Isolation and Characterization of a Small Cryptic Plasmid from a Clinical Isolate of *Burkholderia pseudomallei*

การแยกและการศึกษาคุณลักษณะของพลาสมิดขนาดเล็กจากเชื้อ *Burkholderia pseudomallei* ที่แยกได้จากผู้ป่วย

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ABSTRACT

Melioidosis, an infectious disease caused by *Burkholderia pseudomallei*, is a fulminating and fatal disease of humans and animals mainly in South-East Asia and northern Australia. Plasmid of *B. pseudomallei* has not been characterized. The standard alkaline lysis method was unable to isolate native plasmid unless the concentration of sodium dodecyl sulfate (SDS) in lysis solution was increased to 7%. The modified alkaline lysis method was used to screen one thousand and fifty-four *B. pseudomallei* clinical isolates from 17 provincial hospitals in northeast of Thailand for native plasmids and found that that 2.2 percent of *B. pseudomallei* carry small plasmid. The isolated plasmids could be assigned into two groups based on their size and the restriction profiles. A plasmid in group 2 from *B. pseudomallei* PHB194, designated pPHB194, was sequenced and characterized. The plasmid is 13,482 bp in length with a G+C content of 61% and shows a distinctive nucleotide sequence without homology to other known plasmids. The nucleotide sequence analysis predicts fifteen putative open reading frames (ORFs). Two functional regions have been proposed for the pPHB194 plasmid. The ORF-11, ORF-12, and ORF-14 are responsible for putative conjugative transfer, ORF-10 for plasmid replication and ORF-8, and ORF-9 for plasmid maintenance. The estimated copy number of pPHB194 in *B. pseudomallei* PHB194 was 11 copies per chromosome. This information will be useful for construction of a shuttle vector.

บทคัดย่อ

โรคเมลิออยโดสิสเป็นโรคติดเชื้อแบคทีเรียที่มีสาเหตุมาจากเชื้อ *Burkholderia pseudomallei* ซึ่งสามารถก่อโรคที่มีความรุนแรงถึงแก่ชีวิตได้ในมนุษย์ โรคนี้พบได้ส่วนใหญ่ในแถบเอเชียตะวันออกเฉียงใต้และทางตอนเหนือของประเทศออสเตรเลีย ปัจจุบันยังไม่พบการศึกษาถึงพลาสมิดของเชื้อ *B. pseudomallei* ในเชิงลึก การศึกษาเบื้องต้นพบว่าวิธี alkaline lysis ซึ่งเป็นวิธีมาตรฐานไม่สามารถจะใช้ในการสกัด

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พลาสมิด จากเชื้อ *B. pseudomallei* ได้ เว้นแต่ได้รับการปรับปรุงโดยการเพิ่มความเข้มข้นของ sodium dodecyl sulfate เป็น 7 เปอร์เซ็นต์ เมื่อนำวิธีสกัดพลาสมิดที่ปรับปรุงใหม่นี้ไปใช้ในการตรวจหาพลาสมิดจากเชื้อ *B. pseudomallei* จำนวน 1,054 ตัวอย่าง ที่แยกได้จากผู้ป่วยจากโรงพยาบาลประจำจังหวัด 17 แห่ง ในภาคตะวันออกเฉียงเหนือของประเทศไทย พบเชื้อที่มีพลาสมิดขนาดเล็กจำนวน 23 ตัวอย่าง คิดเป็น 2.2 เปอร์เซ็นต์ โดยสามารถแบ่งกลุ่มพลาสมิดที่พบออกได้เป็น 2 กลุ่ม ตามขนาดของพลาสมิดและรูปแบบการย่อยด้วยเอนไซม์ตัดจำเพาะ จากการศึกษาคุณลักษณะโดยการตรวจหาลำดับเบสของพลาสมิด pPHB194 พบว่ามีขนาด 13,482 คู่เบส โดยมี G + C ทั้งหมด 61 เปอร์เซ็นต์ พบเป็นพลาสมิดที่ยังไม่มีรายงานในฐานข้อมูล โดยประกอบด้วย 15 putative open reading frames (OFR) ซึ่งบริเวณที่มีหน้าที่เกี่ยวข้องกับการแบ่งตัวของพลาสมิดคือ ORF-10 เกี่ยวข้องกับความเสถียรคือ ORF-8 และ ORF-9 และส่วนที่เกี่ยวข้องกับการส่งผ่านพลาสมิดคือ ORF-11, ORF-12 และ ORF-14 โดยพบว่าพลาสมิด pPHB194 มีจำนวนพลาสมิด 11 copies ต่อหนึ่งโครโมโซมของเซลล์แบคทีเรีย ข้อมูลดังกล่าวมีประโยชน์ในการนำไปใช้ในการผลิต shuttle vector

