Genomic Diversity of *Burkholderia pseudomallei* Clinical Isolates: Subtractive Hybridization Reveals a *Burkholderia mallei*-Specific Prophage in *B. pseudomallei* 1026b

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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 septicemic 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.

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

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14. ABSTRACT

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15. SUBJECT TERMS

Burkholderia mallei, pseudomallei, glanders, melioidosis, gemetic diversity, subtractive hybridization, bacteriophage

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TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristics[a]	Source or reference
pCR2.1-TOPO	3.9-kb TA cloning vector; pMB1 *ori*; Km[r] Ap[r]	Invitrogen
pSKM11	Positive selection cloning and suicide vector; IncP *oriT*; ColE1 *ori*; Ap[r] Tc[s]	47
pGEM-7zf(+)	Standard cloning vector; pMB1 *ori*; Ap[r]	Promega
pMOLUC	Vector for cloning large DNA fragments; pBR322 *ori*; Ap[r]	28
pDD80	pGEM-7zf(+) containing 3,625-bp HindIII fragment from φ1026b	This study
pDD81	pGEM-7zf(+) containing 3,105-bp HindIII fragment from φ1026b	This study
pDD82	pGEM-7zf(+) containing 1,068-bp HindIII fragment from φ1026b	This study
pDD83	pGEM-7zf(+) containing 602-bp HindIII fragment from φ1026b	This study
pDD84	pGEM-7zf(+) containing 3,791-bp HindIII fragment from φ1026b	This study
pDD85	pGEM-7zf(+) containing 2,515-bp HindIII fragment from φ1026b	This study
pDD86	pGEM-7zf(+) containing 8,411-bp HindIII fragment from φ1026b	This study
pDD87	pGEM-7zf(+) containing 9,355-bp HindIII fragment from φ1026b	This study
pDD88	pMOLUC containing 12,775-bp HindIII fragment from φ1026b	This study
pDD94	pSKM11 containing 930-bp internal fragment of φ1026b gene *59* generated by PCR with primers MFS-2 and MFS-3; Ap[r] Tc[r]	This study
pDD101	pCR2.1-TOPO containing 126-bp HindIII fragment from φ1026b	This study
pDD5025B	pSKM11 containing 9,492-bp HindIII fragment from φ1026b; Ap[r] Tc[r]	This study

[a] Km[r], kanamycin resistant; Ap[r], ampicillin resistant; Tc[s], tetracycline sensitive; Tc[r], tetracycline resistant.

study: 316c, NCTC 4845, 1026b, WRAIR 1188, USAMRU Malaysia 32, Pasteur 52237, STW 199-2, STW 176, STW 115-2, STW 152, STW 102-3, STW 35-1, K96243, 576a, 295, 296, 503, 506, 112c, 238, 423, 465a, 776, 439a, 487, 644, 713, 730, E8, E12, E13, E24, E25, E40, E203, E210, E214, E215, E250, E272, E277, E279, E280, E283, E284, E300, E301, E302, and E304 (3, 21, 23, 29, 31, 61, 70). *Burkholderia thailandensis* strains E27, E30, E32, E96, E100, E105, E111, E120, E125, E132, E135, E202, E251, E253, E254, E255, E256, E257, E258, E260, E261, E263, E264, E266, E267, E275, E285, E286, E290, E293, E295, and E299 (7, 61, 71) were also utilized in this study. Other *Burkholderia* strains used in this study included *Burkholderia cepacia* LMG 1222 (44), *Burkholderia multivorans* C5568, *B. multivorans* LMG 18823 (44), *Burkholderia cenocepacia* LMG 18863 (44), *B. cenocepacia* 715j (46), *Burkholderia stabilis* LMG 07000, *Burkholderia vietnamiensis* LMG 16232 (44), *B. vietnamiensis* LMG 10929 (44), *Burkholderia gladioli* 2-72 (58), *B. gladioli* 2-75 (58), *B. gladioli* 4-54 (58), *B. gladioli* 5-62 (58), *Burkholderia uboniae* EY 3383 (73), *Burkholderia cocovenans* ATCC 33664, *Burkholderia pyrrocinia* ATCC 15958, *Burkholderia glathei* ATCC 29195, *Burkholderia caryophylli* Pc 102, *Burkholderia andropogonis* PA-133, *Burkholderia kururiensis* KP23 (74), *Burkholderia sacchari* IPT101 (6), *Burkholderia* sp. strain 2.2N (13), and *Burkholderia* sp. strain T-22-8A. *Ralstonia solanacearum* FC228, FC229, and FC230, *Pandoraea apista* LMG 16407 (19), *Pandoraea norimbergensis* LMG 18379 (19), *Pandoraea pnomenusa* LMG 18087 (19), *Pandoraea pulmonicola* LMG 18106 (19), *Stenotrophomonas maltophilia* XM16 (43), *S. maltophilia* XM47 (43), *Pseudomonas aeruginosa* PAO (38), *P. aeruginosa* PA14 (54), *Pseudomonas syringae* DC3000 (66), *Salmonella enterica* serovar Typhimurium SL1344 (37), *Serratia marcescens* H11, *Escherichia coli* TOP10 (Invitrogen), and *E. coli* S17-1λ*pir* (59) were also used in this study. *E. coli* was grown at 37°C on Luria-Bertani (LB) agar (Lennox) or in LB broth (Lennox). *P. syringae*, *B. andropogonis*, *Burkholderia* sp. strain 2.2N, *Burkholderia* sp. strain T-22-8A, *B. glathei*, and *B. caryophylli* were grown at 25°C on LB agar or in LB broth containing 4% glycerol. All other bacterial strains were grown at 37°C on LB agar or in LB broth containing 4% glycerol. When appropriate, antibiotics were added at the following concentrations: 100 μg of ampicillin per ml, 25 μg of kanamycin per ml, and 15 μg of tetracycline per ml for *E. coli*; and 100 μg of streptomycin per ml and 50 μg of tetracycline per ml for *B. pseudomallei* DD5025. In addition, *B. mallei* DD3008 was grown in the presence of 5 μg of gentamicin per ml, and *B. mallei* NCTC 120(pBHR1-*wbiE*) was grown in the presence of 15 μg of polymyxin B per ml and 5 μg of kanamycin per ml.

MLST of *B. pseudomallei* 1026b. The pairs of primers used for PCR amplification and sequencing of the seven housekeeping gene fragments have been described previously (32). The multilocus sequence typing (MLST) database (www.mlst.net) identification number for *B. pseudomallei* 1026b is 208.

Subtractive hybridization. Subtractive hybridization was performed by using *B. pseudomallei* 1026b genomic DNA as the tester and *B. pseudomallei* K96243 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 hybridiza-

tion 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 5×10^6 bacteria in 100 μl of 0.05 M carbonate 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 monoclonal antibody 3D11 (Research Diagnostics, Inc.) was added, and the plate was incubated at 37°C for 1 h. Monoclonal antibody 3D11 is specific for the LPS O antigen of *B. mallei*. The wells were washed, and a 1:1,000 dilution of a peroxidase-labeled goat anti-mouse immunoglobulin G(H+L) [IgG(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′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS) peroxidase substrate system (KPL) for 10 min. The optical density at 410 nm was determined.

