant to serum resistance.

De-acetylation or de-methylation on O-PS moieties of *B. pseudomallei* increased internalization and IL-1β production into human macrophages. *B. pseudomallei* LPS exhibited weaker pyrogenic activity than enterobacterial LPS [Matsuura et. al., 1996]. The LPS impart a strongly negative charge to surface of gram negative bacteria. Alteration on O-PS structure might affect surface charge of bacteria that increase phagocytosis of *B. pseudomallei* to the human monocytes. This increased IL-1β production because IL-1β production is correlated with the number of intracellular bacteria. These results are consistence with the previous report that substitution of O antigen of *E. coli* and *S. enteric* is correlated with pathogenesis by increased activation of alternative complement pathway, leading to increase phagocytosis [Reeves, 1995].

This study showed correlation between colony morphology of *B. pseudomallei* and 9D5 Mab reaction. Alteration on O-PS modification of *B. pseudomallei* avoids 9D5 Mab recognition. O-antigen modification might be a mechanism of bacteria to adapt in particular niches. Escape from antibody recognition of *B. pseudomallei* can block the initiation of alternative pathway of complement activation. This mechanism increase survival of bacteria in the host during infection. Change in colony morphologies is associated with survival adaptation in

stress conditions and particularly environment. Mucoid colonies which lack of 2-*O*-methyl group on O-PS are not stable and try to change to the common non-mucoid colonies. This is the reason why mucoid colonies are observed as a minor population of *B. pseudomallei*. However, there are many gene clusters involved in LPS biosynthesis of *B. pseudomallei*. The further study needs to identify gene involved in regulation and mechanism of LPS modification.

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Table 1 Bacterial strains used for characterization of LPS and mutagenesis

Strains	Relevant characteristics	Date of isolation	Specimens	Colony morphology	9D5 reaction	Source or reference
B. pseudomallei						
4095a (NM)	*Clinical strain from a Thai patient*	19/09/2006	Sputum	Non-mucoid	Positive	Freezer vial
4095c (M)		11/10/2006	Pleural fluid	Mucoid	Negative	Freezer vial
10457A (NM)	*Clinical strain from a Thai patient*	06/07/2011	Sputum	Non-mucoid	Positive	Primary culture
10457A (M)				Mucoid	Negative	Primary culture
10971B (NM)	*Clinical strain from a Thai patient*	10/09/2012	Tracheal suction	Non-mucoid	Positive	Primary culture
10971B (M)				Mucoid	Negative	Primary culture
11017A (NM)	*Clinical strain from a Thai patient*	28/09/2012	Sputum	Non-mucoid	Positive	Primary culture
11017A (M)				Mucoid	Negative	Primary culture
MSHR295 (NM)	*Soil in Australia*	2002	Soil	Non-mucoid	Positive	Freezer vial [Anuntagool et. al.,
MSHR295 (M)				Mucoid	Negative	2006] (8)
K96243	*Wild type*	1996	-	Non-mucoid	Positive	Holden et. al., 2004 (24)
K96243ΔwbiA	*K96243 derivative: ΔwbiA (Mutant defective in acetylation of OPS)*	N/A	-	Non-mucoid	Negative	This study
K96243ΔwbiA complement	*K96243 derivative: ΔwbiA*	N/A	-	Non-mucoid	Positive	This study

	complement (wbiA complement)					
K96243ΔwbiD	*K96243 derivative: ΔwbiD (Mutant defective in OPS synthesis)*	N/A	-	Non-mucoid	Negative	This study
K96243ΔoacA	*K96243 derivative: ΔoacA (Mutant defective in acetylation of OPS)*	N/A	-	Nonmucoid	Positive	This study
4095a (NM)*ΔwcbB*	*4095a (NM) derivative: ΔwcbB (Mutant defective in CPS)*	N/A	-	Mucoid	Positive	This study
4095c (M)*ΔwcbB*	*4095c (M) derivative: ΔwcbB (Mutant defective in CPS synthesis)*	N/A	-	Mucoid	Negative	This study
E. coli						
HB101	ATCC® 33694™	N/A	-	N/A	N/A	ATCC
DH5α	F' competent cells	N/A	-	N/A	N/A	Liss, 1987 (25)
RHO3	Km^s; SM10 (λ*pir*)Δ*asd*::*FRT* Δ*aphA*::*FRT*	N/A	-	N/A	N/A	Lopez et. al., 2009 (16)