Key Words: *Melioidosis, B. pseudomallei, Plasmid, Alkaline lysis method.*
คำสำคัญ: โรคเมลิออยโดสิส เชื้อ *B. pseudomallei* พลาสมิด วิธี Alkaline lysis

1. Introduction

Melioidosis, an infectious disease caused by *Burkholderia pseudomallei* usually found in soil and water, is of public health importance in endemic areas, particularly in northeast Thailand and northern Australia. It exists in acute and chronic forms. The fatality rate in acute septicemic melioidosis can be as high as 60% within 48 hours of admission [1]. Like many pathogenic bacteria, *B. pseudomallei* has been shown to produce toxin that may contribute to fatal melioidosis [2]. Some of genes that encode for a toxin were located within plasmid and related with virulence of the bacteria [3]. In 1998, Zamaraev and colleagues have reported the cryptic plasmid from *B. pseudomallei* [4]. These plasmids can be mobilized in heterologous *B. mallei* species [5]. In 2000, Radu and colleagues have reported the small cryptic plasmid from Thai patients with melioidosis [6]. However, plasmids of *B. pseudomallei* have not been systematically characterized to see if they contain a virulence gene of clinical significance. Therefore, the objective of the present study is to isolate and characterize a small cryptic plasmid of *B. pseudomallei* from melioidosis patients in northeast of Thailand.

2. Materials and Methodology

2.1 Bacterial strains and culture conditions.

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown at 37°C in LB medium. *B. pseudomallei* strains were cultured at 37°C in 2YT medium. Antibiotics were added at the following final concentrations: tetracycline (40 μg/ml), ampicillin (100 μg/ml).

2.2 Plasmid DNA extraction by the modified alkaline lysis protocol

B. pseudomallei K96243 harboring pMR4 plasmid was cultured in 2YT broth plus 40 μg/ml tetracycline at 37°C with 200 rpm shaking for 18 hr. Two milliliters of overnight culture was pelleted and used for plasmid extraction using a standard alkaline lysis protocol [7]. For the optimization step, concentration of SDS in solution II of the standard protocol was varied to determine the optimal conditions for plasmid purity and yield.

2.3 Screening of plasmid from *B. pseudomallei* clinical isolates

One thousand and fifty-four *B. pseudomallei* clinical isolates from 17 hospitals in northeast of Thailand were used for screening of a native plasmid by the modified alkaline lysis method. The plasmid DNA was digested with restriction enzymes following the manufacturer's recommended protocols (Promega, USA) to confirm that it is a real plasmid. All plasmid harboring strains were stocked at -20°C until use.

Table 1 Bacterial strains and plasmids used

Bacterial strains or plasmids	Relevant properties	Sources
Escherichai coli DH5 alpha	Cloning host	Gift from Prof. Jim Pittard Australia
B. pseudomallei K96243	*B. pseudomallei*, K 96243, isolated from a patient in Khon Kaen hospital	Gift from Assoc. Prof. Dr. Sunee Korbsrisate
Clinical isolates	1,054 *B. pseudomallei* clinical isolates from 17 hospitals in northeast of Thailand	Provided by Assoc. Prof. Dr. Surasak Wongratanacheewin
Plasmids		
pMR4	A broad host range plasmid, tet^r, able to replicate in *B. cenocepacia* and *E. coli*, 9.0 kb	Gift from Prof. Christian Mohr
pPHB 194	Group 2 native plasmid isolated from *B. pseudomallei* PHB194	This study
pPHE 210	~6 kb fragment of pPHB194 cloned into *EcoR*I/*Sma*I linearized pUC18, Ap^R	This study
pPHE 220	~8 kb fragment of pPHB194 cloned into *EcoR*I/*Sma*I linearized pUC18, Ap^R	This study

2.4 Whole sequence of the selected native plasmid

To sequence the entire plasmid, the 6 kb and the 8 kb EcoRI/SspI digested fragments of pPHB194 were cloned into pUC18 digested with restriction enzymes indicated in Table 1 and the correct clones designated pPHE210 and pPHE220 respectively. These clones were the initial source for sequencing the entire plasmid using the universal sequencing primers M13F and M13R [8]. The complete nucleotide sequence was resolved directly by primer walking using several internal primers (Table 2). Analysis of the DNA sequence was performed primarily on the webpage of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). Analysis of the open reading frames (ORFs) was done by using the web based version of the NCBI ORF finder (http://www.ncbi.nlm.nih.gov/projects/gorf/orfig.cgi) program. Those ORFs predicted to encode proteins were analyzed further to determine their homology to proteins of known function using BLAST and BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi). G+C density analysis was performed with NEB cutter.

