Appendix A:

USAMRIID FORM 360-1-1
CLEARANCE OF PUBLICATIONS & PRESENTATIONS

Date: January 6, 2004

Submitting Author: David DeShazer, Ph.D.

Title: Genomic Diversity of *Burkholderia pseudomallei* Clinical Isolates: Subtractive Hybridization Reveals a Temperate Bacteriophage Present in *B. pseudomallei* 1026b

Submission Information:

Type of Submission: Manuscript

Recipient Organization: Journal of Bacteriology

For Presentations:

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For Resubmissions, Indicate Previous RPP Tracking #:

Plan/Protocol Reference Numbers (if applicable):

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Animal Use Protocol Number: B03-02 and B99-06

Human Use Protocol Number:

CDC Select Agent(s) Described: *Burkholderia pseudomallei* and *Burkholderia mallei*

Submitting Author Acknowledgement (for USAMRIID-sponsored work):

I acknowledge that the information contained in this submission accurately reflects the content of the proposed publication or presentation, and that I have given proper consideration to authorship and intellectual property issues.

David DeShazer
### Abstract

Burkholderia pseudomallei is the etiologic agent of the disease melioidosis and is a category B biological threat agent. The genomic sequence of B. pseudomallei K96243 was recently determined, but little is known about the overall genetic diversity of this species. Suppression subtractive hybridization was employed to assess the genetic variability between two distinct clinical isolates of B. pseudomallei, 1026b and K96243. Numerous mobile genetic elements, including a temperate bacteriophage designated phi1026b, were identified among the 1026b-specific suppression subtractive hybridization products. Bacteriophage phi1026b was spontaneously produced by 1026b, and it had a restricted host range, infecting only Burkholderia mallei. It possessed a noncontractile tail, an isometric head, and a linear 54,865-bp genome. The mosaic nature of the phi1026b genome was revealed by comparison with bacteriophage phiE125, a B. mallei-specific bacteriophage produced by Burkholderia thailandensis. The phi1026b genes for DNA packaging, tail morphogenesis, host lysis, integration, and DNA replication were nearly identical to the corresponding genes in phiE125. On the other hand, phi1026b genes involved in head morphogenesis were similar to head morphogenesis genes encoded by Pseudomonas putida and Pseudomonas aeruginosa bacteriophages. Consistent with this observation, immunogold electron microscopy demonstrated that polyclonal antiserum against phiE125 reacted with the tail of phi1026b but not with the head. The results presented here suggest that B. pseudomallei strains are genetically heterogeneous and that bacteriophages are major contributors to the genomic diversity of this species. The bacteriophage characterized in this study may be a useful diagnostic tool for differentiating B. pseudomallei and B. mallei, two closely related biological threat agents.
15. SUBJECT TERMS
Burkholderia mallei, pseudomallei, glanders, melioidosis, genetic diversity, subtractive hybridization, bacteriophage

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Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18
U.S. Army Medical Research Institute of Infectious Diseases
Standard Operating Procedure

SOP Title:  
SOP No:  
Revision:  
Effective Date:  
Page: 15 of 19

Co-Author Acknowledgement:

I am aware that I am listed as a co-author on the above-named submission, and I have reviewed the information contained in the enclosed publication or presentation and agree with its content.

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-----Original Message-----
From: Hoffman, Kay L Ms USAMRIID
Sent: Wednesday, January 21, 2004 8:56 AM
To: Cavazos, Jaime Mr MEDCOM HQ
Cc: Dasey, Chuck F Mr USAMRMC
Subject: FW: MANUSCRIPT RPP-04-214

Jaime,

Manuscript entitled “Genomic Diversity of Burkholderia pseudomallei Clinical Isolates: Subtractive Hybridization Reveals a Temperate Bacteriophage Present in B. pseudomallei 1026B” submitted for approval. The manuscript will be submitted to the Journal of Bacteriology, if approved. Thanks. Kay Hoff

-----Original Message-----
From: DeShazer, David Dr USAMRIID
Sent: Wednesday, January 21, 2004 9:41 AM
To: Hoffman, Kay L Ms USAMRIID
Subject: RE: MANUSCRIPT RPP-04-214

<< File: manuscript1.doc >>

Dr. DeShazer;

Please send me an electronic copy of your manuscript entitled “genomic Diversity of Burkholderia pseudomallei Clinical Isolates: Subtractive Hybridization Reveals a Temperate Bacteriophage Present in B. pseudomallei 1026B”. Your tracking number is RPP-04-214. Thank You. Kay Hoff

Kay Hoff, Program Assistant
Research Plans and Programs
(301-619-2767)
Genomic Diversity of *Burkholderia pseudomallei* Clinical Isolates: Subtractive Hybridization Reveals a *Burkholderia mallei*-Specific Prophage in *B. pseudomallei* 1026b