Table 2 Primer pairs used in this study

Primers	Sequence (5'-3')	Position[§]	Product size (bp)
wbiA(BPSL2680)			
wbiA-F1	CGATTCGATGCCGCCGACGT	34-53	297
wbiA-R1	CAACGCATCCGCTCGCATGC	311-330	
wbiA-F2	GCATGCGAGCGGATGCGTTGTGCTGCTTGGCTATCCGGCG	911-930	305
wbiA-R2	TCCATCCTTGTCCGGGCCCC	1176-1190	
wbiA-F1	CGATTCGATGCCGCCGACGT	34-53	1162
wbiA-R2	TCCATCCTTGTCCGGGCCCC	1176-1190	
wbiA-Fex	GGAATCTGCGTCTCCGGCTT	256-275	300
wbiA-Rex	ATAGGGTGTCGTGTCTCGCAG	535-555	
wbiA-seqA	AATGGCTGCACGATGCGGTGT	(-104)-(-124)	814
wbiA-seqB	CACGGCAAGCAACACCCTGC	671-690	
wbiA-seqC	TGCTGCGAGACACGACACCC	533-552	783
wbiA-seqD	GCGCAGCCGATAAAGCCAGC	(+57)-(+76)	
wbiD (BPSL2677)			
wbiD-F1	CGGTGTACAGCAATGTCGTT	41-60	206
wbiD-R1	CAGACGGTGCAGGTCGATTC	227-246	
wbiD-F2	GAATCGACCTGCACCGTCTGTCTACCGAAAGTGGCGTTCC	1409-1428	259
wbiD-R2	CGGATGCCTGACAAAGAACC	1628-1647	
wbiD-F1	CGGTGTACAGCAATGTCGTT	41-60	1607
wbiD-R2	CGGATGCCTGACAAAGAACC	1628-1647	
oacA (BPSL1936)			
oacA-F1	AAAGGCCGACGCATTCCGGG	(-162)-(-144)	311
oacA-R1	AATGCCCGCTGGAGCGTGTC	130-149	
oacA-F2	GACACGCTCCAGCGGGCATTCCCCAAGCACCATTGGCGGT	995-1014	344
oacA-R2	ATGTGGGCATGGGGAAGCGC	(+114)-(+133)	
oacA-F1	AAAGGCCGACGCATTCCGGG	(-162)-(-144)	1480
oacA-R2	ATGTGGGCATGGGGAAGCGC	(+114)-(+133)	
wcbB (BPSL2808)			
wcbB-F1	CCGGCACTATGGCAGCCGAG	96-115	218
wcbB-R1	CCATTCCGCTGTGGCTTGTATGC	291-313	
wcbB -F2	GCATACAAGCCACAGCGGAATGGAGCGACCTGGACGTGTTCCG	940-959	188
wcbB -R2	CCACGTCGGTTCGCGGAAGT	1085-1104	
wcbB -F1	CCGGCACTATGGCAGCCGAG	96-115	1009
wcbB -R2	CCACGTCGGTTCGCGGAAGT	1085-1104	
16S			
Univ_*16S*_F	TGGCTCAGAACGAACGCTGGCGGC	21-44	336
Univ_*16S*_R	CCCACTGCTGCCTCCCGTAGGAGGAGT	327-356	
oriT-F	TCCGCTGCATAACCCTGCTTC	598-578	236
oriT-R	CAGCCTCGCAGAGCAGGATTC	368-383	

[§] Positions corresponding to the nucleotide sequence of *wbiA, wbiD, oacA and wcbB genes of B. pseudomallei* K96243 chromosome 1 (NCBI Reference Sequences , accession number 3094050, 3093124, 3091646 and 3093047 *for wbiA, wbiD, oacA*and *wcbB*respectively)

Position (-xxx) = gene at 5'UTR and (+xxx) = gene at 3'UTR of the genes

Table 3 Colonies of *B. pseudomallei* on agar plates and LPS antigenic types

***B. pseudomallei* and source**	**Total isolates**	**Observed colonies on plates (No. of isolates), %**	**Colony types (No.)**	**LPS type and reaction with Mab**					
				A		**B**		**Rough**	
				Positive	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**
Set 1: Clinical strains from Thai patients No relapse (200 patients)	200	Single (191) 95.5%	Mucoid (5)	0	5	0	0	0	0
			Non-mucoid (186)	183	0	0	2	0	1
		Mixed (9) 4.5%	Mucoid (9)	0	9	0	0	0	0
			Non-mucoid (9)	9	0	0	0	0	0
Set 2: Clinical strains from Thai patients Relapse (78 patients)	166	Single (153) 92.2%	Mucoid (5)	0	5	0	0	0	0
			Non-mucoid (148)	142	0	0	4	0	2
		Mixed (13) 7.8%	Mucoid (15)	0	13	0	0	0	0
			Non-mucoid (15)	13	0	0	0	0	0
Set 3: Clinical and environmental strains from Australia (38 patients, 10 animals, 4 environments)	52	Single (40) 76.9%	Mucoid (0)	0	0	0	0	0	0
			Non-mucoid (40)	33	0	0	4	0	3
		Mixed (12) 23.1%	Mucoid (12)	0	9	0	3	0	0
			Non-mucoid (12)	9	0	0	3	0	0
Set 4: Primary isolates from Thai patients (40 patients)	40	Single (32) 80.0%	Mucoid (0)	0	0	0	0	0	0
			Non-mucoid (32)	32	0	0	0	0	0
		Mixed (8) 20.0%	Mucoid (8)	0	8	0	0	0	0
			Non-mucoid (8)	8	0	0	0	0	0

Table 4 Intensity comparisons of the 6dTal anomeric signals in the HSQC spectra of *B. pseudomallei* OPS as a percent of the sum of all 6dTal anomerics

Residue	4095a OPS	4095c OPS	1026b
→3)-2-*O*-Me-4-*O*-Ac-α-L-6dTal*p*-(1→	ND	ND	14.7
→3)-α-L-6dTal*p*-(1→	13.1	18.5	8.8
→3)-2-*O*-Ac-α-L-6dTal*p*-(1→	53.4	70.4	43.0
→3)-2,4-di-*O*-Ac-α-L-6dTal*p*-(1→	ND	ND	5.4
3-*O*-Me-2,4-di-*O*-Ac-α-L-6dTal*p*-(1→	8.2	8.7	11.3
→3)-2-*O*-Me-α-L-6dTal*p*-(1→	23.6[a]	ND	14.6[a]
→3)-2-*O*-Ac-α-L-6dTal*p*-(1→	1.7[b]	2.4[b]	2.2[b]
Total	100	100	100

ND - not detected; [a]residues are 1-3-linked to Glc; [b]residues are 1-3-linked to GlcNAc.