φ1026b sensitivity testing. Approximately 10^2 PFU of φ1026b 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 φ1026b 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 φ1026b after incubation at 25 and 37°C. No bacterial species tested formed plaques in the absence of φ1026b.

Negative staining of φ1026b. The procedure used for negatively staining bacteriophage φ1026b with 1% phosphotungstic acid (PTA) (pH 6.6) has been described previously (70).

DNA manipulation and plasmid conjugation. Restriction enzymes and T4 DNA ligase were purchased from Roche Molecular Biochemicals and were used according to the manufacturer's instructions. DNA fragments used in cloning procedures were excised from agarose gels and purified with a GeneClean III kit (Q · BIOgene). Bacterial genomic DNA was prepared by a previously described protocol (68). Plasmids were purified from overnight cultures by using Wizard Plus SV Minipreps (Promega). The suicide vector pDD94 was electroporated into *E. coli* S17-1λ*pir* (12.25 kV/cm) and conjugated with *B. pseudomallei* 1026b for 8 h, as described elsewhere (23). The resulting strain, *B. pseudomallei* DD5025, contained pDD94 integrated into gene *59* of the φ1026b prophage. Chromosomal DNA was isolated from DD5025 and digested with restriction endonuclease BamHI, and the bacteriophage attachment site and flanking bacterial DNA were obtained by self-cloning (23).

DNA sequencing and analysis. DNA sequencing was performed at ACGT, Inc. (Wheeling, Ill.) and at the LMT Sequencing Lab (Frederick, Md.). Most φ1026b

TABLE 2. Subtractive hybridization products present in *B. pseudomallei* 1026b but not in *B. pseudomallei* K96243

Plasmid	Insert size (bp)	G+C content (%)	Protein function	Best BLASTP hit	E value	Accession no.
pSH3	756	53.6	Hypothetical protein	*Helicobacter pylori* J99	1e-04	NP_224110
				Helicobacter pylori 26695	3e-04	NP_208290
pSH4	235	55.7	Phage-related protein	Phage φE125	7e-05	NP_536410
pSH5	1,113	57.8	Transposase B of insertion sequence IS*Bp*1	*Burkholderia pseudomallei*	4e-77	AAG39072
pSH6	326	50.6	Putative Rossmann fold nucleotide-binding protein involved in DNA uptake	*Novosphingobium aromaticivorans*	2e-35	ZP_00093056
				Thermoanaerobacter tengcongensis	2e-14	NP_623068
pSH7	350	50.3	Putative ATP binding protein	*Ralstonia solanacearum*	2e-26	NP_521299
pSH8	321	52.0				
pSH10	1,217	47.6				
pSH11	517	52.2				
pSH13	432	44.2	Hypothetical protein	*Ralstonia metallidurans*	9e-64	ZP_00022203
				Nostoc sp. strain PCC 7120	3e-34	NP_487474
pSH18	1,440	49.2	Phage-related and hypothetical proteins	*Magnetococcus* sp. strain MC-1	3e-13	ZP_00042491
				Nostoc punctiforme	1e-42	ZP_00112321
				Phage φE125		NP_536385
pSH19	478	48.9				
pSH21	379	43.5				
pSH23	270	53.7	Putative ATP binding protein	*Ralstonia solanacearum*	2e-13	NP_521299
pSH25	434	49.5	Phage-related integrase	*Xanthomonas axonopodis*	5e-05	NP_642500
				Ralstonia metallidurans	8e-05	ZP_00023697
pSH26	419	42.7				
pSH29	167	50.3				
pSH31	490	53.8	Putative acetyltransferase	*Methanosarcina mazei* Goe1	0.008	NP_632505
				Pseudomonas aeruginosa PAO1	0.019	NP_253367
pSH35	268	54.5				
pSH36	321	52.0	Putative site-specific DNA methyltransferase	*Cenarchaeum symbiosum*	5e-04	T31327
				Pseudomonas syringae	6e-04	ZP_00127033
pSH37	888	59.7	Phage-related and hypothetical proteins	Phage φE125	3e-52	NP_536387
				Bordetella parapertussis	0.25	NP_884214
pSH38	741	46.8				
pSH40	441	50.3	Putative AraC family transcriptional regulator	*Vibrio vulnificus*	2e-06	NP_935090
				Bradyrhizobium japonicum	4e-06	NP_773722
pSH42	389	49.9	Putative ATP binding protein	*Ralstonia solanacearum*	1e-05	NP_521299
pSH47	790	45.1	Phage-related integrase	*Xanthomonas axonopodis*	7e-15	NP_643606
				Vibrio parahaemolyticus	3e-14	NP_797022
pSH49	235	55.7				
pSH51	531	46.1	Putative plasmid mobilization protein	*Zymomonas mobilis* plasmid ZM2	1.0	P15255
				Treponema denticola plasmid pTS1	2.9	NP_073756
				Bartonella grahamii plasmid pBGR1	3.4	NP_696963
pSH53	335	61.5				

genes were identified by using GeneMark.hmm (http://opal.biology.gatech.edu/GeneMark/gmhmm2_prok.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 gapped BLASTX and BLASTP programs were used to search the nonredundant sequence database for homologous proteins (2). The φ1026b and φE125 genomes were aligned by using BLAST 2 SEQUENCES (http://www.ncbi.nlm.nih.gov/BLAST/bl2seq/bl2.html) with the Mega BLAST option selected.

Animal studies. Syrian hamsters (five animals per group) were infected intraperitoneally with 10^2, 10^3, and 10^4 1026b cells and 10^2, 10^3, and 10^4 DD5025 cells. The deaths in each group were monitored for 2 days, and the 50% lethal doses (LD_{50}) were determined. All of the animals died within 48 h of infection. This research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the *Guide for the Care and Use of Laboratory Animals* (http://oacu.od.nih.gov/regs/guide/guidex.htm). The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Phenotype microarray studies. PM1 and PM2 MicroPlates were purchased from BIOLOG (www.biolog.com) and were used according to the instructions supplied by the manufacturer.

Production of polyclonal antiserum against φE125. One New Zealand rabbit was immunized with 1 ml of a 1:1 mixture of bacteriophage φE125 (~10^5 PFU) and the RIBI R-700 adjuvant system (Corixa). Five hundred microliters of the antigen-adjuvant mixture was injected intramuscularly into each hind leg on days 1 and 28. Antiserum was obtained by cardiac puncture on day 39 and was stored at −20°C until it was used.

Immunogold electron microscopy. 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).

PCR amplifications. The sizes of PCR products were determined by agarose electrophoresis, and the products were cloned by using a pCR2.1 TOPO TA cloning kit (Invitrogen) and chemically competent *E. coli* TOP10 (Invitrogen). PCR amplifications were performed in 100-μl (final volume) mixtures containing 1× Taq PCR master mix (QIAGEN), each oligodeoxyribonucleotide primer at a concentration of 1 μM, and approximately 200 ng of genomic DNA. PCR mixtures were transferred to a PTC-150 MiniCycler with a Hot Bonnet accessory (MJ Research) and heated to 97°C for 5 min. This was followed by 30 cycles of a three-temperature cycling protocol (97°C for 30 s, 55°C for 30 s, and 72°C for 2 min) and one cycle at 72°C for 10 min. Genomic DNA from φ1026b was used to PCR amplify an internal fragment of gene *59* with the following oligodeoxyribonucleotide primer pair: MFS-2 (5′-ACAACCTGTCTCTGTTGCTG-3′) and MFS-3 (5′-CTGGAAACATGTCGCTAAGC-3′).