David DeShazer*

Bacteriology Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland 21702

Received 9 January 2004/Accepted 8 March 2004

*Burkholderia pseudomallei* is the etiologic agent of the disease melioidosis and is a category B biological threat agent. The genomic sequence of *B. pseudomallei* K96243 was recently determined, but little is known about the overall genetic diversity of this species. Suppression subtractive hybridization was employed to assess the genetic variability between two distinct clinical isolates of *B. pseudomallei*, 1026b and K96243. Numerous mobile genetic elements, including a temperate bacteriophage designated φ1026b, were identified among the 1026b-specific suppression subtractive hybridization products. Bacteriophage φ1026b was spontaneously produced by 1026b, and it had a restricted host range, infecting only *Burkholderia mallei*. It possessed a noncontractile tail, an isometric head, and a linear 54,865-bp genome. The mosaic nature of the φ1026b genome was revealed by comparison with bacteriophage φE125, a *B. mallei*-specific bacteriophage produced by *Burkholderia thailandensis*. The φ1026b genes for DNA packaging, tail morphogenesis, host lysis, integration, and DNA replication were nearly identical to the corresponding genes in φE125. On the other hand, φ1026b genes involved in head morphogenesis were similar to head morphogenesis genes encoded by *Pseudomonas putida* and *Pseudomonas aeruginosa* bacteriophages. Consistent with this observation, immunogold electron microscopy demonstrated that polyclonal antiserum against φE125 reacted with the tail of φ1026b but not with the head. The results presented here suggest that *B. pseudomallei* strains are genetically heterogeneous and that bacteriophages are major contributors to the genomic diversity of this species. The bacteriophage characterized in this study may be a useful diagnostic tool for differentiating *B. pseudomallei* and *B. mallei*, two closely related biological threat agents.

*Burkholderia pseudomallei* is the causative agent of the glanders-like disease melioidosis (21, 22, 67). This organism is endemic in Southeast Asia and northern Australia, where it can be isolated from moist soil and surface water. Humans and animals can be infected by *B. pseudomallei* by direct inoculation of soil or water into skin abrasions or by inhalation of contaminated material. Underlying diseases such as diabetes mellitus and chronic renal failure are risk factors for melioidosis, but apparently healthy individuals can also develop clinical melioidosis (18). The clinical manifestations of melioidosis are protean and often include fever and abscess formation. The clinical spectra of melioidosis in endemic regions are similar, but brainstem encephalitis and genitourinary infections are more common in northern Australia while suppurative parotitis is more common in Southeast Asia (21, 22, 67). The basis for geographic differences in disease presentation is currently unknown, but the differences may be due to genetic differences in patients and/or in the *B. pseudomallei* strains present in the different regions.

Capsular polysaccharide and lipopolysaccharide (LPS) O antigen are important for *B. pseudomallei* virulence in animal models of melioidosis (4, 24, 55). The recently completed genome sequence of *B. pseudomallei* K96243 (http://www.sanger.ac.uk/) has facilitated identification of several new virulence gene candidates. In particular, K96243 harbors multiple genomic islands with relatively low G+C contents, suggesting that there was recent acquisition by lateral gene transfer (34, 35, 49, 50). Lateral gene transfer is a process in which genetic material is transferred from a donor to a recipient via mobile genetic elements, such as plasmids, transposons, integrons, or bacteriophages. The laterally acquired genetic material can alter the phenotype of the recipient and promote adaptation to its environment. Further studies are required to elucidate the biology of *B. pseudomallei* mobile genetic elements and to examine their contribution to genomic diversity, niche adaptation, and virulence.

The goal of this study was to examine the genomic diversity of *B. pseudomallei* clinical isolates by performing subtractive hybridization between *B. pseudomallei* 1026b (tester) and K96243 (driver). *B. pseudomallei* 1026b was isolated in Thailand from a human case of septiemic melioidosis with skin, soft tissue, and spleen involvement and has been studied extensively in the laboratory (26). In this study, numerous mobile genetic elements in 1026b that were not present in K96243 were identified. One of the 1026b-specific mobile genetic elements was a temperate bacteriophage (φ1026b) that was spontaneously produced during growth in liquid broth. The morphology, host range, genomic sequence, and immunological reactivity of bacteriophage φ1026b are reported here.

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**MATERIALS AND METHODS**

*Bacterial plasmids, strains, and growth conditions*. The plasmids used in this study are described in Tables 1 and 2. The *Burkholderia mallei* strains used in this study are listed in Table 3. The following *B. pseudomallei* strains were used in this...
The hybridization temperature was 73°C.

Subtractive hybridization. Subtractive hybridization was performed by using B. pseudomallei 1026b genomic DNA as the tester and B. pseudomallei K926245 genomic DNA as the driver. The protocol described in the CLONTECH PCR-Select bacterial genome subtraction kit user manual was followed, except that the hybridization temperature was 73°C instead of 63°C. The subtractive hybridization products were cloned into pCR2.1-TOPO and transformed into chemically competent E. coli TOP10 cells.

Bacteriophage production, propagation, and DNA purification. The procedures used for bacteriophage production, propagation, and DNA purification have been described previously (70).

Enzyme-linked immunosorbent assay. The wells of a round-bottom microtiter plate were coated with approximately 5 x 10^5 bacteria in 100 μl of 0.05 M carbonate-bicarbonate buffer (pH 9.6), and the plate was incubated for 1 h at 37°C. The wells were washed with phosphate-buffered saline containing 0.05% Tween 20 and blocked with a 3% solution of skim milk in phosphate-buffered saline—Tween 20 for 1 h at 37°C. The wells were washed, a 1:1,000 dilution of a peroxidase-labeled goat anti-mouse immunoglobulin G(H+ L) antibody (KPL) was added to each well. The plate was incubated for 1 h at 37°C, washed, and developed with the 2,2'-azino-bis(3-ethylbenzthiazolinesulfonic acid) (ABTS) peroxidase substrate system (KPL) for 10 min. The optical density at 410 nm was determined.