Figure legends

Figure 1 Comparison of the appearance of non-mucoid colonies of *B. pseudomallei* and the mucoid colony variant on blood agar (A, B and C) and TSA (D, E and F). Non-mucoid colonies (A and D), mucoid colonies (B and E) and mixed colony morphologies (C and F). Colony morphology was observed after spread-plating on blood agar (A, B and C) and TSA (D, E and F) for 4 days at 37^{o}C in air.

Figure 2 Effect of 10 laboratory conditions on colony morphotype switching between non-mucoid and mucoid colonies of 5 paired *B. pseudomallei* isolates. (A) Colony switching starting from non-mucoid to mucoid types. (B) Colony switching starting from mucoid to non-mucoid types. Two hundred microliters of 1 x 10^{8} CFU/ml suspension were inoculated into 2 ml DW or TSB and incubated with one of these following conditions: (i) DW at 37^{o}C for 24h, (ii) TSB pH 7.4 at 37^{o}C for 24h, (iii) TSB pH 4.0 at 37^{o}C for 24h, (iv) TSB pH 8.5 at 37^{o}C for 24h, (v) TSB pH 7.4 at 42^{o}C for 24h, (vi) TSB pH 7.4 + 350 mMNaCl at 37^{o}C for 24h, (vii) TSB pH 7.4 + 50 mM $NaNO_2$ at 37^{o}C for 24h, (viii) TSB pH 7.4 + 2 mM H_2O_2 at 37^{o}C for 24h, (ix) TSB pH 7.4 at 37^{o}C for 24h in anaerobic jar (Oxoid, UK) and (x) TSB pH 7.4 at 37^{o}C for 7 days. Morphotype switching was presented as the proportion (%) of alternative types in relation to the total colonies present. Error bars are standard deviation.

Figure 3 Non-mucoid isolates were more resistance to internalization into primary human monocytes than mucoid paired isolates. THP1 cells at 3x10^{5}cells were incubated for 2h with *B. pseudomallei* at a MOI 1:1, after which non-adherent bacteria were killed by incubating 2 h with kanamycin. The bacteria count was enumerated at 4 h by cell lysis and plating onto TSA. The data represent mean values CFU /ml ± standard deviations of each isogenic pair from 5 *B. pseudomallei* isolates and is expressed as the percent internalisation at 4 h compared with the number of inoculum.

Figure 4 (A) Mutants of *wbiA, wbiD, oacA* and *wcbB* genes exhibited PCR products of corresponding deletion alleles. M represents 100 bp ladder marker. Three LPS mutants were constructed on K96243 wild *type* (WT), Δ*wbiA;* mutant defective in *wbiA (BPSL2680),* Δ*wbiD;*

mutant defective in *wbiD (BPSL2677)* and Δ*oacA;* mutant defective in *(BPSL1936).* Δ*wbiA* complement is a complemented strain of *wbiA* mutant. Two capsule mutants were constructed on 4095a(NM) WT and 4095c(M)WT. Δ*wcbB;* mutant defective in Δ*wcbB (BPSL2808).*PCR products size using specific primers for each gene are shown.
(B) SDS-PAGE and silver stain of LPS extracts of *wbiA, oacA* and *wcbB* mutants but not *wbiD* mutant exhibited identical LPS ladder pattern to wild types. M represents protein standard marker.
(C) Western blot of LPS extracts of *wbiA, wbiD, oacA* and *wcbB* mutants probed with OPS specific Mab, 9D5 demonstrated *B. pseudomallei* mutant without OPS (Δ*wbiD*) and 2-O-acetylation moiety (Δ*wbiA)* and isolates of 4095c did not react with Mab.
(D) Western blot of LPS extracts of *wbiA, wbiD, oacA* and *wcbB* mutants probed with capsule specific Mab, 4B11 demonstrated that only *wcbB* capsule mutants (Δ*wcbB)* did not react with capsule specific Mab, 4B11.

Figure 5 Mucoid colony variants are not recognised by OPS specific monoclonal antibody. (A) SDS-PAGE and silver stain of paired LPS extracts of two colony types of *B. pseudomallei* from 4 representative paired isolates (4095, MSHR, 10457A and 10971B). (B) Western blot of LPS extracts of the same paired isolates probed with 9D5 Mab. M represents protein standard marker.

Figure 6 Chemical structure of O-polysaccharide expressed by non-mucoid and mucoid *B. pseudomallei* strain 4095a (NM) and 4095c (M), and a previous described OPS structure of strain1026b.