In order to determine the order and orientation of the HindIII fragments in the intact φ1026b genome, outward-oriented primers specific for the ends of each HindIII fragment (except the 126-, 602-, and 1,068-bp fragments) were synthesized, and PCRs were performed with φ1026b genomic DNA and all possible primer combinations. It was hypothesized that two HindIII fragments were adjacent if a PCR product was obtained with primer pairs specific for the

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

Strain	Relevant characteristics	Plaque formation	Reference or source
NCTC 120	LPS O-antigen mutant; *wbiE*::IS*407A*	−	70
NCTC 120 (pBHR1-*wbiE*)	LPS O antigen positive; *wbiE* provided in trans on pBHR1	+	70
NCTC 10248		+	70
NCTC 10229		+	70
NCTC 10260		+	70
NCTC 10247		+	70
NCTC 3708		+	70
NCTC 3709		+	70
ATCC 23344	Type strain; genomic sequence completed[a]	+	70
ATCC 10399		+	70
ATCC 15310	Produces LPS O antigen	+	70
DB110795	Laboratory-passaged ATCC 15310; LPS O-antigen mutant; *wbiG*::IS*407A*	−	70
BML1	Lysogen; ATCC 23344 (φ1026b)	−	This study
BML10	Lysogen; ATCC 23344 (φE125)	−	70
DD3008	ATCC 23344 derivative; capsule mutant	+	27
2002721273		+	30
2002721274		+	30
2002721276		+	30
2002721277		+	30
2002721278		+	30
2002721279		+	30
2002721280		+	30
2000031064		+	30
2000031065		+	30
2000031066		+	30
Turkey 1		+	USDA[b]
Turkey 2		+	USDA
Turkey 3		+	USDA
Turkey 4	Produces LPS O antigen	−	USDA
Turkey 5	Produces LPS O antigen	−	USDA
Turkey 6		+	USDA
Turkey 7		+	USDA
Turkey 8		+	USDA
Turkey 9		+	USDA
Turkey 10		+	USDA
ISU	LPS O-antigen mutant; genetic mutation unknown	−	USDA

[a] http://www.tigr.org/.
[b] USDA, United States Department of Agriculture.

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: 8.4R, 5′-GTGCTGTC GCACTAATCATG-3′; 3.6L, 5′-CAACGGAAGAGTCGCGATTG-3′; 3.6R, 5′-CCGACGATCTGATCAAGATC-3′; 3.1L, 5′-TGCTGCTGAAACGATATT GC-3′; 3.1R, 5′-ATCGTGAAACTCGGCGTGTC-3′; 12.8L, 5′-AACGCGCTT TGTCGATCGTG-3′; 12.8R, 5′-ACCATCTCGAAGAGTTCGTG-3′; 9.3L, 5′-TCAAGGTAGAACAGCGTGTG-3′; 9.3R, 5′-CAGCGCTCACGTAGTTCA AG-3′; 98A, 5′-TCTGACAATTCGATACGCGTG-3′; 96B, 5′-AAGCTCGAG ACGTTTCTTGG-3′; 2.5L-2, 5′-TAGCCACTCGCGAAACATCG-3′; 2.5R, 5′-TGGTTTATCGTTCGCGCATG-3′; 3.8L, 5′-GCCCCTTACTTCATTGAAC C-3′; 3.8R, 5′-AAGAGGACTCGCCGATCAAC-3′; and 8.4L, 5′-ATCGCAGT TCGCCATGCAAC-3′.
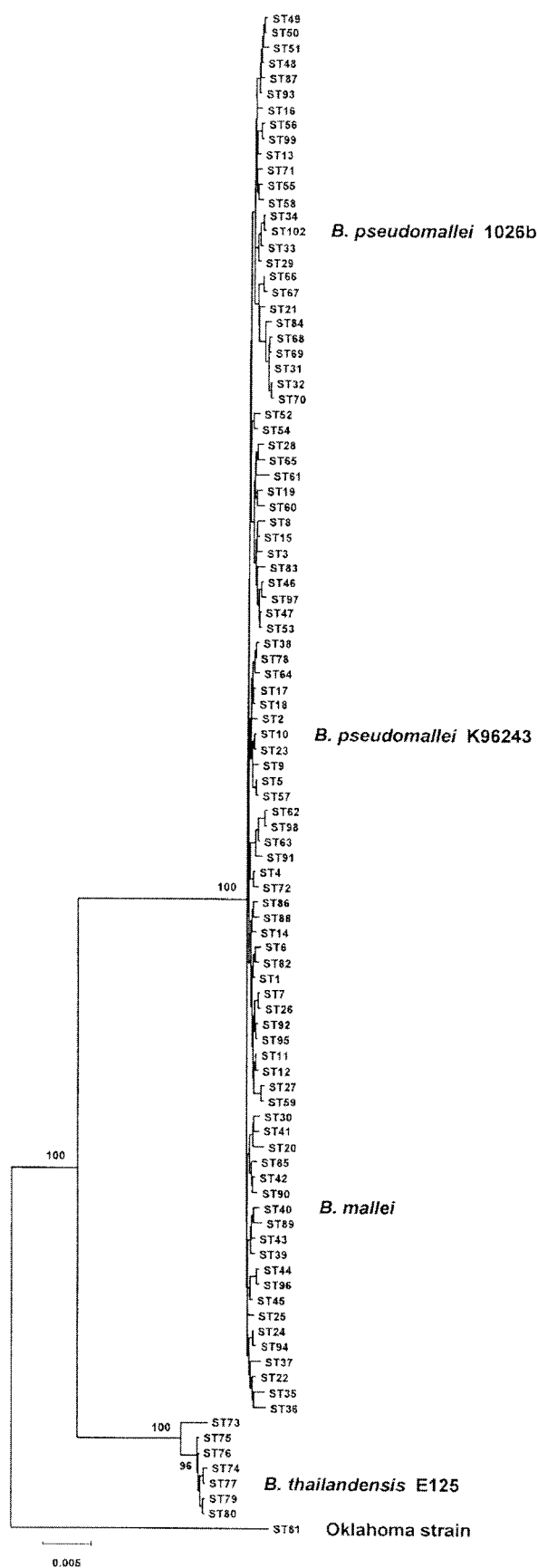
PCRs were performed with genomic DNAs from *B. pseudomallei* K96243 and 1026b, *B. mallei* ATCC 23344 and BML1, and with primers Int2 (5′-CACCGACGAGAAGATGACTG-3′) and Int5 (5′-TTGAATCGCACCGT TTGGTG-3′) to determine if φ1026b integrated into the tRNA^{Pro-3} gene. A single PCR product of the expected 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

FIG. 1. Minimum-evolution tree constructed from the concatenated sequences of seven MLST loci. The seven housekeeping genes used for the MLST scheme are *ace*, *gltB*, *gmhD*, *lepA*, *lipA*, *narK*, and *ndh* (32). The concatenated sequences from 100 sequence types, representing isolates of *B. pseudomallei*, *B. mallei*, and *B. thailandensis* and the Oklahoma strain (72), were used to construct the minimum-evolution tree. The positions of the *B. mallei* clone (ST40), *B. pseudomallei* 1026b (ST102), *B. pseudomallei* K96243 (ST10), *B. thailandensis* E125 (ST77), and the Oklahoma strain (ST81) are indicated. The levels of recovery of the major nodes in 1,000 bootstrap replicates (expressed as percentages) are also indicated. Bar = differences at 0.5% of the nucleotide sites.