**1026b sensitivity testing.** Approximately 10^2 PFU of 1026b was added to a saturated bacterial culture and incubated at 25°C for 20 min, and 4.8 ml of molten LB top agar (0.7%) containing 4% glycerol was added. The mixture was immediately poured onto an LB agar plate containing 4% glycerol and incubated overnight at 25 or 37°C, depending on the bacterial species being tested. Bacteria were considered to be sensitive to 1026b if they formed plaques under these conditions and resistant if they did not. The positive control, B. mallei ATCC 23344, formed plaques in the presence of 1026b after incubation at 25 and 37°C. No bacterial species tested formed plaques in the absence of 1026b.

**Negative staining of 1026b.** The procedure used for negatively staining bacteriophage 1026b with 1% phosphotungstic acid (PTA) (pH 6.6) has been described previously (70).

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant characteristics</th>
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<tr>
<td>pCR2.1-TOPO</td>
<td>3.9-kb TA cloning vector; pMB1 ori; Km' Ap'</td>
<td>Invitrogen 47</td>
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<tr>
<td>pSKM11</td>
<td>Positive selection cloning and suicide vector; IncP oriT; ColE1 ori; Ap' Tc'</td>
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<tr>
<td>pMOLUC</td>
<td>Vector for cloning large DNA fragments; pBR322 oriA'</td>
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<tr>
<td>pDD80</td>
<td>pGEM-7f(+) containing 3.625-bp HindIII fragment from 1026b</td>
<td>This study</td>
</tr>
<tr>
<td>pDD81</td>
<td>pGEM-7f(+) containing 3.105-bp HindIII fragment from 1026b</td>
<td>This study</td>
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<tr>
<td>pDD82</td>
<td>pGEM-7f(+) containing 1.068-bp HindIII fragment from 1026b</td>
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<td>pMOLUC containing 12.775-bp HindIII fragment from 1026b</td>
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<td>pSKM11 containing 9,492-bp HindIII fragment from 1026b; Ap' Tc'</td>
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**a** Km', kanamycin resistant; Ap', ampicillin resistant; Tc', tetracycline sensitive; Tc', tetracycline resistant.

**TABLE 1. Plasmids used in this study**
TABLE 2. Subtractive hybridization products present in B. pseudomallei 1026b but not in B. pseudomallei K926243

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genes were identified by using GeneMark.hmm (http://opal.biology.gatech.edu/GeneMark/gmhmm2_prot.cgi); other genes were identified by visual inspection, guided by BLAST (2) results. DNA and protein sequences were analyzed with GeneJockeyII and MacVector 7.2 software for the Macintosh computer. The methods used for immunogold electron microscopy have been described previously (24). Briefly, φE125 and φ1026b were reacted with polyclonal rabbit antiserum directed against φE125, washed, and reacted with goat anti-rabbit IgG gold conjugate (Sigma).
corresponding ends of those fragments. All PCR products were cloned and sequenced to confirm the PCR results. The sequences of the 16 oligodeoxyribonucleotide primers used in this analysis were as follows: 8.4L, 5'-GGTCTGTGC
GCACAAATCATG-3'; 3.6L, 5'-CAACGAAGAGTCGCACTG-3'; 3.6R, 5'-GCACCATGACACGATATT
TGCTGCTGCAATTCGATACGCGTG-3'; 12.8L, 5'-AACGCGCTTCGCGATCCT
TGTCGATCGTG-3'; 12.8R, 5'-AAGCTCGAGACCATTACCT
AGCGCTTATTGAC-3'; 9.3L, 5'-CAAGCTGACTACGAGTGTG
CAAGCTGACTACGAGTGTG-3'; 9.3R, 5'-GCCGCTGACCGACATACGAT
GCCGCTGACCGACATACGAT-3'; 2.5L-2, 5'-TAGCCACTCGGACGACAT
ACGTTTTCATTG-3'; 2.5R, 5'-TTAGCCACTCGGACGACAT
ACGTTTTCATTG-3'; 3.1L, 5'-AAGGACTCGCCGAGTACAC-3'; 3.1R, 5'-TGCGCTGA
TGCGCTGA-3'; 2.5L-2, 5'-ACGCGCTTGCGGAAATTCATCG
ACGTTTTCATTG-3'; 2.5R, 5'-TTAGCCACTCGGACGACAT
ACGTTTTCATTG-3'; 3.6L, 5'-GCCGCTGACCGACATACGAT
GCCGCTGACCGACATACGAT-3'; and 8.4L, 5'-ATCACGACT
TGCCCATGCAAC-3'.

PCRs were performed with genomic DNAs from B. pseudomallei K96243 and 1026b, B. mallei ATCC 23344 and BML1, and φ1026b and with primers Int2 (5'-CAACGAAGAGAGGTACTG-3') and Int5 (5'-TTGAATCGCACCGT
TTCGAC-3') to determine if φ1026b integrated into the tRNA^Pro-3 gene. A single PCR product of the expected size (447 bp) was obtained with B. mallei BML1 DNA and B. pseudomallei 1026b DNA. This product was cloned, and its nucleotide sequence was determined. As expected, no PCR products were obtained when genomic DNAs from B. pseudomallei K96243, B. mallei ATCC 23344, and φ1026b were used in the PCR.