Table 2 Primers used for complete sequencing of pPHB194

Primers name	Primer Sequence (5′-3′)	Location in pPHB194 (nt start)
PHE210forward-1	GACGATGATGAGCCAAT	665
PHE210forward-2	GACTCTGTATCGTAGCCT	1353
PHE210forward-3	CTGGCGAGGATCGTAT	2109
PHE210reverse-1	CAGAATCGACCAGAAGC	4907
PHE210reverse-2	TTCGCGCAGATAGGCC	4279
PHE210reverse-3	CAGTGGAAGGGGCGTT	3488
PHE210reverse-4	ACATCGTCTACATCAAGGAG	2731
PHE220forward-1	GTTGGCTGCACGCATGA	12,838
PHE220forward-2	GATGTCAGCCAGGCG	12,074
PHE220forward-3	TCGACTCGCAGCCATTC	11,308
PHE220forward-4	AACCTGGTAGCGGGCATT	10,626
PHE220forward-5	CAAACGCGCCTTCTCGT	9895
PHE220reverse-1	CAGTGCAGCAGTTCGAC	6332
PHE220reverse-2-1	CGCTGTGGAAAACCTCAATAC	7036
PHE220reverse-3	AACACCGTCCATAAGCATCT	7834
PHE220reverse-4	GTCGAATGATTACGCGCT	8604

2.5 Determination of the plasmid copy number

PHB194 harboring pPHB194 was used to prepared total DNA using the method of Pushnova, *et al.* [9]. Serial twofold dilutions of bacterial DNA sample (2, 4, 8, 16, 32, 64, 128, and 256-folds) were made with TE buffer. Ten microliters of undiluted and each diluted sample were loaded into the wells of 0.8% agarose gel. The gel was stained with ethidium bromide (0.5 mg/ml) for 20 min and destained in water for 10 min. The photographs of the ethidium bromide-stained gels were taken under UV light. The negative photo of gel was used to determine the intensity of DNA band by using One-Dscan program. Plasmid copy number was deduced with the following formula [9].

$$\text{Plasmid copy number per genome} = \frac{\text{Size of chromosome} \times \text{Total amount of plasmid DNA}}{\text{Size of plasmid} \times \text{Total amount of chromosomal DNA}}$$

3. Results

3.1 Modified alkaline lysis for extraction of plasmid from *B. pseudomallei*

Preliminary studies indicate that plasmid DNA yield from *B. pseudomallei* was poor using the standard alkaline lysis method. By increasing the concentration of SDS in the lysis solution (solution II), at least 4% of SDS must be used to isolate the plasmid pMR4 from B. pseudomallei. SDS at concentration of 6%-8% resulted in a much higher yield of plasmid when compared with the standard method (Fig 1). Therefore, the modified alkaline lysis using 7% SDS in solution II was used to screen native plasmids from 1,054 *B. pseudomallei* clinical isolates.

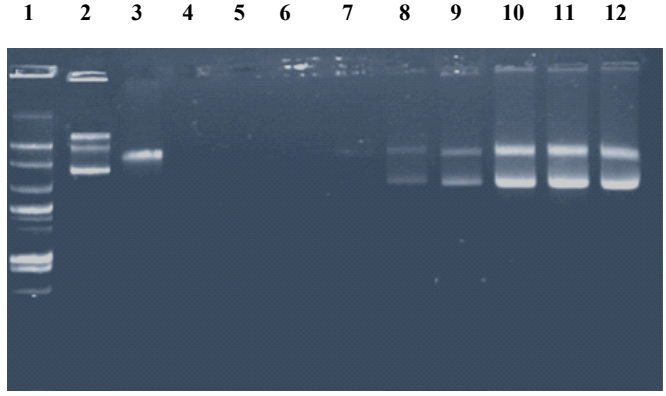


Fig 1 Agarose gel electrophoresis of pMR4 plasmid extracted by varying the concentration of SDS in solution II from 1%-8%. Lane1: E. coli V517 plasmid, Lane2: pMR4, Lane3: genomic DNA from B.pseudomallei strain K96243, Lane5: 1% SDS (standard method), Lane6: 2% SDS, Lane7: 3% SDS, Lane8: 4% SDS, Lane9: 5% SDS, Lane10: 6% SDS, Lane11: 7% SDS, Lane12: 8% SDS