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 φE125 proteins (pSH4, pSH18, and pSH37). The IS*3* family insertion sequence IS*Bp1* (69) was present in plasmid pSH5 (Table 2). Woo et al. previously demonstrated that IS*Bp1* was present in ~65% of *B. pseudomallei* strains but was absent from K96243 (69). The nucleotide sequence of the pSH5 DNA insert suggests that IS*Bp1* 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-*wbiE* 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. cenocepacia*, *B. stabilis*, *B. vietnamiensis*, *B. gladioli*, *B. uboniae*, *B. cocovenans*, *B. pyrrocinia*, *B. glathei*, *B. caryophylli*, *B. andropogonis*, *B. kururiensis*, *Burkholderia* sp. strain 2.2N, *Burkholderia* sp. strain T-22-8A, *P. apista*, *P. norimbergensis*, *P. pnomenusa*, *P. pulmonicola*, *P. aeruginosa*, *P. syringae*, *R. solanacearum*, *S. maltophilia*, *S. enterica* serovar Typhimurium, *S. marcescens*, and *E. coli*. Bacteriophage φ1026b formed plaques with none of these bacteria. These results demonstrate that bacteriophage φ1026b forms plaques only on *B. mallei* strains and that LPS O antigen is required but is not sufficient for plaque formation by φ1026b. Note that the host range of φ1026b is identical to the host range of bacteriophage φE125 (70). These results suggest that bacteriophage φ1026b may be a useful diagnostic tool for differentiating *B. pseudomallei* and *B. mallei*, two closely related biological threat agents (56). However, there is no advantage to using φ1026b rather than φE125 for this application (70).

Bacteriophage φ1026b has an isometric head and a long, noncontractile tail. Bacteriophages may be tailed, polyhedral, filamentous, or pleomorphic and can be classified by morphotype and by the nature of the nucleic acid (1). Numerous negatively stained bacteriophages 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′-GCGGGCGAAG-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 BLASTP 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 tRNA[Pro-3] 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
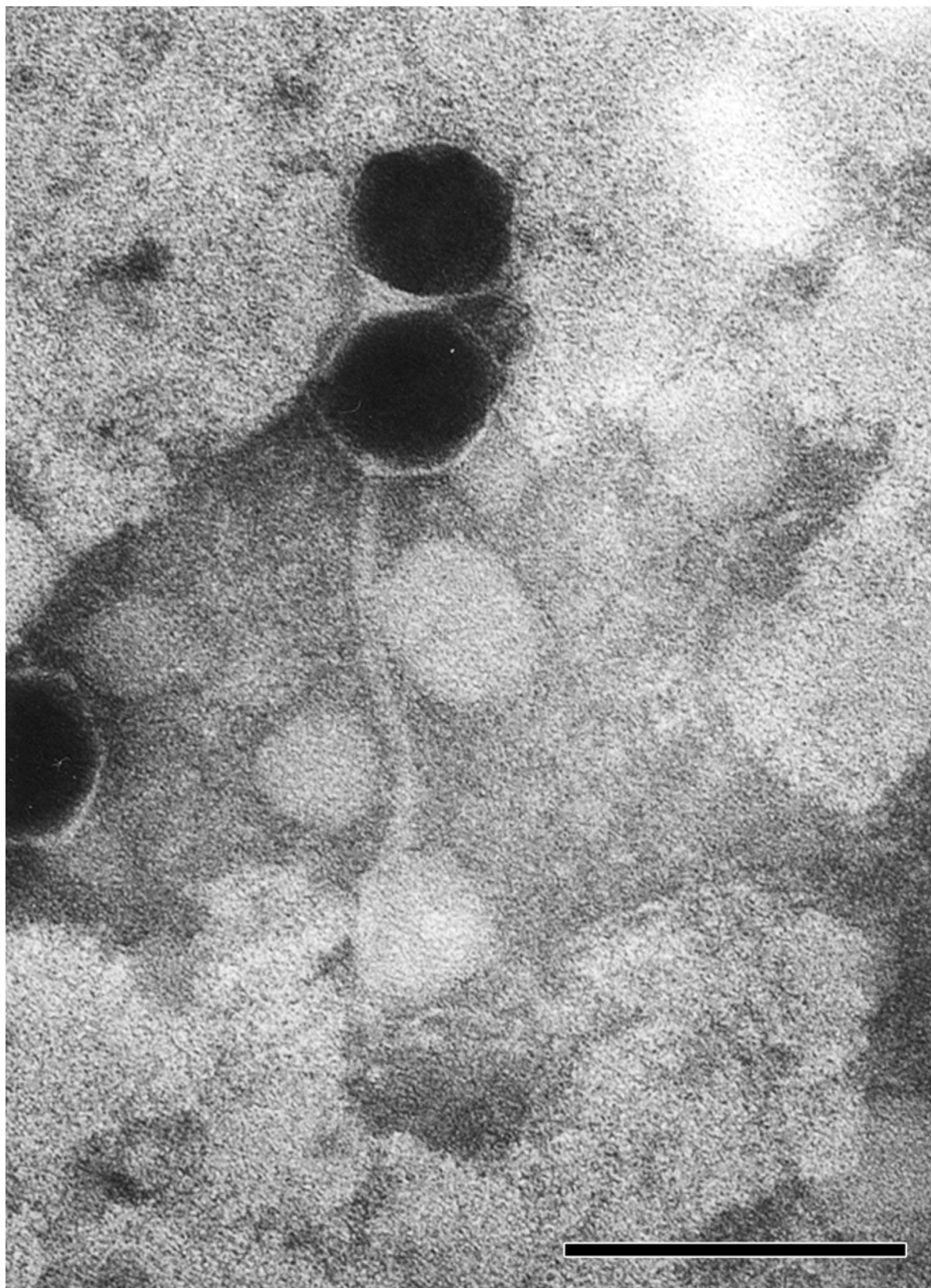
FIG. 2. Transmission electron micrograph of bacteriophage φ1026b negatively stained with 1% PTA. One intact bacteriophage (head and tail) and one bacteriophage head without an attached tail are shown. Scale bar = 100 nm.
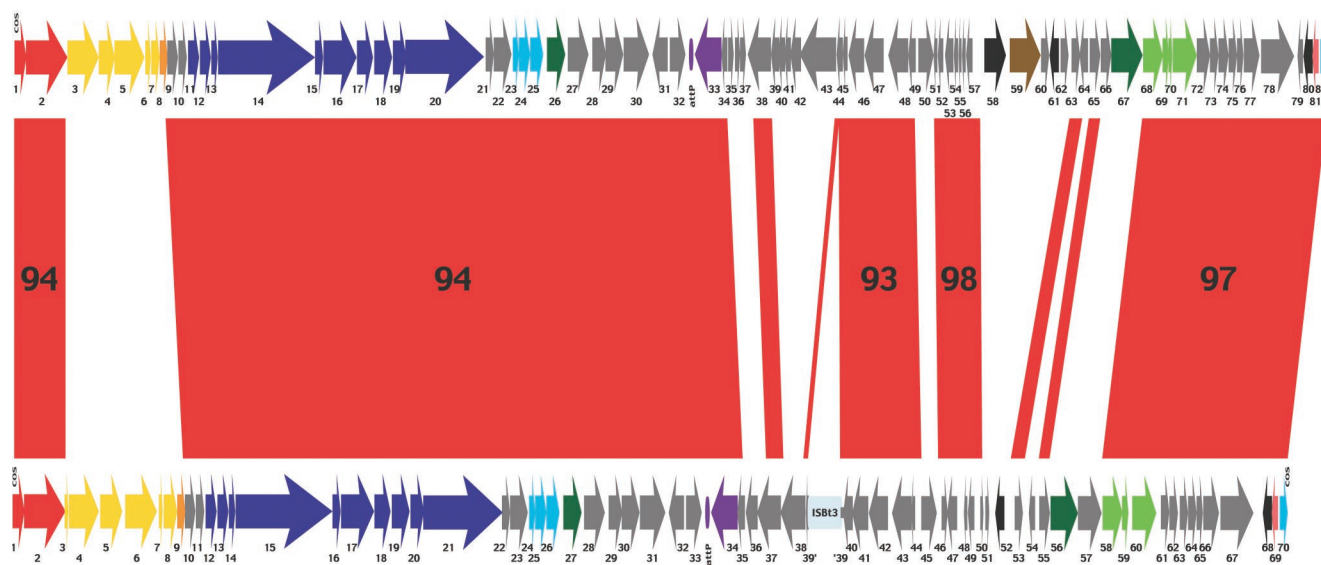
φ1026b