Figure 7 LPS from non-mucoid colonies reduced LPS-induced NF-κB activation through TLR4 receptor. HEK 293, 5 x 10^4 cells were transfected with 1 ng of human TLR4, 1 ng of MD2, 1 ng of CD14, 10 ng of pNiFty-Luc, 1 ng of pRL-TK,. The cells were stimulated with 0, 1, 10 and 100 ng/ml LPS in triplicate for 6 or 18 h before harvesting. *E. coli* K12 LPS was used as a control. NF-κB activity was quantified by dual luciferase assay. The data represent mean values RLU ± standard deviations of one of three experiments in triplicate.

Figure1

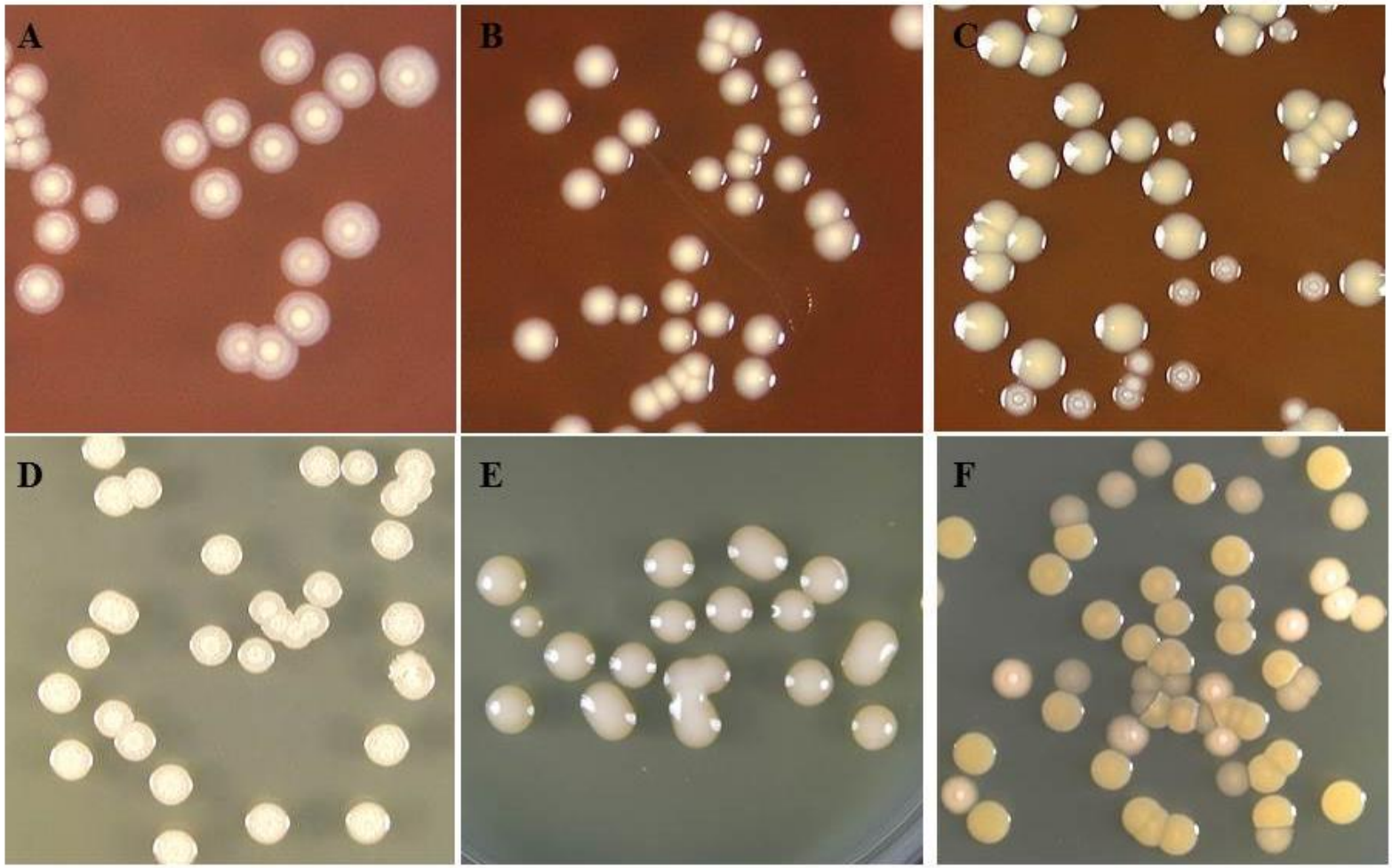


Figure2

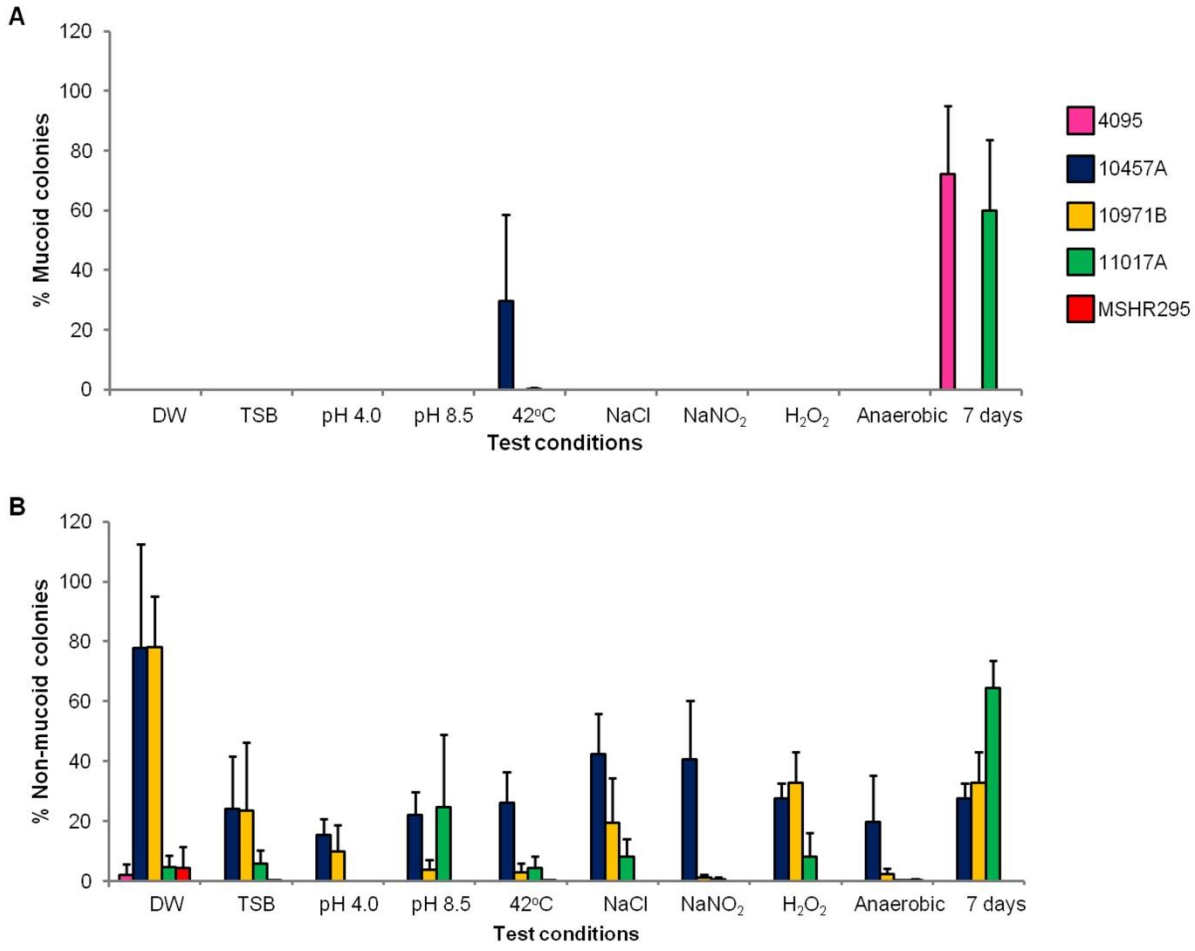

A
% Mucoid colonies
120
100
80
60
40
20
0
DW
TSB
pH 4.0
pH 8.5
42°C
NaCl
NaNO2
H2O2
Anaerobic
7 days
Test conditions
4095
10457A
10971B
11017A
MSHR295
B
% Non-mucoid colonies
120
100
80
60
40
20
0
DW
TSB
pH 4.0
pH 8.5
42°C
NaCl
NaNO2
H2O2
Anaerobic
7 days
Test conditions

