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Burkholderia thailandensis E125 Harbors a Temperate Bacteriophage Specific for *Burkholderia mallei*

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***Burkholderia thailandensis* is a nonpathogenic gram-negative bacillus that is closely related to *Burkholderia mallei* and *Burkholderia pseudomallei*. We found that *B. thailandensis* E125 spontaneously produced a bacteriophage, termed φE125, which formed turbid plaques in top agar containing *B. mallei* ATCC 23344. We examined the host range of φE125 and found that it formed plaques on *B. mallei* but not on any other bacterial species tested, including *B. thailandensis* and *B. pseudomallei*. Examination of the bacteriophage by transmission electron microscopy revealed an isometric head and a long noncontractile tail. *B. mallei* NCTC 120 and *B. mallei* DB110795 were resistant to infection with φE125 and did not produce lipopolysaccharide (LPS) O antigen due to IS*407*A insertions in *wbiE* and *wbiG*, respectively. *wbiE* was provided in *trans* on a broad-host-range plasmid to *B. mallei* NCTC 120, and it restored LPS O-antigen production and susceptibility to φE125. The 53,373-bp φE125 genome contained 70 genes, an IS*3* family insertion sequence (IS*Bt*3), and an attachment site (*attP*) encompassing the 3′ end of a proline tRNA (UGG) gene. While the overall genetic organization of the φE125 genome was similar to λ-like bacteriophages and prophages, it also possessed a novel cluster of putative replication and lysogeny genes. The φE125 genome encoded an adenine and a cytosine methyltransferase, and purified bacteriophage DNA contained both N6-methyladenine and N4-methylcytosine. The results presented here demonstrate that φE125 is a new member of the λ supergroup of *Siphoviridae* that may be useful as a diagnostic tool for *B. mallei*.**

The disease glanders is caused by *Burkholderia mallei*, a host-adapted pathogen that does not persist in nature outside of its horse host (32). Glanders is a zoonosis, and humans whose occupations put them into close contact with infected animals can contract the disease. There have been no naturally occurring cases of glanders in North America in the last 60 years, but laboratory workers are still at risk of infection with *B. mallei* via cutaneous (68) and inhalational (31) routes. Human glanders has been described as a painful and loathsome disease from which few recover without antibiotic intervention (33, 51). There is little known about the virulence factors of this organism, but a recent report indicates that the capsular polysaccharide is essential for virulence in hamsters and mice (24).

Burkholderia pseudomallei is the etiologic agent of the glanders-like disease melioidosis (21). As the names suggest, *B. mallei* and *B. pseudomallei* are closely related species (19, 56, 59, 69). These β-*Proteobacteria* can now be directly compared at the genomic level because the *B. pseudomallei* K96243 genomic sequence is available at the Sanger Institute website (http://www.sanger.ac.uk/) and the *B. mallei* ATCC 23344 genomic sequence is available at the TIGR (The Institute for Genomic Research) website (http://www.tigr.org/). Preliminary BLAST (4) comparisons indicate that the genes conserved between these species are ~99% identical at the nucleotide level. This high level of nucleotide identity makes it challenging to use nucleic acid-based assays to discriminate between *B. mallei* and *B. pseudomallei* (6, 71).

There are legitimate concerns that *B. mallei* and *B. pseudomallei* may be misused as biological weapons (16, 46, 51, 60), and there is compelling evidence that *B. mallei* has already been used in this manner (3, 74). Diagnostic assays should be developed to discriminate between these microorganisms in the event that they are misused in the future. The use of a combination of diagnostic assays may be necessary to discriminate between these species, including nucleic acid-based assays, phenotypic assays (colony morphology, motility, and carbohydrate utilization), enzyme-linked immunosorbent assay, intact cell matrix-assisted laser desorption ionization–time of flight, and bacteriophage susceptibility.