**Nucleotide sequence accession numbers.** The nucleotide sequences reported in this paper have been deposited in the GenBank database under accession numbers AY471580 to AY471606 (1026b-K96243 subtractive hybridization products) and AY453853 (bacteriophage φ1026b).

### RESULTS

**MLST of B. pseudomallei 1026b.** Godoy et al. developed an MLST scheme for B. pseudomallei, B. mallei, and B. thailandensis based on sequence variations in seven housekeeping genes, but B. pseudomallei 1026b was not one of the isolates examined (32). In this study, the allelic profile of 1026b was determined to be 3-4-12-1-1-4-1, which corresponds to a new sequence type (ST102). Figure 1 shows a minimum-evolution tree based on the concatenated sequences of the seven MLST loci for 92 sequence types of B. pseudomallei. Note that B. mallei isolates (ST40) cluster with B. pseudomallei isolates on the minimum-evolution tree and are considered to be a distinct clone of B. pseudomallei (32). Clinical isolates 1026b and K96243 were resolved into two genetically distinct clones, ST102 and ST10, based on the MLST analysis (Fig. 1). The MLST results suggest that 1026b and K96243 are excellent candidates for examining the genomic diversity of B. pseudomallei.

**B. pseudomallei 1026b-specific subtractive hybridization library contains multiple mobile genetic elements.** The goal of

### TABLE 3. Bacteriophage φ1026b plaque formation on B. mallei strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Plaque formation</th>
<th>Reference or source</th>
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^a http://www.tigr.org/.

^b USDA, United States Department of Agriculture.
this study was to identify genetic determinants present in *B. pseudomallei* 1026b but not in *B. pseudomallei* K96243 by subtractive hybridization. Forty plasmid inserts from a 1026b-K96243 subtractive hybridization library were identified and used to perform BLASTN searches with the completed K96243 genome (http://www.sanger.ac.uk/). Twenty-seven of the subtractive hybridization products were not present in K96243 (Table 2). The sizes of the 1026b-specific subtractive hybridization products ranged from 167 to 1,440 bp, and these products had relatively low G+C contents compared to the G+C content of the K96243 genome (68.1%). The putative functions of proteins encoded by genes in the subtractive hybridization library included several mobile genetic elements (51), including phage-related proteins, an insertion sequence element, and a plasmid-like mobilization protein (Table 2). Plasmids pSH4, pSH18, pSH25, pSH37, and pSH47 encode phage-related proteins. These proteins include phage-related integrases (pSH25 and pSH47) and several proteins that are similar to bacteriophage pE125 proteins (pSH4, pSH18, and pSH37). The IS3 family insertion sequence ISBp1 (69) was present in plasmid pSH5 (Table 2). Woo et al. previously demonstrated that ISBp1 was present in ~65% of *B. pseudomallei* strains but was absent from K96243 (69). The nucleotide sequence of the pSH5 DNA insert suggests that ISBp1 is also present in the genome of *B. pseudomallei* 1026b. The 531-bp subtractive hybridization insert of plasmid pSH51 encodes a putative plasmid mobilization protein that was also isolated from a *B. pseudomallei* 1026b-*B. thailandensis* E264 subtractive library (55). No plasmids have been described in *B. pseudomallei* 1026b, and the pSH51 insert may represent an integrated plasmid that is not present in *B. pseudomallei* K96243 or *B. thailandensis* E264. Three plasmid inserts, pSH7, pSH23, and pSH42, encoded distinct regions of a putative ATP binding protein (Table 2). Finally, 13 subtractive hybridization products encoded hypothetical proteins (pSH3 and pSH13) or novel proteins (pSH8, pSH10, pSH11, pSH19, pSH21, pSH26, pSH29, pSH35, pSH38, pSH49, and pSH53) (Table 2). Taken together, the results demonstrate the genomic diversity of these two clinical isolates of *B. pseudomallei*, especially with respect to mobile genetic elements and novel gene sequences.

*B. pseudomallei* 1026b spontaneously produces a bacteriophage that is specific for *B. mallei*. The subtractive hybridization product library contained multiple 1026b-specific bacteriophage sequences, and it was of interest to see if this strain actually produced a bacteriophage. *B. mallei* was chosen as a host because previous studies demonstrated that it is suscep-
tible to infection with *B. pseudomallei* and *B. thailandensis* bacteriophages (45, 62, 70). 1026b spontaneously produced a bacteriophage, designated φ1026b, that formed turbid plaques with a diameter of 1.5 to 2.0 mm on *B. mallei* ATCC 23344. No other plaque types were identified, which suggests that 1026b produces only one bacteriophage under the growth conditions used. However, it is possible that 1026b produces additional bacteriophages that cannot use *B. mallei* as a host. Bacteriophage production was only slightly increased by brief exposure to UV light (470 versus 540 PFU/ml). After infection, the φ1026b genome integrated into the *B. mallei* chromosome at a specific site and became a prophage (see below). *B. mallei* ATCC 23344 was infected with φ1026b, and a lysogenic derivative was isolated and designated BML1 (Table 3).