3.2 Screening of native plasmids from clinical isolates of *B. pseudomallei*

The modified alkaline lysis method was used to screen 1,054 clinical isolates of *B. pseudomallei* from 17 provincial hospitals in northeast of Thailand for native plasmids. As shown in Table 3, 23 out of 1,054 isolates were found to harbor a medium sized plasmid. Frequency of *B. pseudomallei* clinical isolates harboring a native plasmid is 2.2%. Following digestion with either *Eco*RI or *Xho*I, the isolated plasmid can be classified into 2 groups, group 1 (G1) and group 2 (G2) as shown in Fig 2. Most of plasmids belong to group G1 (21 isolates), while group G2 contains 2 isolates. The smallest plasmid from group G2, pPHB 194, was used for further characterization.

Table 3 Plasmids of Burkholderia pseudomallei from 1,054 clinical isolates

Hospital	No. of Samples	No. of Plasmid	Plasmid group (name)
Chaiyaphum	54	2	Group 1
Maha Sarakham	65	0	-
Maharacha	52	2	Group 1
Bureeram	39	1	Group 1
Surin	85	0	-
Sisaket	43	0	-
Mukdahan	80	1	Group 1
Nakhon Panom	74	2	Group 1
Amnatjaroen	78	1	Group 1
Sakon Nakhon	80	2	Group 1
Nong Khai	91	3	Group 1
Roi-Et	80	4	Group 1
Nongbualumphu	59	0	-
Kalasin	17	0	-
Srinagarind	47	3	Group 1
Udonthani	61	1	Group 2 (pPHB193)
Yasothon	49	1	Group 2 (pPHB194)

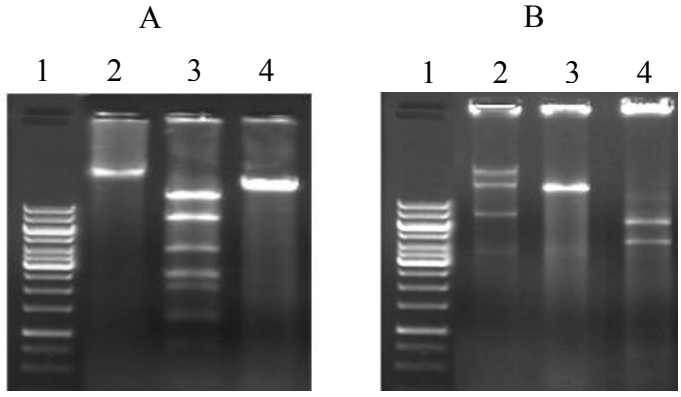


Fig 2 Agarose gel electrophoresis of G1 and G2 plasmid digested with *Eco*RI and *Xho*I
A; Lane1: 1 Kb DNA ladder, Lane2: G1 plasmid, Lane3: G1 plasmid digested with *Eco*RI, Lane4: G1 plasmid digested with XhoI
B; Lane1: 1 Kb DNA ladder, Lane2: G2 plasmid, Lane3: G2 plasmid digested with *Eco*RI, Lane4: G2 plasmid digested with *Xho*I

3.3 Whole sequence analysis of pPHB194 native plasmid

The sequence results show that pPHB194 is 13,482 bp in length, with a G+C content of 61%, which is lower than that of *B. pseudomallei* K96243 chromosomal DNA (68%) [10]. Fifteen putative open reading frames (ORFs) with at least 400 nucleotides in length were found in the pPHB194 DNA sequence (Fig 3), (Table 4). However, only two functional regions have been proposed for the pPHB194 plasmid. The ORF-11, ORF-12, ORF-14 are responsible for putative conjugative transfer, ORF-10 for plasmid replication while ORF-8 and ORF-9 are for plasmid maintenance. This information will be useful for construction of a stable shuttle vector (submitted for publication).

The accession number of the complete nucleotide sequence of pPHB194 plasmid deposited in the GenBank databases is GQ401131.