FIG. 3. Comparative analysis of the genomes of *Burkholderia* bacteriophages φ1026b and φE125. The genomes of temperate bacteriophages φ1026b and φE125 are depicted schematically at the top and bottom, respectively. Red indicates DNA sequences that are present in both bacteriophages, and the numbers in the red areas indicate the percentages of nucleotide identity in conserved regions that are 1 kb long or longer. The putative functions of proteins encoded by φ1026b genes are color coded, and insertion sequence IS*Bt3* in gene *39* of φE125 is indicated by pale blue. Gene *25a* (φ1026b) and gene *26a* (φE125) are not shown for clarity.

to the *attP* site of φE125 (70). It is worth emphasizing that *B. pseudomallei* 1026b and *B. thailandensis* E125 both contain bacteriophages integrated at tRNA^{Pro-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 IS*Bt3* 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) can 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 bacterioprophages 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 190 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 10^2, 10^3, and 10^4 cells of 1026b and DD5025, and the LD_{50}s were determined 2 days postinfection. The LD_{50} for both strains was $<10^2$ 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
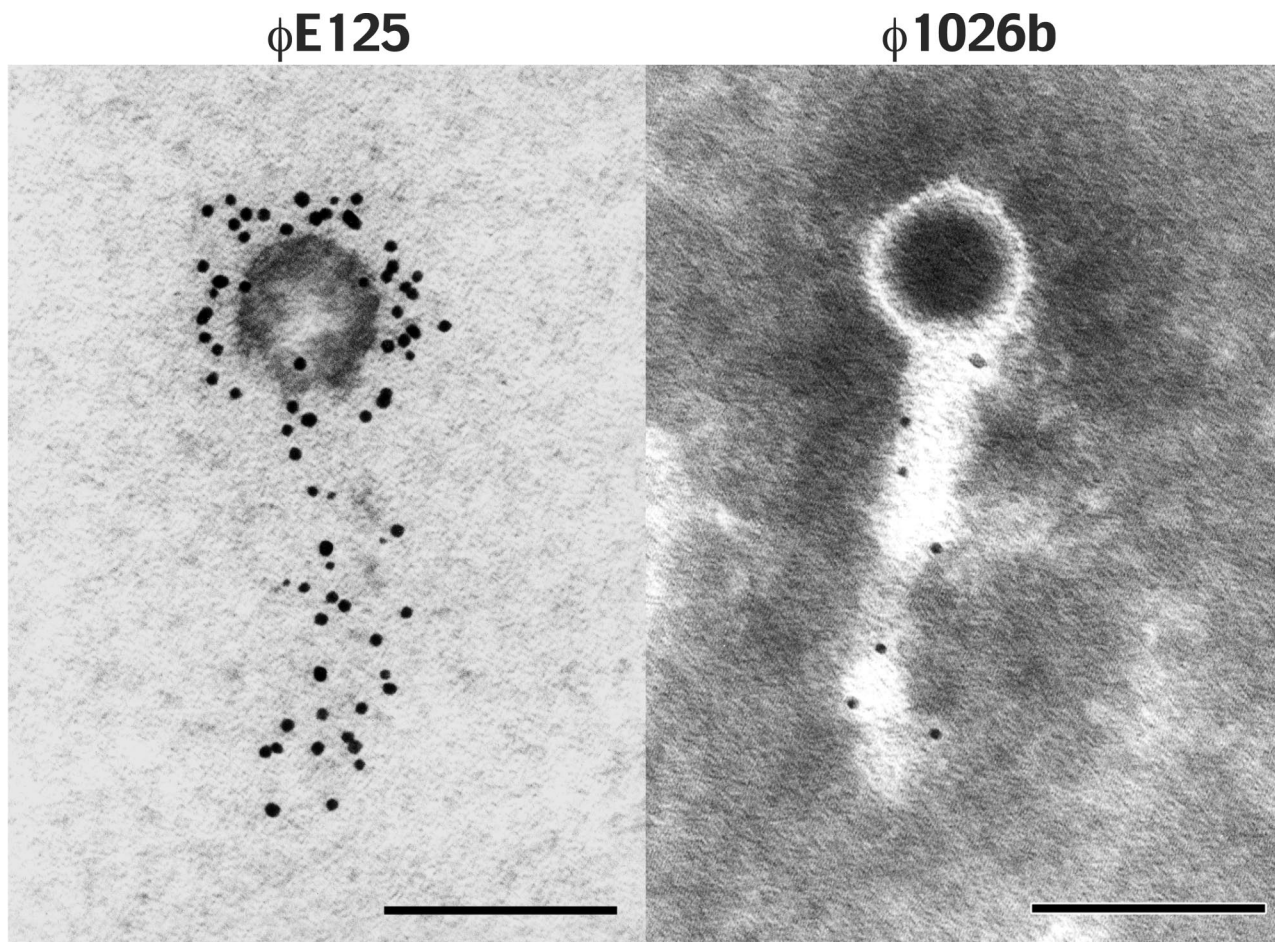
φE125 φ1026b

FIG. 4. Immunogold electron microscopy of bacteriophages φE125 and φ1026b. The bacteriophages were reacted with polyclonal rabbit antiserum directed against φE125, washed, and reacted with goat anti-rabbit IgG gold conjugate (5 nm). Bacteriophage φ1026b was subsequently negatively stained with 1% PTA. Scale bar = 100 nm.