Bacteriophage φ1026b formed plaques on 29 of 36 *B. mallei* strains used in this study (Table 3). Bacteriophages initiate infection by specifically binding to a surface receptor on the bacterial host, such as LPS O antigen and capsular polysaccharide. LPS O-antigen production by *B. mallei* strains was examined by an enzyme-linked immunosorbent assay by using monoclonal antibody 3D11 (Table 3). Three of the φ1026b-resistant *B. mallei* strains, NCTC 120, DB110795, and ISU, did not produce LPS O antigen. When LPS O-antigen production in *B. mallei* NCTC 120 was complemented by providing pBHR1-whIe in trans, the resulting strain was susceptible to infection with bacteriophage φ1026b (Table 3). Surprisingly, *B. mallei* strains Turkey 4 and Turkey 5 were resistant to infection with φ1026b even though they produced LPS O antigen (Table 3). The *B. mallei* lysogens BML1 and BML10 produced LPS O antigen and were resistant to infection with φ1026b, presumably due to immunity or superinfection exclusion proteins encoded by the prophages that they harbor. Capsular polysaccharide was not required for plaque formation as φ1026b formed plaques on DD3008, a capsule-deficient mutant derived from ATCC 23344 (Table 3).

The host range of φ1026b was further examined by using *B. pseudomallei*, *B. thailandensis*, *B. cepacia*, *B. multivorans*, *B. canescens*, *B. stabilis*, *B. vietnamiensis*, *B. glathei*, *B. pseudomallei* Turkey 4 and Turkey 5, *B. multivorans* ATCC 23344, *B. mallei* ATCC 23344, *B. cocovenans*, *B. thailandensis* K96243, *B. uboniae*, *B. apista*, *B. thailandensis* ATCC 23344 and *B. mallei* K96243 were examined, and a representative image of φ1026b is shown in Fig. 2. φ1026b possessed an isometric head that was 56 nm in diameter and a long, noncontractile tail that was approximately 200 nm long and 8 nm in diameter. Based on its morphotype, φ1026b can be classified as a member of the order Caudovirales and the family Siphoviridae (1).

**Molecular characterization of the bacteriophage φ1026b genome.** The φ1026b genome was digested with HindIII, and 11 fragments were generated; these fragments were 0.1, 0.6, 1.1, 2.5, 3.1, 3.6, 3.8, 8.4, 9.4, 9.5, and 12.7 kb long. The fragments were heated to 80°C, and the 8.4-kb fragment dissociated into two fragments (2.3 and 6.1 kb), suggesting that a cohesive (cos) site was present (data not shown). The 11 HindIII fragments were cloned, and their nucleotide sequences were determined. The nucleotide sequencing results are shown schematically in Fig. 3. The subtractive hybridization DNA insert of pSH4 was identical to bacteriophage φ1026b from position 44417 to position 44651, which included the 3’ ends of gene 63 and gene 64 (Table 2 and Fig. 3).

The φ1026b genome is a linear molecule that is 54,865 bp long, and it contains 10-base 3’ single-stranded extensions on the left (3’-GCGGGGCGAGG-5’) and right (5’-CGCCCGCTT C-3’), as shown in Fig. 3. The cos site of φ1026b is identical to the cos site of bacteriophage φE125 (70). The G+C content of the φ1026b genome is 60.7%, which is lower than the G+C content of the *B. pseudomallei* K96243 genome (68.1%) (http://www.sanger.ac.uk/). The φ1026b genome encodes 83 proteins, and 58 of these proteins generated best hits to bacteriophage φE125 proteins when the BLAST search algorithm was used.

Bacteriophage genomes are composed of a mosaic of multigene modules, each of which encodes a group of proteins involved in a common function, such as DNA packaging, head biosynthesis, tail biosynthesis, host lysis, lysogeny, or replication (10, 36, 40). The φ1026b genome contains multigene modules involved in DNA packaging, head morphogenesis, tail morphogenesis, host lysis, and DNA replication (Fig. 3). The relative order of these modules in the φ1026b genome is similar to the order in other Siphoviridae genomes (10, 40, 70). φ1026b also encodes a LysR family transcriptional regulator (57) and a major facilitator superfamily (MFS) transporter (52), encoded by gene 58 and gene 59 (Fig. 3). It is interesting that these genes have been found in tandem in several recently completed bacterial genomes, including those of *R. solanacearum*, *B. fungorum*, and *P. syringae*. gp59 is a member of the metabolite:H+ symporter family of MFS proteins which function by proton symport and allow the uptake of a wide variety of metabolites (52).