In 1957 Smith and Cherry described eight lysogenic *B. pseudomallei* strains that produced bacteriophage that were more active on *B. mallei* than on *B. pseudomallei* (67). In fact, bacteriophage E attacked *B. mallei* strains exclusively. Manzenink et al. (45a) found that 91% of their *B. pseudomallei* strains were lysogenic and that three bacteriophages, PP19, PP23, and PP33, could be used in combination to identify *B. mallei*. Unfortunately, these *B. mallei*-specific bacteriophage were not further characterized and are not readily available. It is interesting that neither study identified bacteriophage production by *B. mallei* strains.

The purpose of this work was to identify and characterize a *B. mallei*-specific bacteriophage and make it available to the scientific community. *Burkholderia thailandensis* is a nonpathogenic soil saprophyte that has been described as *B. pseudomallei*-like (9, 10), and there are no published reports describing bacteriophage production by this species. *B. thailandensis*

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TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristics[a]	Source or reference
pBluescript KS	General cloning vector; ColE1; Ap^r	Stratagene
pDW1	pBluescript KS containing 1,068-bp *Hin*dIII fragment from ϕE125	This study
pDW3.2	pBluescript KS containing 3,231-bp *Hin*dIII fragment from ϕE125	This study
pDW4.4	pBluescript KS containing 4,351-bp *Hin*dIII fragment from ϕE125	This study
pDW5.5	pBluescript KS containing 5,448-bp *Hin*dIII fragment from ϕE125	This study
pDW7.5	pBluescript KS containing 7,325-bp *Hin*dIII fragment from ϕE125	This study
pDW9.5	pBluescript KS containing 9,025-bp *Hin*dIII fragment from ϕE125	This study
pDW11	pBluescript KS containing 9,942-bp *Hin*dIII fragment from ϕE125	This study
pDW18	pBluescript KS containing 12,983-bp *Hin*dIII fragment from ϕE125	This study
pSKM11	Positive selection cloning and suicide vector; IncP oriT; ColE1 ori; Ap^r Tc^s	50
pSKM3.2	pSKM11 containing 3,231-bp *Hin*dIII fragment from ϕE125; Ap^r Tc^r	This study
pDD5003B	37.4-kb *Bam*HI fragment from DD5003 obtained by self-cloning; Ap^r Tc^r	This study
pCR2.1	3.9-kb TA cloning vector; pMB1 oriR; Km^r Ap^r	Invitrogen
pAM1	pCR2.1 containing ϕE125 gene*27* downstream of the *lac* promoter	This study
pCM1	pCR2.1 containing ϕE125 gene*56* downstream of the *lac* promoter	This study
pDD70	pCR2.1 containing 3.2-kb NCTC 120 *wbiE*::IS*407*A PCR fragment	This study
pDD71	pCR2.1 containing 3.2-kb DB110795 *wbiG*::IS*407*A PCR fragment	This study
pDD72	pCR2.1 containing ATCC 23344 *wbiE*	This study
pSPORT 1	General cloning vector; ColE1; Ap^r	Life Technologies
pSPORT 8.1	pSPORT 1 containing 8.1-kb *Hin*dIII-*Eco*RI fragment from pDD5003B	This study
pBHR1	Mobilizable broad-host-range vector; Km^r Cm^r	MoBiTec
pBHR1-*wbiE*	pBHR1 containing 1.8-kb *Eco*RI fragment from pDD72; Km^r Cm^s	This study

[a] Abbreviations: Ap, ampicillin; Tc, tetracycline; Km, kanamycin; Cm, chloramphenicol.

E125, isolated in 1991 from soil in northeastern Thailand (70), spontaneously produced a temperate bacteriophage (ϕE125) that attacked *B. mallei* but not any other bacterial species examined. The gene order and modular organization of the ϕE125 genome is reminiscent of lambdoid bacteriophages (11, 34), and it contains several interesting features, including an insertion sequence, two DNA methylase genes, and a novel cluster of putative replication and lysogeny genes. Bacteriophage ϕE125 exhibits a B1 morphotype and therefore is a new member of the family *Siphoviridae* (phage with long noncontractile tails) (1, 2).