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 φ1026b and φE125 revealed regions with high sequence similarity interspersed with regions displaying no sequence similarity (Fig. 3). This mosaic genetic relationship indicates that recombination between φ1026b or φE125 and an unrelated bacteriophage(s) occurred during the evolution of these *Burkholderia* bacteriophages, which resulted in acquisition of new head and lysogeny genes. The φ1026b head morphogenesis genes more closely resemble the head morphogenesis genes of *P. aeruginosa* and *P. putida* bacteriophages than the corresponding genes in φE125. In addition, the host lysis cassettes of φ1026b (genes *23* to *25*) and φE125 (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-

ule-host lysis cassette module organization seems to be an ancestral trait in at least a subgroup of *Burkholderia* and *Pseudomonas Siphoviridae*.

It is curious that bacteriophages φ1026b and φE125 specifically infect *B. mallei* but are harbored by *B. pseudomallei* and *B. thailandensis*. What is the mechanism by which *B. pseudomallei* and *B. thailandensis* strains are resistant to infections with φ1026b and φE125? First, *B. pseudomallei* and *B. thailandensis* strains may be immune to superinfection with these bacteriophages because they harbor similar prophages. The genomic sequence of *B. pseudomallei* K96243 contains eight genes that are nearly identical to φ1026b genes (http://www.sanger.ac.uk/), and Woods et al. (70) found that 31% of *B. thailandensis* strains harbor a φE125-like prophage. Thus, it is clear that some *B. pseudomallei* and *B. thailandensis* strains are lysogenic and may be immune to superinfection with φ1026b and φE125. However, 69% of *B. thailandensis* strains did not possess an φE125-like prophage, suggesting that superinfection immunity alone is not responsible for their resistance to infection with φE125 (70). Second, there may be differences in the bacteriophage receptors present on *B. mallei* and on *B. pseudomallei* and *B. thailandensis*. LPS O antigen is required for plaque formation on *B. mallei*, indicating that this is the

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 L-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 L-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 stabile 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.

REFERENCES

1. **Ackermann, H.-W.** 2003. Bacteriophage observations and evolution. Res. Microbiol. **154:**245–251.
2. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
3. **Anuntagool, N., P. Aramsri, T. Panichakul, V. Wuthiekanun, R. Kinoshita, N. J. White, and S. Sirisinha.** 2000. Antigenic heterogeneity of lipopolysaccharide among *Burkholderia pseudomallei* clinical isolates. Southeast Asian J. Trop. Med. Public Health **31:**146–152.
4. **Atkins, T., R. Prior, K. Mack, P. Russell, M. Nelson, J. Prior, J. Ellis, P. C. Oyston, G. Dougan, and R. W. Titball.** 2002. Characterization of an acapsular mutant of *Burkholderia pseudomallei* identified by signature tagged mutagenesis. J. Med. Microbiol. **51:**539–547.
5. **Boyd, E. F., and H. Brussow.** 2002. Common themes among bacteriophage-encoded virulence factors and diversity among the bacteriophages involved. Trends Microbiol. **10:**521–529.
6. **Brämer, C. O., P. Vandamme, L. F. da Silva, J. G. C. Gomez, and A. Steinbüchel.** 2001. *Burkholderia sacchari* sp. nov., a polyhydroxyalkanoate-accumulating bacterium isolated from soil of a sugar-cane plantation in Brazil. Int. J. Syst. Bacteriol. Evol. Microbiol. **51:**1709–1713.
7. **Brett, P. J., D. DeShazer, and D. E. Woods.** 1998. *Burkholderia thailandensis* sp. nov., description of a *Burkholderia pseudomallei*-like species. Int. J. Syst. Bacteriol. **48:**317–320.

8. **Brown, J. M., and K. J. Shaw.** 2003. A novel family of *Escherichia coli* toxin-antitoxin gene pairs. J. Bacteriol. **185:**6600–6608.

9. **Brown, N. F., and I. R. Beacham.** 2000. Cloning and analysis of genomic differences unique to *Burkholderia pseudomallei* by comparison with *B. thailandensis.* J. Med. Microbiol. **49:**993–1001.

10. **Brussow, H., and F. Desiere.** 2001. Comparative phage genomics and the evolution of *Siphoviridae*: insights from dairy phages. Mol. Microbiol. **39:**213–222.

11. **Brussow, H., and R. W. Hendrix.** 2002. Phage genomics: small is beautiful. Cell **108:**13–16.

12. **Burtnick, M. N., P. J. Brett, and D. E. Woods.** 2002. Molecular and physical characterization of *Burkholderia mallei* O antigens. J. Bacteriol. **184:**849–852.

13. **Cain, C. C., A. T. Henry, R. H. Waldo III, L. J. Casida, Jr., and J. O. Falkinham III.** 2000. Identification and characteristics of a novel *Burkholderia* strain with broad-spectrum antimicrobial activity. Appl. Environ. Microbiol. **66:**4139–4141.

14. **Campbell, A.** 2003. Prophage insertion sites. Res. Microbiol. **154:**277–282.

15. **Canchaya, C., G. Fournous, S. Chibani-Chennoufi, M.-L. Dillmann, and H. Brussow.** 2003. Phage as agents of lateral gene transfer. Curr. Opin. Microbiol. **6:**417–424.

16. **Canchaya, C., C. Proux, G. Fournous, A. Bruttin, and H. Brussow.** 2003. Prophage genomics. Microbiol. Mol. Biol. Rev. **67:**238–276.

17. **Casjens, S.** 2003. Prophages and bacterial genomics: what have we learned so far? Mol. Microbiol. **49:**277–300.

18. **Chaowagul, W., N. J. White, D. A. Dance, Y. Wattanagoon, P. Naigowit, T. M. Davis, S. Looareesuwan, and N. Pitakwatchara.** 1989. Melioidosis: a major cause of community-acquired septicemia in northeastern Thailand. J. Infect. Dis. **159:**890–899.

19. **Coenye, T., E. Falsen, B. Hoste, M. Ohlen, J. Goris, J. R. W. Govan, M. Gillis, and P. Vandamme.** 2000. Description of *Pandoraea* gen. nov. with *Pandoraea apista* sp. nov., *Pandoraea pulmonicola* sp. nov., *Pandoraea pnomenusa* sp. nov., *Pandoraea sputorum* sp. nov. and *Pandoraea norimbergensis* comb. nov. Int. J. Syst. Evol. Microbiol. **50:**887–899.

20. **Craig, N. L.** 1988. The mechanism of conservative site-specific recombination. Annu. Rev. Genet. **22:**77–105.

21. **Currie, B. J., D. A. Fisher, D. M. Howard, J. N. Burrow, D. Lo, S. Selva-Nayagam, N. M. Anstey, S. E. Huffam, P. L. Snelling, P. J. Marks, D. P. Stephens, G. D. Lum, S. P. Jacups, and V. L. Krause.** 2000. Endemic melioidosis in tropical northern Australia: a 10-year prospective study and review of the literature. Clin. Infect. Dis. **31:**981–986.

22. **Dance, D. A. B.** 2002. Melioidosis. Curr. Opin. Infect. Dis. **15:**127–132.

23. **DeShazer, D., P. J. Brett, R. Carlyon, and D. E. Woods.** 1997. Mutagenesis of *Burkholderia pseudomallei* with Tn5-OT182: isolation of motility mutants and molecular characterization of the flagellin structural gene. J. Bacteriol. **179:**2116–2125.