Temperate bacteriophage genomes often contain an attachment site (*attP*) utilized for integration into a homologous region within the bacterial genome (*attB*) via site-specific recombination (20). The *attP* site of φ1026b was adjacent to the site encoding gp33, a site-specific integrase (Fig. 3). The nucleotide sequence of *attP* contained a 49-bp sequence that was identical to *attB* sequences present in the genomes of *B. mallei* ATCC 23344 and *B. pseudomallei* K96243. This sequence corresponded to the 3’ end of the *tRNAPro* gene on chromosome 1 of *B. mallei* (positions 830691 to 830615) and chromosome 1 of *B. pseudomallei* (positions 1604091 to 1604043), *tRNA* genes often serve as target sequences for site-specific integration of temperate bacteriophages, plasmids, and pathogenicity islands (14, 34). The *attP* site of φ1026b was identical...
FIG. 2. Transmission electron micrograph of bacteriophage φ1026b negatively stained with 1% PTA. One intact bacteriophage (head and tail) and one bacteriophage head without an attached tail are shown. Scale bar = 100 nm.
to the attP site of φE125 (70). It is worth emphasizing that *B. pseudomallei* 1026b and *B. thailandensis* E125 both contain bacteriophages integrated at tRNAPro-3, while *B. pseudomallei* K96243 does not (Fig. 1). However, *B. pseudomallei* K96243 does have a prophage-like region on chromosome 2 that is 98% identical to φ1026b gene 48 to gene 52 and 97% identical to φ1026b gene 55 to gene 57 (http://www.sanger.ac.uk/). It is not known if this is a functional or defective prophage.

**Comparative analysis of the genomes of temperate bacteriophages φ1026b and φE125.** The host range and morphology of φ1026b are remarkably similar to the host range and morphology of φE125 (70), a temperate bacteriophage harbored by *B. thailandensis* E125 (Fig. 1). As mentioned above, the two genomes contain identical cos and attP sites, and 70% of the φ1026b proteins generate best hits to φE125 proteins when the BLASTP search algorithm is used. The genome of φ1026b is marginally larger (1.5 kb) than the genome of φE125. Figure 3 shows a comparative analysis of the genomes of φ1026b and φE125 generated by using the BLAST 2 SEQUENCES program (63). Large segments of DNA are shared by the two genomes, and the levels of nucleotide identity are 93 to 98%. These conserved regions are interspersed with DNA segments that exhibit little or no sequence similarity (Fig. 3). The mosaic nature of the genomes is illustrated by the head morphogenesis and head-tail joining genes in φ1026b (gene 3 to gene 8) and φE125 (gene 3 to gene 9). The φ1026b genes more closely resemble head morphogenesis and head-tail joining genes of *P. aeruginosa* and *Pseudomonas putida* bacteriophages than the corresponding genes in φE125. However, the DNA packaging and tail morphogenesis genes flanking this region in φ1026b and φE125 are 94% identical (Fig. 3). The most likely explanation for this finding is that recombination between one of these bacteriophages and an unrelated bacteriophage (or prophage) resulted in acquisition of a different set of head morphogenesis and head-tail joining genes (11, 36). Because the proteins involved in head morphogenesis interact with one another, it is not surprising that the genes encoding them are laterally acquired as a group. The putative crossover points for this recombination event and those described below occur at or near gene boundaries. The modular exchange of head mor-
phogenesis genes suggests that DNA packaging proteins (gp1 and gp2) can associate with two distinct head protein sets, while head-to-tail association seems to require the mediation of a specific head-tail joining protein (gp8 in φ1026b and gp9 in φE125).

Genetic mosaicism was readily evident in the region spanning the site-specific integrase and DNA replication genes of φ1026b and φE125 (Fig. 3). This large mosaic region includes five modules of conserved genes and six modules of genes with no sequence similarity. Note that one of the conserved modules in φE125 is disrupted by an ISBt3 insertion in gene 39 (70), which corresponds to gene 44 in φ1026b (Fig. 3). The biological function(s) of this large mosaic region probably includes lysogeny, lysogenic conversion, and superinfection immunity (10, 39). As mentioned above, bacteriophage φ1026b cannot form plaques on the lysogens BML1 and BML10 (Table 3). In comparison, bacteriophage φE125 can form plaques on BML1 but not on BML10. This indicates that the φE125 lysogen (BML10) can prevent superinfection with both φE125 and φ1026b but that the φ1026b lysogen (BML1) cannot prevent superinfection only with φ1026b. It seems likely that one or more of the novel gene modules in the mosaic region are responsible for the differences in superinfection immunity, but further studies are required to prove this.

Several additional features of the large mosaic region should be mentioned here. First, φ1026b gene 66 and gene 67 were replaced in φE125 by gene 56 and gene 57 (Fig. 3). This modular replacement occurred precisely at the gene boundaries, suggesting that these gene pairs perform analogous functions. The biological function likely involves DNA methylation because both gene 67 (φ1026b) and gene 56 (φE125) encode DNA methyltransferases. Interestingly, DNA methyltransferases are relatively common in bacteriophages from gram-positive bacteria but not in bacteriophages from gram-negative bacteria. Second, single-gene modular replacement between φ1026b gene 50 and φE125 gene 45 also occurred, but the biological importance of this exchange is not known because it involved genes with no known functions (Fig. 3). Finally, the large mosaic region of φ1026b includes gene 58 and gene 59, genes that encode a LysR family transcriptional regulator and an MFS transporter (Fig. 3). These genes were not present in the φE125 genome, supporting the notion that they were acquired by lateral gene transfer from a bacterial genome (see above).