MATERIALS AND METHODS

Bacterial plasmids, strains, and growth conditions. The plasmids used in this study are described in Table 1. The *B. mallei* strains used in this study are listed in Table 2. The following *B. pseudomallei* strains were used in this 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, 275, 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 (5, 20, 22, 25, 26, 66, 76). *B. 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 (10, 66, 76) were also utilized in this study. Other *Burkholderia* species used in this study include *B. cepacia* LMG 1222 (genomovar I) (44), *B. multivorans* C5568, *B. multivorans* LMG 18823 (44), *B. cepacia* LMG 18863 (genomovar III) (44), *B. cepacia* 715j (genomovar III) (47), *B. stabilis* LMG 07000, *B. vietnamiensis* LMG 16232 (44), *B. vietnamiensis* LMG 10929 (44), *B. gladioli* 2-72 (62), *B. gladioli* 2-75 (62), *B. gladioli* 4-54 (62), *B. gladioli* 5-62 (62), *B. uboniae* EY 3383 (77), *B. cocovenans* ATCC 33664, *B. pyrrocinia* ATCC 15958, *B. glathei* ATCC 29195, *B. caryophylli* Pc 102, *B. andropogonis* PA-133, *B. kururiensis* KP23 (79), *B. sacchari* IPT101 (8), *Burkholderia* sp. strain 2.2N (13), and *Burkholderia* sp. strain T-22-8A. *Ralstonia solanacearum* FC228, *R. solanacearum* FC229, *R. solanacearum* FC230, *Pandoraea apista* LMG 16407 (17), *Pandoraea norimbergensis* LMG 18379 (17), *Pandoraea pnomenusa* LMG 18087 (17), *Pandoraea pulmonicola* LMG 18106 (17), *Stenotrophomonas maltophilia* XM16 (39), *S. maltophilia* XM47 (39), *Pseudomonas aeruginosa* PAO (30), *P. aeruginosa* PA14 (55), *Pseudomonas syringae* DC3000 (73), *Salmonella enterica* serovar Typhimurium SL1344 (29), *Serratia marcescens* H11, *Escherichia coli* TOP10 (Invitrogen), S17-1λ*pir* (65), HB101 (7), MC4100 (15), DH5α (Gibco BRL), JM105 (78), E2348/69 (41), and DB24 (36) 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, 30 μg of chloramphenicol, 25 μg of kanamycin, and 15 μg of tetracycline per ml for *E. coli* and 100 μg of streptomycin and 50 μg of tetracycline per ml for *B. thailandensis. B. mallei* DD3008 was grown in the presence of 5 μg of gentamicin per ml, and *B. mallei* NCTC 120 (pBHR1) was grown in the presence of 15 μg of polymyxin B and 5 μg of kanamycin per ml.

Spontaneous bacteriophage production by lysogenic *B. thailandensis* strains and UV induction experiments. *B. thailandensis* strains E264, E275, E202, E125, and E251 were grown in LB broth for 18 h at 37°C with shaking (250 rpm). One hundred microliters of each saturated culture was used to inoculate two LB broth (3-ml) subcultures. One set of subcultures was incubated for 5 h under the same conditions. The other set of subcultures was incubated for 3 h, poured into sterile petri dishes in a class II biological safety cabinet, subjected to a hand-held UV light source (254 nm) for 20 s (25 cm above the sample), pipetted back into culture tubes, and incubated for an additional 2 h. Both sets of subcultures were briefly centrifuged to pellet the cells, and the supernatants were filter sterilized (0.45-μm-pore-size filters). The samples were serially diluted in suspension medium (SM) (40), and the numbers of PFU were assessed by using *B. mallei* ATCC 23344 as the host strain as described below. Bacteriophage was considered to be induced if the titer increased twofold (or more) after exposure to UV light. If bacteriophage titers did not increase twofold, the bacteriophage was not considered to be induced by UV light.