Figure 3

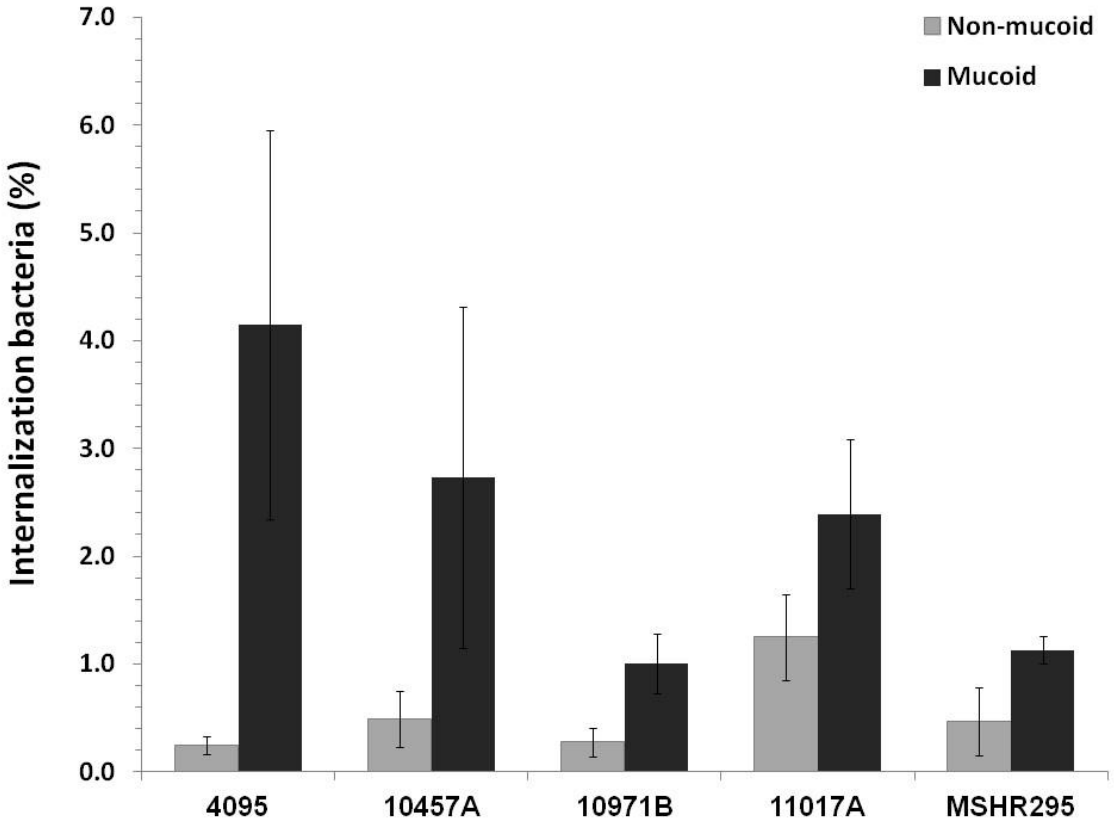
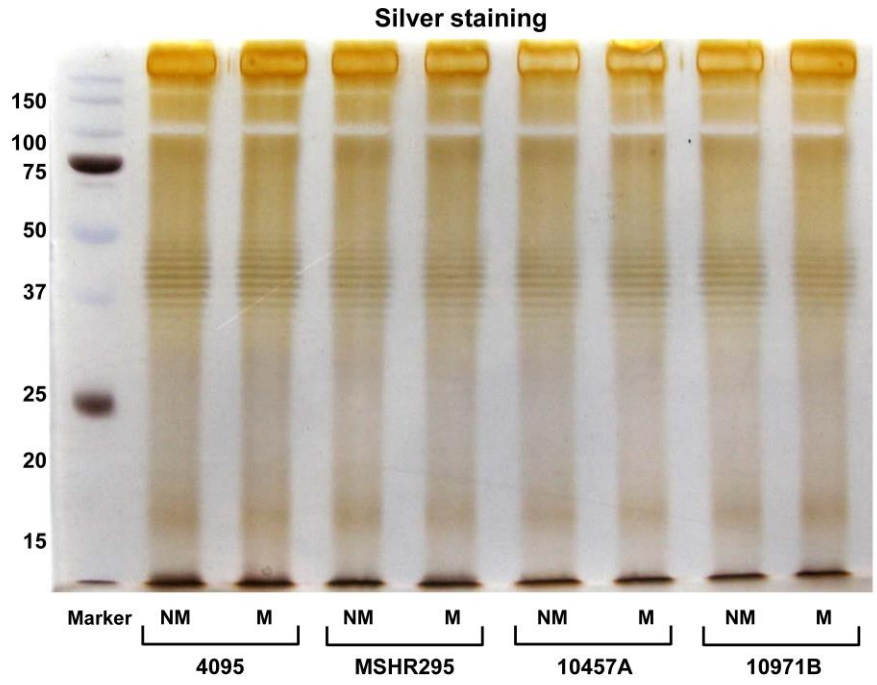

Non-mucoid
Mucoid
Internalization bacteria (%)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
4095
10457A
10971B
11017A
MSHR295

Figure 4

A


Silver staining
150
100
75
50
37
25
20
15
Marker
NM
M
NM
M
NM
M
NM
M
4095
MSHR295
10457A
10971B

B

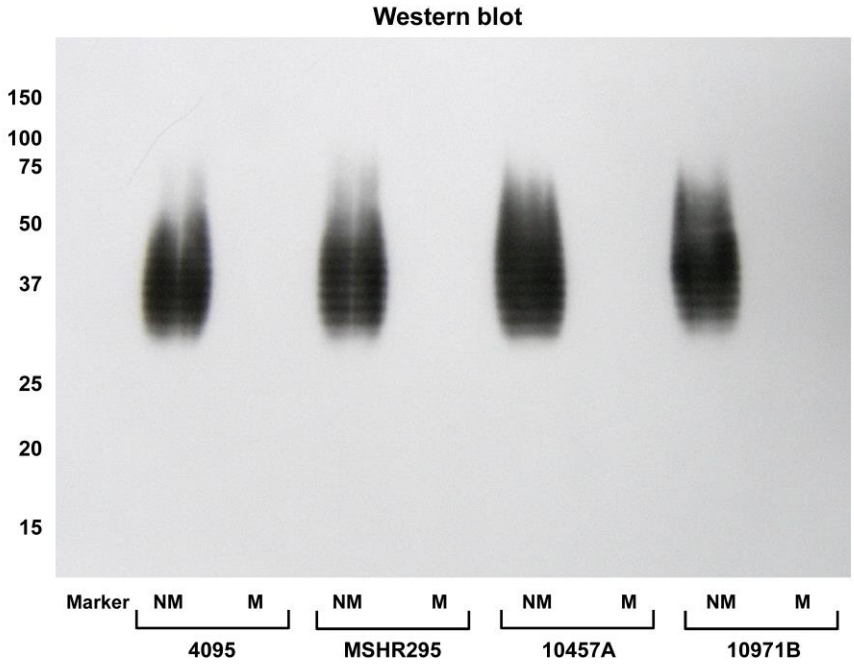

Western blot
150
100
75
50
37
25
20
15
Marker
NM
M
NM
M
NM
M
NM
M
4095
MSHR295
10457A
10971B