24. **DeShazer, D., P. J. Brett, and D. E. Woods.** 1998. The type II O-antigenic polysaccharide moiety of *Burkholderia pseudomallei* lipopolysaccharide is required for serum resistance and virulence. Mol. Microbiol. **30:**1081–1100.

25. **DeShazer, D., and D. E. Woods.** 1999. Animal models of melioidosis, p. 199–203. *In* O. Zak and M. Sande (ed.), Handbook of animal models of infection. Academic Press Ltd., London, United Kingdom.

26. **DeShazer, D., and D. E. Woods.** 1999. Pathogenesis of melioidosis: use of Tn5-OT182 to study the molecular basis of *Burkholderia pseudomallei* virulence. J. Infect. Dis. Antimicrob. Agents **16:**91–96.

27. **DeShazer, D., D. M. Waag, D. L. Fritz, and D. E. Woods.** 2001. Identification of a *Burkholderia mallei* polysaccharide gene cluster by subtractive hybridization and demonstration that the encoded capsule is an essential virulence determinant. Microb. Pathog. **30:**253–269.

28. **Feng, T., Z. Li, W. Jiang, B. Breyer, L. Zhou, H. Cheng, R. C. Haydon, A. Ishikawa, M. A. Joudeh, and T.-C. He.** 2002. Increased efficiency of cloning large DNA fragments using a lower copy number plasmid. BioTechniques **32:**992–998.

29. **Finkelstein, R. A., P. Atthasampunna, and M. Chulasamaya.** 2000. *Pseudomonas* (*Burkholderia*) *pseudomallei* in Thailand, 1964–1967; geographic distribution of the organism, attempts to identify cases of active infection, and presence of antibody in representative sera. Am. J. Trop. Med. Hyg. **62:**232–239.

30. **Gee, J. E., C. T. Sacchi, M. B. Glass, B. K. De, R. S. Weyant, P. N. Levett, A. M. Whitney, A. R. Hoffmaster, and T. Popovic.** 2003. Use of 16S rRNA gene sequencing for rapid identification and differentiation of *Burkholderia pseudomallei* and *B. mallei.* J. Clin. Microbiol. **41:**4647–4654.

31. **Godfrey, A. J., S. Wong, D. A. Dance, W. Chaowagul, and L. E. Bryan.** *Pseudomonas pseudomallei* resistance to beta-lactam antibiotics due to alterations in the chromosomally encoded beta-lactamase. Antimicrob. Agents Chemother. **35:**1635–1640.

32. **Godoy, D., G. Randle, A. J. Simpson, D. M. Aanensen, T. L. Pitt, R. Kinoshita, and B. G. Spratt.** 2003. Multilocus sequence typing and evolutionary relationships among the causative agents of melioidosis and glanders, *Burkholderia pseudomallei* and *B. mallei.* J. Clin. Microbiol. **41:**2068–2079. (Erratum, **41:**4913.)

33. **Gotfredsen, M., and K. Gerdes.** 1998. The *Escherichia coli relBE* genes belong to a new toxin-antitoxin gene family. Mol. Microbiol. **29:**1065–1076.

34. **Hacker, J., G. Blum-Oehler, I. Muldorfer, and H. Tschape.** 1997. Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. Mol. Microbiol. **23:**1089–1097.

35. **Hacker, J., and E. Carniel.** 2001. Ecological fitness, genomic islands and bacterial pathogenicity. EMBO Rep. **2:**376–381.

36. **Hendrix, R. W., G. F. Hatfull, and M. C. M. Smith.** 2003. Bacteriophage with tails: chasing their origins and evolution. Res. Microbiol. **154:**253–257.

37. **Hoiseth, S. K., and B. A. D. Stocker.** 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature **291:**238–239.

38. **Holloway, B. W., U. Romling, and B. Tummler.** 1994. Genomic mapping of *Pseudomonas aeruginosa* PAO. Microbiology **140:**2907–2929.

39. **Iandolo, J. J., V. Worrell, K. H. Groicher, Y. Qian, R. Tian, S. Kenton, A. Dorman, H. Ji, S. Lin, P. Loh, S. Qi, H. Zhu, and B. A. Roe.** 2002. Comparative analysis of the genomes of the temperate bacteriophages phi 11, phi 12 and phi 13 of *Staphylococcus aureus* 8325. Gene **289:**109–118.

40. **Juhala, R. J., M. E. Ford, R. L. Duda, A. Youlton, G. F. Hatfull, and R. W. Hendrix.** 2000. Genomic sequences of bacteriophages HK97 and HK022: pervasive genetic mosaicism in the lambdoid bacteriophages. J. Mol. Biol. **299:**27–51.

41. **Knirel, Y. A., N. A. Paramonov, A. S. Shashkov, N. K. Kochetkov, R. G. Yarullin, S. M. Farber, and V. I. Efremenko.** 1992. Structure of the polysaccharide chains of *Pseudomonas pseudomallei* lipopolysaccharides. Carbohydr. Res. **233:**185–193.

42. **Kropinski, A. M.** 2000. Sequence of the genome of the temperate, serotypeconverting, *Pseudomonas aeruginosa* bacteriophage D3. J. Bacteriol. **182:**6066–6074.

43. **Laing, F. P. Y., K. Ramotar, R. R. Read, N. Alfieri, A. Kureishi, E. A. Henderson, and T. J. Louie.** 1995. Molecular epidemiology of *Xanthomonas maltophilia* colonization and infection in the hospital environment. J. Clin. Microbiol. **33:**513–518.

44. **Mahenthiralingam, E., T. Coenye, J. W. Chung, D. P. Speert, J. R. W. Govan, P. Taylor, and P. Vandamme.** 2000. Diagnostically and experimentally useful panel of strains from the *Burkholderia cepacia* complex. J. Clin. Microbiol. **38:**910–913.

45. **Manzeniuk, O. I., N. V. Volozhantsev, and E. A. Svetoch.** 1994. Identification of *Pseudomonas mallei* bacteria with the help of *Pseudomonas pseudomallei* bacteriophages Mikrobiologiya **63:**537–544. (In Russian.)

46. **McKevitt, A. I., S. Bajaksouzian, J. D. Klinger, and D. E. Woods.** 1989. Purification and characterization of an extracellular protease from *Pseudomonas cepacia.* Infect. Immun. **57:**771–778.

47. **Mongkolsuk, S., S. Rabibhadana, P. Vattanaviboon, and S. Loprasert.** 1994. Generalized and mobilizabile positive-selection cloning vectors. Gene **143:**145–146.

48. **Nakagawa, I., K. Kurokawa, A. Yamashita, M. Nakata, Y. Tomiyasu, N. Okahashi, S. Kawabata, K. Yamazaki, T. Shiba, T. Yasunaga, H. Hayashi, M. Hattori, and S. Hamada.** 2003. Genomic sequencing of an M3 strain of *Streptococcus pyogenes* reveals a large-scale genomic rearrangement in invasive strains and new insights into phage evolution. Genome Res. **13:**1042–1055.

49. **Ochman, H., J. G. Lawrence, and E. A. Groisman.** 2000. Lateral gene transfer and the nature of bacterial innovation. Nature **405:**299–304.