Phenotypic analysis of B. pseudomallei DD5025. The prophage-encoded MFS transporter (gp59) may provide B. pseudomallei 1026b with a selective advantage over other B. pseudomallei strains by allowing the uptake of a nutrient(s) from the environment (36, 52). In order to examine the function of gene 59, a strain harboring a mutation in this gene was constructed. An internal gene fragment of gene 59 was PCR amplified and cloned into the suicide vector pSKM11 (Table 1). Plasmid pDD94 was mobilized into B. pseudomallei 1026b, and the resulting merodiploid strain was designated DD5025. There were no detectable differences between the growth of 1026b and the growth of DD5025 in complex or defined media (data not shown). Both strains were examined to determine their abilities to metabolize 19 different carbon sources by using PM1 and PM2 phenotype microarrays (www.biolog.com), but no differences were observed. Prophage-encoded virulence factors in other bacterial species have been described (5), and it was of interest to see if gene 59 provided a selective benefit to 1026b in an animal model of melioidosis (25). Syrian hamsters were infected intraperitoneally with 10², 10³, and 10⁴ cells of 1026b and DD5025, and the LD₉₀ values were determined 2 days postinfection. The LD₉₀ for both strains was <10⁵ cells, suggesting that gene 59 is not important for the pathogenesis of 1026b in this animal model of melioidosis.

Immunogold electron microscopy of φE125 and φ1026b. The comparative genomics analysis of φE125 and φ1026b predicted that these phages contain antigenically related tails but antigenically distinct heads (Fig. 3). Immunogold electron microscopy was performed to see if polyclonal antiserum against φE125 reacted with φ1026b (Fig. 4). The bacteriophages were reacted with polyclonal rabbit antiserum directed against φE125, washed, and reacted with a goat anti-rabbit IgG gold conjugate. As expected, the antibodies reacted with the head and tail of bacteriophage φE125 (Fig. 4). The anti-φE125 antibodies did not react with the head of φ1026b but did react with the tail (Fig. 4). These results corroborate the comparative genomics results and demonstrate that the tails of bacteriophages φE125 and φ1026b are antigenically related but the heads are antigenically distinct. Tailed bacteriophages bind to the surfaces of their bacterial hosts by using their tails, and the genetic and antigenic relatedness of the tails of φE125 and φ1026b probably accounts for their specificity for B. mallei.

DISCUSSION

The results presented here demonstrate that clinical isolates of B. pseudomallei exhibit genetic diversity, especially with regard to the mobile genetic elements that they harbor. It should be emphasized that only two clinical strains were compared in this study, but it is likely that future B. pseudomallei genome sequencing and comparative genome hybridization projects will yield similar results. At least five prophages (or prophage-like elements) were identified in B. pseudomallei 1026b but not in B. pseudomallei K96243 (http://www.sanger.ac.uk/). Prophages are a major source of strain-specific differences in several pathogenic bacteria, including Shiga toxin-producing E. coli, Streptococcus pyogenes, Staphylococcus aureus, S. enterica, and Xylella fastidiosa (11, 16, 17). The genomic sequencing results for multiple strains of Streptococcus and Xylella suggest that different disease pathologies may be due to differences in the prophage contents of the infecting strains (48, 64). Prophages are responsible for much of the laterally transferred DNA in bacteria, and they play a major role in the evolution of bacterial pathogens by providing new virulence determinants (5, 15). Manzeniuk et al. found that 92% of B. pseudomallei strains produced temperate bacteriophages, demonstrating that prophages are relatively common in this bacterial species (45). Brown and Beacham performed subtractive hybridization between B. pseudomallei and B. thailandensis and identified multiple B. pseudomallei-specific mobile genetic elements, including a P2-like prophage (9). Taken together, the results demonstrate that there is considerable diversity in the mobile genetic elements that B. pseudomallei strains harbor. It is tempting to speculate that the variable clinical presentation of melioidosis is due, at least in part, to the prophage contents of the infecting B. pseudomallei strains. The genomic sequences
of additional *B. pseudomallei* strains isolated from melioidosis patients with defined clinical manifestations are needed to further explore this possibility.

It is widely accepted that tailed bacteriophage genomes are a mosaic collection of genetic material resulting from recombination between bacteriophages (or prophages) (11, 16, 17, 36). A comparative genome analysis of \( \Phi H9278 \text{1026b} \) and \( \Phi H9278 \text{E125} \) revealed regions with high sequence similarity interspersed with regions displaying no sequence similarity (Fig. 3). This mosaic genetic relationship indicates that recombination between \( \Phi H9278 \text{1026b} \) or \( \Phi H9278 \text{E125} \) and an unrelated bacteriophage(s) occurred during the evolution of these *Burkholderia* bacteriophages, which resulted in acquisition of new head and lysogeny genes. The \( \Phi H1026b \) head morphogenesis genes more closely resemble the head morphogenesis genes of *P. aeruginosa* and *P. putida* bacteriophages than the corresponding genes in \( \Phi H1026b \). In addition, the host lysis cassettes of \( \Phi 1026b \) (genes 23 to 25) and \( \Phi E125 \) (genes 24 to 26) are located directly downstream of the putative tail fiber module, which is similar to the genetic organization of *P. aeruginosa* bacteriophage D3 (42). This genetic organization is commonly found in *Siphoviridae* from low-G+C-content gram-positive bacteria (10) but not in *Siphoviridae* from gram-negative bacteria. The tail fiber mod-