Figure 5

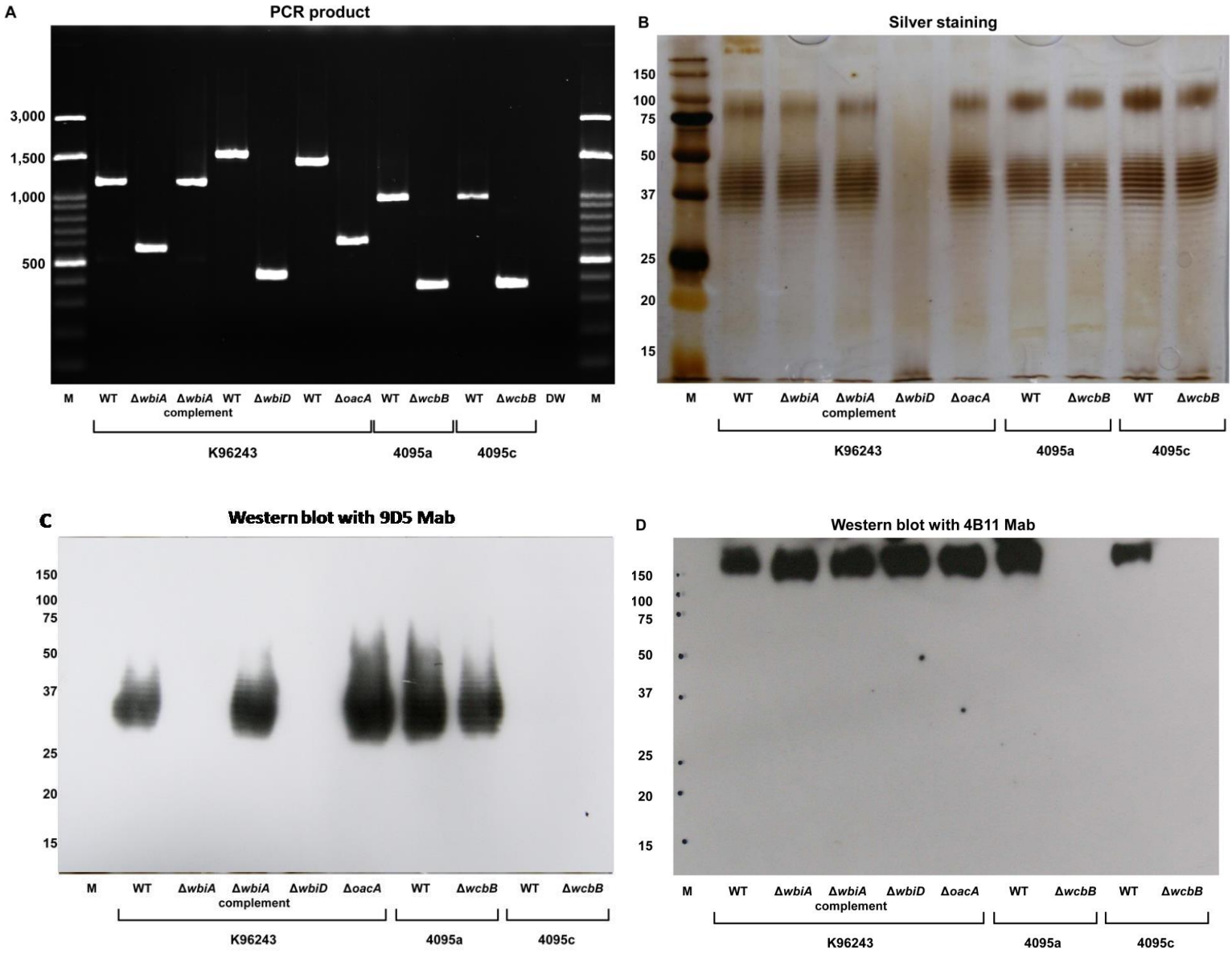

A
PCR product
3,000
1,500
1,000
500
M WT ΔwbiA ΔwbiA complement WT ΔwbiD WT ΔoacA WT ΔwcbB WT ΔwcbB DW M
K96243
4095a
4095c
B
Silver staining
150
100
75
50
37
25
20
15
M WT ΔwbiA ΔwbiA complement ΔwbiD ΔoacA WT ΔwcbB WT ΔwcbB
K96243
4095a
4095c
C
Western blot with 9D5 Mab
150
100
75
50
37
25
20
15
M WT ΔwbiA ΔwbiA complement ΔwbiD ΔoacA WT ΔwcbB WT ΔwcbB
K96243
4095a
4095c
D
Western blot with 4B11 Mab
150
100
75
50
37
25
20
15
M WT ΔwbiA ΔwbiA complement ΔwbiD ΔoacA WT ΔwcbB WT ΔwcbB
K96243
4095a
4095c