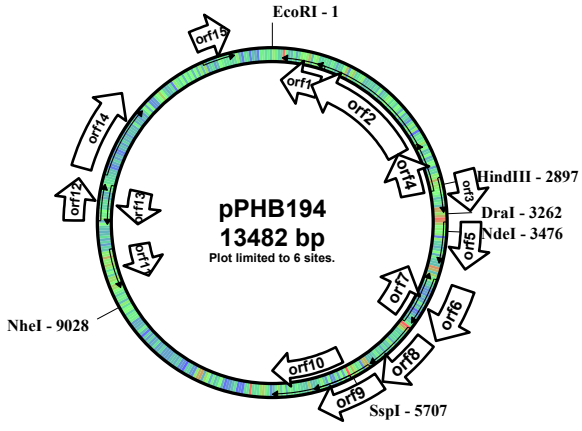


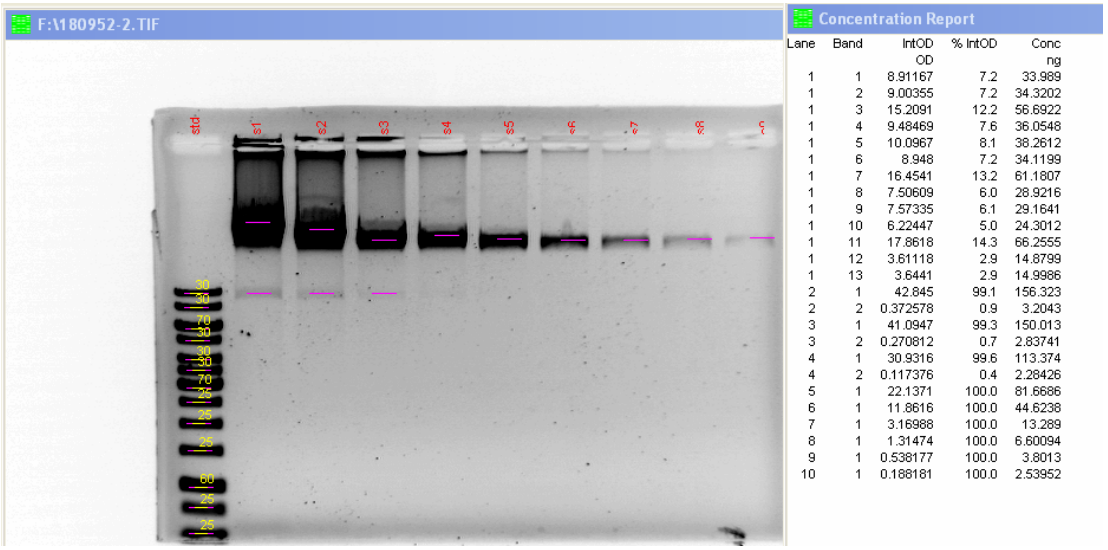
Fig 3 Organization of pPHB194 harboring 15 open reading frames (orf). Unique restriction sites within pPHB194 were indicated

Table 4 NCBI Blast protein sequence of 15 ORFs of pPHB194

ORF	% similarity	Protein encoded	Source
1	78%	integral membrane protein	*Burkholderia pseudomallei* 112
2	84%	integral membrane protein	*Burkholderia pseudomallei* 14
3	27%	unnamed protein product	Homo sapiens
4	84%	hypothetical protein	*Burkholderia pseudomallei* 14
5	38%	GH12405	*Drosophila grimshawi*
6	75%	invertase/recombinase-like protein	uncultured bacterium
7	-	No significant similarity found	-
8	90%	ParA	Binary vector pLH9000
9	84%	ParB	plasmid stabilization protein [*Salmonella enterica* subsp. enterica serovar Westhampton].
10	82%	RepA	*Pseudomonas aeruginosa*
11	32%	MobC mobilisation protein	*Pseudomonas aeruginosa*
12	79%	MobA mobilisation protein	*Pseudomonas aeruginosa*
13	33%	DEAD/DEAH box helicase-like	*Anabaena variabilis* ATCC 29413
14	62%	mobA protein	pTF-FC2 plasmid from *Acidithiobacillus ferrooxidans*
15	76%	Hypothetical protein BURPS305_4324	*Burkholderia pseudomallei* 305

3.4 Plasmid copy number

Plasmid can be categorized as low-copy (1 to 10 copies)-, moderate-copy (11-20 copies)-, or high-copy (>50 copies)-number plasmid. The intensity of DNA band of total DNA of PHB194 was analyzed by One-Dscan (Fig 4). Based on the complete sequence of whole genome of *B. pseudomallei* K96243, the chromosome size of the bacterium was reported to be ~ 7.24 Mb [10]. Accordingly, copy number of the plasmid pPHB194 was determined to be 11 in its natural host, *B. pseudomallei* PHB194 and classified into a moderate-copy number plasmid.