50. **Ochman, H., and N. A. Moran.** 2001. Genes lost and genes found: evolution of bacterial pathogenesis and symbiosis. Science **292:**1096–1098.

51. **Osborn, A. M., and D. Boltner.** 2002. When phage, plasmids, and transposons collide: genomic islands, and conjugative- and mobilizable-transposons as a mosaic continuum. Plasmid **48:**202–212.

52. **Pao, S. S., I. T. Paulsen, and M. H. Saier, Jr.** 1998. Major facilitator superfamily. Microbiol. Mol. Biol. Rev. **62:**1–34.

53. **Perry, M. B., L. L. MacLean, T. Schollaardt, L. E. Bryan, and M. Ho.** 1995. Structural characterization of the lipopolysaccharide O antigens of *Burkholderia pseudomallei.* Infect. Immun. **63:**3348–3352.

54. **Rahme, L. G., E. J. Stevens, S. F. Wolfort, J. Shao, R. G. Tompkins, and F. M. Ausubel.** 1995. Common virulence factors for bacterial pathogenicity in plants and animals. Science **268:**1899–1902.

55. **Reckseidler, S. L., D. DeShazer, P. A. Sokol, and D. E. Woods.** 2001. Detection of bacterial virulence genes by subtractive hybridization: identification of capsular polysaccharide of *Burkholderia pseudomallei* as a major virulence determinant. Infect. Immun. **69:**34–44.

56. **Rotz, L. D., A. S. Khan, S. R. Lillibridge, S. M. Ostroff, and J. M. Hughes.** 2002. Public health assessment of potential biological terrorism agents. Emerg. Infect. Dis. **8:**225–230.

57. **Schell, M. A.** 1993. Molecular biology of the LysR family of transcriptional regulators. Annu. Rev. Microbiol. **47:**597–626.

58. **Segonds, C., T. Heulin, N. Marty, and G. Chabanon.** 1999. Differentiation of *Burkholderia* species by PCR-restriction fragment length polymorphism analysis of the 16S rRNA gene and application to cystic fibrosis isolates. J. Clin. Microbiol. **37:**2201–2208.

59. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1:**784–791.

60. **Sleator, R. D., and C. Hill.** 2002. Bacterial osmoadaptation: the role of osmolytes in bacterial stress and virulence. FEMS Microbiol. Rev. **26:**49–71.
61. **Smith, M. D., V. Wuthiekanun, A. L. Walsh, and N. J. White.** 1995. Quantitative recovery of *Burkholderia pseudomallei* from soil in Thailand. Trans. R. Soc. Trop. Med. Hyg. **89:**488–490.
62. **Smith, P. B., and W. B. Cherry.** 1957. Identification of *Malleomyces* by specific bacteriophages. J. Bacteriol. **74:**668–672.
63. **Tatusova, T. A., and T. L. Madden.** 1999. BLAST 2 Sequences, a new tool for comparing protein and nucleotide sequences. FEMS Microbiol. Lett. **174:**247–250.
64. **Van Sluys, M. A., M. C. de Oliveira, C. B. Monteiro-Vitorello, C. Y. Miyaki, L. R. Furlan, L. E. Camargo, A. C. da Silva, D. H. Moon, M. A. Takita, E. G. Lemos, M. A. Machado, M. I. Ferro, F. R. da Silva, M. H. Goldman, G. H. Goldman, M. V. Lemos, H. El-Dorry, S. M. Tsai, H. Carrer, D. M. Carraro, R. C. de Oliveira, L. R. Nunes, W. J. Siqueira, L. L. Coutinho, E. T. Kimura, E. S. Ferro, R. Harakava, E. E. Kuramae, C. L. Marino, E. Giglioti, I. L. Abreu, L. M. Alves, A. M. do Amaral, G. S. Baia, S. R. Blanco, M. S. Brito, F. S. Cannavan, A. V. Celestino, A. F. da Cunha, R. C. Fenille, J. A. Ferro, E. F. Formighieri, L. T. Kishi, S. G. Leoni, A. R. Oliveira, V. E. Rosa, Jr., F. T. Sassaki, J. A. Sena, A. A. de Souza, D. Truffi, F. Tsukumo, G. M. Yanai, L. G. Zaros, E. L. Civerolo, A. J. Simpson, N. F. Almeida, Jr., J. C. Setubal, and J. P. Kitajima.** 2003. Comparative analysis of the complete genome sequence of Pierce's disease and citrus varigated chlorosis strains of *Xylella fastidiosa.* J. Bacteriol **185:**1018–1026.
65. **Wang, I.-N., D. L. Smith, and R. Young.** 2000. Holins: the protein clocks of bacteriophage infections. Annu. Rev. Microbiol. **54:**799–825.
66. **Whalen, M. C., R. W. Innes, A. F. Bent, and B. J. Staskawicz.** 1991. Identi-fication of *Pseudomonas syringae* pathogens of *Arabidopsis* and a bacterial locus determining avirulence on both *Arabidoposis* and soybean. Plant Cell **3:**49–59.
67. **White, N. J.** 2003. Melioidosis. Lancet **361:**1715–1722.
68. **Wilson, K.** 1987. Preparation of genomic DNA from bacteria, p. 2.4.1–2.4.5. *In* F. M. Ausubel, R. Brent, R. E. Kingston, et al. (ed.), Current protocols in molecular biology. John Wiley & Sons, New York, N.Y.
69. **Woo, P. C. Y., P. K. L. Leung, H.-W. Tsoi, B. Y. L. Chan, T.-L. Que, and K.-Y. Yuen.** 2002. Characterization of a novel insertion sequence, IS*Bp1*, in *Burkholderia pseudomallei.* Arch. Microbiol. **177:**267–273.
70. **Woods, D. E., J. A. Jeddeloh, D. F. Fritz, and D. DeShazer.** 2002. *Burkholderia thailandensis* E125 harbors a temperate bacteriophage specific for *Burkholderia mallei.* J. Bacteriol. **184:**4003–4017.
71. **Wuthiekanun, V., M. D. Smith, D. A. Dance, and N. J. White.** 1995. Isolation of *Pseudomonas pseudomallei* from soil in north-eastern Thailand. Trans. R. Soc. Trop. Med. Hyg. **89:**41–43.
72. **Yabuuchi, E., Y. Kosako, M. Arakawa, H. Hotta, and I. Yano.** 1992. Identi-fication of Oklahoma isolate as a strain of *Pseudomonas pseudomallei.* Microbiol. Immunol. **36:**1239–1249.
73. **Yabuuchi, E., Y. Kawamura, T. Ezaki, M. Ikedo, S. Dejsirilert, N. Fujiwara, T. Naka, and K. Kobayashi.** 2000. *Burkholderia uboniae* sp. nov., L-arabinose-assimilating but different from *Burkholderia thailandensis* and *Burkholderia vietnamiensis.* Microbiol. Immunol. **44:**307–317.
74. **Zhang, H., S. Hanada, T. Shigematsu, K. Shibuya, Y. Kamagata, T. Kanagawa, and R. Kurane.** 2000. *Burkholderia kururiensis* sp. nov., a trichloroeth-ylene (TCE)-degrading bacterium isolated from an aquifer polluted with TCE. Int. J. Syst. Evol. Microbiol. **50:**743–749.