Figure 6

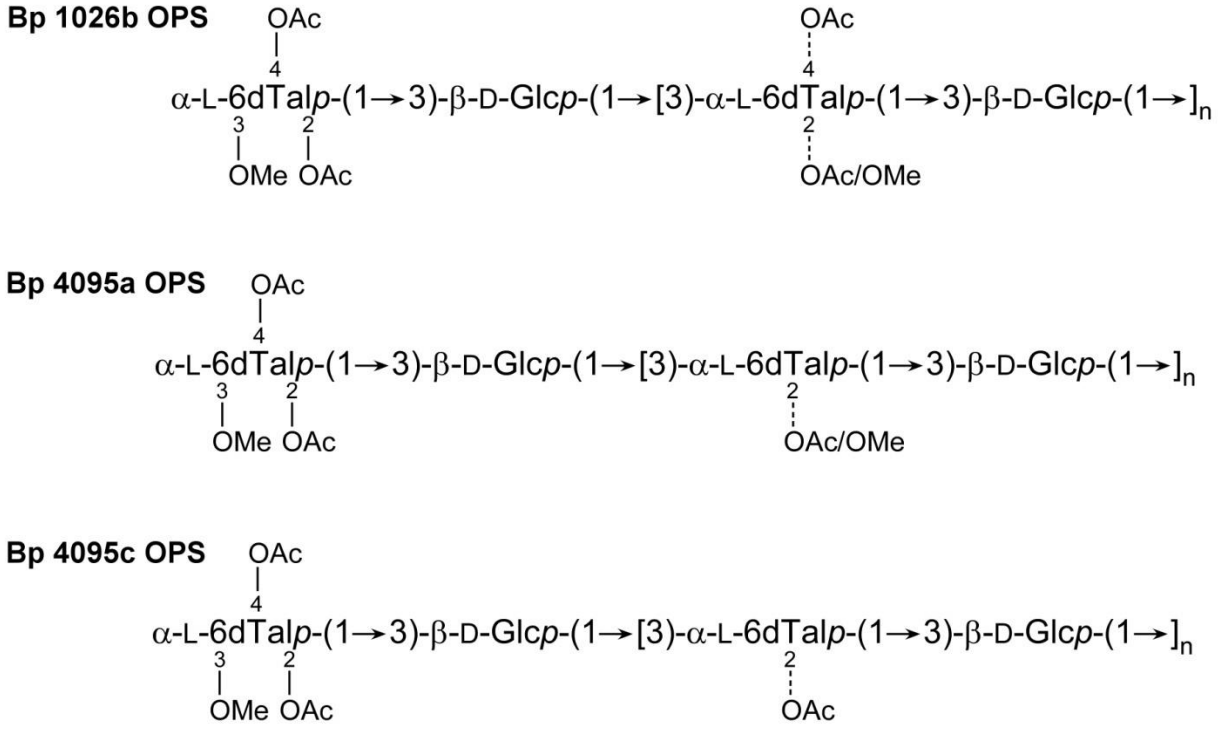
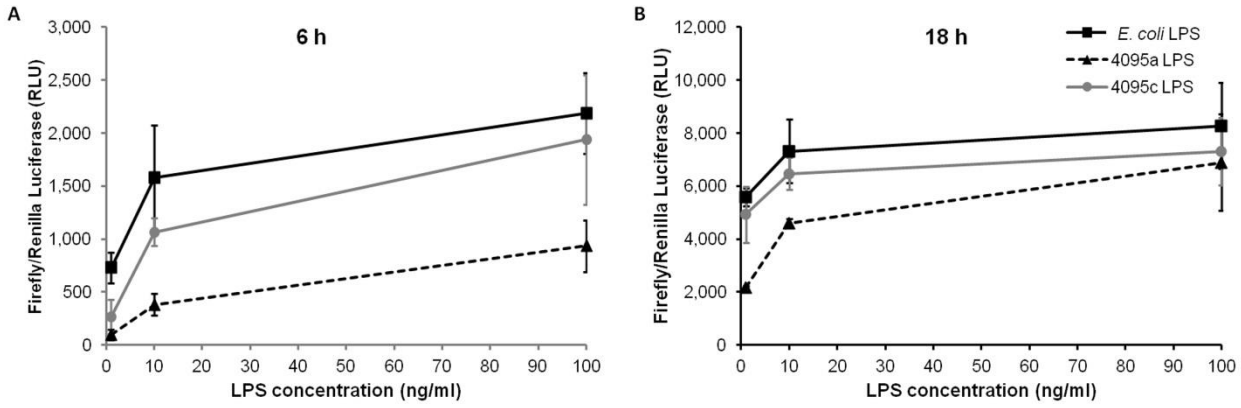

Bp 1026b OPS
OAc
4
α-L-6dTalp-(1→3)-β-D-Glcp-(1→[3)-α-L-6dTalp-(1→3)-β-D-Glcp-(1→]n
3
2
OMe OAc
OAc
4
2
OAc/OMe
Bp 4095a OPS
OAc
4
α-L-6dTalp-(1→3)-β-D-Glcp-(1→[3)-α-L-6dTalp-(1→3)-β-D-Glcp-(1→]n
3
2
OMe OAc
2
OAc/OMe
Bp 4095c OPS
OAc
4
α-L-6dTalp-(1→3)-β-D-Glcp-(1→[3)-α-L-6dTalp-(1→3)-β-D-Glcp-(1→]n
3
2
OMe OAc
2
OAc

Figure 7


A
6 h
Firefly/Renilla Luciferase (RLU)
3,000
2,500
2,000
1,500
1,000
500
0
0 10 20 30 40 50 60 70 80 90 100
LPS concentration (ng/ml)
B
18 h
E. coli LPS
4095a LPS
4095c LPS
Firefly/Renilla Luciferase (RLU)
12,000
10,000
8,000
6,000
4,000
2,000
0
0 10 20 30 40 50 60 70 80 90 100
LPS concentration (ng/ml)