Lane	Band	IntOD OD	% IntOD	Conc ng
1	1	8.91167	7.2	33.989
1	2	9.00355	7.2	34.3202
1	3	15.2091	12.2	56.6922
1	4	9.48469	7.6	36.0548
1	5	10.0967	8.1	38.2612
1	6	8.948	7.2	34.1199
1	7	16.4541	13.2	61.1807
1	8	7.50609	6.0	28.9216
1	9	7.57335	6.1	29.1641
1	10	6.22447	5.0	24.3012
1	11	17.8618	14.3	66.2555
1	12	3.61118	2.9	14.8799
1	13	3.6441	2.9	14.9986
2	1	42.845	99.1	156.323
2	2	0.372578	0.9	3.2043
3	1	41.0947	99.3	150.013
3	2	0.270812	0.7	2.83741
4	1	30.9316	99.6	113.374
4	2	0.117376	0.4	2.28426
5	1	22.1371	100.0	81.6686
6	1	11.8616	100.0	44.6238
7	1	3.16988	100.0	13.289
8	1	1.31474	100.0	6.60094
9	1	0.538177	100.0	3.8013
10	1	0.188181	100.0	2.53952

Fig 4 The intensity of DNA band of total DNA of PHB194 was analyzed by One-Dscan program to estimate the copy number of pPHB194

4. Discussion

An alkaline lysis, which is more simple, rapid and reproducible protocol for small-scale isolation of plasmid DNA, is regarded as the standard method for extracting plasmid [7]. However, preliminary studies from this study indicate that plasmid DNA yield from *B. pseudomallei* was poor when the standard alkaline lysis method was used. This could be due to the fact that cell walls of *Burkholderia spp.* contain exopolysaccharide that render cells more tolerant to lysis by 1% SDS/0.1NaOH of solution II used in the standard protocol. Although SDS at 7.0-8.0% is optimal for plasmid yield, it also gives rise to some degree of chromosomal DNA contamination. However, it is convenient and appropriate for the purpose of rapid and large-scale screening of clinical isolates of *B. pseudomallei.* for native plasmids.

In this study, clinical isolates of *B. pseudomallei* from northeast of Thailand were screened for native plasmids. A total of 1,054 clinical bacterial isolates isolated from melioidosis patients from 17 hospitals were screened by using the modified alkaline lysis method. Twenty three samples from 12 hospitals were found to carry a small plasmid. The plasmid from *B. pseudomallei* clinical isolates were identified with frequency of only 2.2%. Previously document on the detection of *B. pseudomallei* plasmids reported higher prevalences at 12% [4] or 6% [6] of the strains examined. A number of variables may account for these differences, including the inherently distinctive microbial populations isolated in different geographic regions, the limitation of extraction method, and presence of plasmid in low copy number

and natural occurring plasmid of *B. pseudomallei.* The plasmids thus far identified in *B. pseudomallei* are relatively small in size and cryptic, and do not appear to be essential for viability because the majority of strains lack identifiable plasmids.

One of our objectives was to investigate if the plasmid contains a virulence gene. The strategy to test this hypothesis is to compare virulence in an animal model between a plasmid harboring strain and a constructed plasmid-free strain. Whether the dominant *B. pseudomallei* isolates harboring group 1 plasmid are more pathogenic than the other strain harboring group 2 plasmid will remain a mystery. We chose the strain harboring group 2 plasmid for further characterization since it is of smaller size. A number of curing methods were explored unsuccessfully since the number of plasmid present inside the bacterial cell is of a moderate copy-number. Therefore, the entire pHB194 plasmid was sequenced for structural characterization. However, the 15 ORFs found in this plasmid appear to contain no virulence gene nor antibiotic resistance gene, indicating that virulence of this pathogen is chromosome-mediated. The ORF 7 with no homology to any published sequence might hold the key as to whether it is of pathogenesis significance. The presence of chromosomal protein encoding sequences in a plasmid (ORF 1, 2 4, 15) supports the speculation on how bacterial genomes have evolved [11]. The idea that the original genome may have consisted of multiple, small, self-replicating molecule propagating beneficial traits fits with the diversity being discovered within the sequences of bacterial genomes. However, with the entire sequence known, this plasmid is a valuable material for construction of a shuttle vector essential for genetic study in this important human and animal pathogens. In fact, we have successfully constructed a *B.pseudomallei-E.coli* shuttle vector that is very stable both *in intro* and *in vivo* (manuscript submitted for publication).

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