![FIG. 4. Immunogold electron microscopy of bacteriophages \( \Phi E125 \) and \( \Phi 1026b \). The bacteriophages were reacted with polyclonal rabbit antiserum directed against \( \Phi E125 \), washed, and reacted with goat anti-rabbit IgG gold conjugate (5 nm). Bacteriophage \( \Phi 1026b \) was subsequently negatively stained with 1% PTA. Scale bar = 100 nm.](image-url)
surface-exposed bacteriophage receptor (Table 3). B. mallei LPS O antigen is similar to the antigen previously described for B. pseudomallei and B. thailandensis except that it is devoid of an O-acetyl group at the 4’ position of the 1-talose residue (7, 12, 41, 53). B. pseudomallei and B. thailandensis strains may be resistant to infection with φ1026b and φE125 because the O-acetyl group at the 4’ position of the 1-talose residue alters the conformation of the LPS O antigen and/or blocks the bacteriophage binding site. Finally, B. pseudomallei and B. thailandensis may be resistant to infection with these bacteriophages because they do not produce a coreceptor. φ1026b and φE125 do not form plaques on B. mallei strains Turkey 4 and Turkey 5, two strains that produce LPS O antigen (Table 3). Taken together, the results indicate that LPS O antigen is required, but is not sufficient, for infection with these bacteriophages. It is possible that B. mallei strains Turkey 4 and Turkey 5 do not produce a coreceptor that participates with LPS O antigen in the initial interaction with φ1026b and φE125. Further studies are required to identify and characterize this putative coreceptor and examine if it is present in B. pseudomallei and B. thailandensis.

φ1026b gene 58 and gene 59 encode a LysR family transcriptional regulator (57) and an MFS transporter (52), respectively. These genes are not present in the φE125 genome, but similar gene pairs are present in several bacterial genomes (Fig. 3). Given this information, it is feasible that φ1026b gene 58 and gene 59 were acquired together by lateral transfer from a bacterial genome. The tandem arrangement of these genes in diverse genomes suggests that they may function together. One obvious possibility is that expression of the MFS transporter is regulated by the LysR family transcriptional regulator. It is hypothesized that gene 58 and gene 59 provide a selective advantage to B. pseudomallei 1026b by allowing it to take up a solute(s) from the environment that may not be accessible to other bacteria, including other strains of B. pseudomallei. In addition, the genes may also benefit the prophage by ensuring that it is maintained in the chromosome of its host. Unfortunately, no phenotype was observed for a strain (DD5025) harboring a mutation in gene 59. There was no difference in the growth, virulence, or catabolism of 190 carbon sources between 1026b and DD5025. Preliminary studies have indicated that while these two strains have similar growth rates in brain heart infusion broth, DD5025 grows noticeably slower in brain heart infusion broth containing 3.5% NaCl. One of the strategies used by bacteria to cope with environments with elevated osmolarity is to take up osmoprotective compounds, termed compatible solutes (60). gp59 is a member of the metabolite:H+ symporter family of transporters, and the metabolites transported by this family include compatible solutes (52). Thus, the biological function of gp59 may be to transport a compatible solute into the cell and allow B. pseudomallei 1026b to overcome environmental salt stress (60).

φ1026b and φE125 encode a RelE-like toxin that is flanked by a transcriptional regulator and a class I holin (Fig. 3). The presence of both a class I holin (gp82 in φ1026b, gp70 in φE125) and a class II holin (gp23 in φ1026b, gp24 in φE125) in these bacteriophages is unusual, and it is not known if one or both of these holins are required for the programmed release of lysozyme from the cytoplasm prior to the bacteriophage burst (65). RelE toxin and RelB antitoxin are members of an E. coli toxin-antitoxin protein system that reversibly inhibits protein synthesis in response to nutrient limitation (33). The genes encoding toxin-antitoxin systems are widespread in bacteria and are typically adjacent to one another on plasmids or chromosomes (8). The antitoxin binds the toxin and prevents it from killing the bacterial host by binding to essential enzymes or disrupting important cellular functions. The antitoxin component is typically less stable than the toxin component, and decreased transcription or translation of the antitoxin results in death of the bacterial host. Toxin-antitoxin systems were first identified on plasmids, where they play an important role in plasmid stabilization (8). The presence of toxin-antitoxin genes in bacteriophage genomes is uncommon and may be a mechanism by which prophages maintain their genomes in their bacterial hosts. However, φ1026b and φE125 do not harbor an obvious antitoxin gene, and future experiments should explore if there is a novel antitoxin gene and what, if any, function the putative toxin-antitoxin system plays in these bacteriophages.

In conclusion, bacteriophages are significant contributors to the genomic diversity of B. pseudomallei isolates. The bacteriophage described in this study was specific for B. mallei, and it exhibited a mosaic genetic relationship with bacteriophage φE125, another B. mallei-specific bacteriophage produced by B. thailandensis (70). Thus, it appears that B. mallei may be an ideal host for the study of additional bacteriophages produced by B. pseudomallei and B. thailandensis. Direct comparisons of the bacteriophages produced by these species may reveal virulence genes that are present in B. pseudomallei bacteriophages but not in B. thailandensis bacteriophages.

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REFERENCES