Bacteriophage ϕE125 propagation and DNA purification. The protocols followed for picking plaques, titrating bacteriophage stocks, and preparing plate lysates were the same as those used for bacteriophage λ (61), with a few minor modifications. Briefly, 0.1 ml of ϕE125 and 0.1 ml of a saturated culture of *B. mallei* ATCC 23344 ($\sim 5 \times 10^8$ bacteria) were mixed 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 LB plates containing 4% glycerol and incubated overnight at 37°C. For preparation of plate lysate stocks, 5 ml of SM was added to the plate, and bacteriophage was eluted overnight at 4°C without shaking. SM was harvested, bacterial debris was separated by centrifugation, and the resulting supernatant was filter sterilized (0.45-μm-pore-size filters) and stored at 4°C. Bacteriophage ϕE125 DNA was purified from a plate culture lysate using the Wizard Lambda Preps DNA Purification System (Promega). The ϕE125 lysogen BML10 was isolated from a single turbid plaque

TABLE 2. Bacteria used to examine the host range of bacteriophage φE125

Bacterium	Relevant information	Plaque formation[a]
Burkholderia mallei		
NCTC 120	LPS O-antigen mutant; *wbiE*::IS*407*A	−
NCTC 10248		+
NCTC 10229		+
NCTC 10260		+
NCTC 10247		+
NCTC 3708		+
NCTC 3709		+
ATCC 23344		+
ATCC 10399		+
ATCC 15310		+
DB110795	Laboratory-passaged ATCC 15310; LPS O-antigen mutant; *wbiG*::IS*407*A	−
BML10	ATCC 23344 (φE125)	−
DD3008	ATCC 23344::pGSV3008; capsule mutant	+
Burkholderia pseudomallei	50 strains	−
Burkholderia thailandensis	32 strains	−
Burkholderia cepacia	Genomovar I; 1 strain	−
Burkholderia multivorans	2 strains	−
Burkholderia cepacia	Genomovar III; 2 strains	−
Burkholderia stabilis	1 strain	−
Burkholderia vietnamiensis	2 strains	−
Burkholderia gladioli	4 strains	−
Burkholderia uboniae	1 strain	−
Burkholderia cocovenans	1 strain	−
Burkholderia pyrrocinia	1 strain	−
Burkholderia glathei	1 strain	−
Burkholderia caryophylli	1 strain	−
Burkholderia andropogonis	1 strain	−
Burkholderia kururiensis	1 strain	−
Burkholderia sacchari	1 strain	−
Burkholderia spp.	2 strains	−
Ralstonia solanacearum	3 strains	−
Pandoraea apista	1 strain	−
Pandoraea norimbergensis	1 strain	−
Pandoraea pnomenusa	1 strain	−
Pandoraea pulmonicola	1 strain	−
Stenotrophomonas maltophilia	2 strains	−
Pseudomonas aeruginosa	2 strains	−
Pseudomonas syringae	1 strain	−
Salmonella enterica serovar Typhimurium	1 strain	−
Serratia marcescens	1 strain	−
Escherichia coli	8 strains	−

[a] +, present; −, absent.

formed on ATCC 23344. The plaque was picked with a Pasteur pipette, transferred to a tube containing 3 ml of broth media, and incubated overnight. The saturated culture was spread onto solid media with an inoculating loop, and 10 isolated colonies were tested for their ability to form plaques with φE125. All of the colonies were resistant to infection with φE125, and one was selected and designated BML10.

φE125 sensitivity testing. Approximately 10^2 PFU 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 a LB 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 φE125 if they formed plaques under these conditions and resistant if they did not. It should be noted that the positive control, *B. mallei* ATCC 23344, formed plaques in the presence of φE125 after incubation at 25 and 37°C. No bacterial species tested formed plaques in the absence of φE125.

Negative staining of φE125. Bacteriophage φE125 was prepared from 20 ml of a plate culture lysate (see above), incubated at 37°C for 15 min with Nuclease Mixture (Promega), precipitated with Phage Precipitant (Promega), and resuspended in 1 ml of Phage Buffer (Promega). The bacteriophage solution (~100 μl) was added to a strip of parafilm M (Sigma), and a formvar-coated nickel grid (400 mesh) was floated on the bacteriophage solution for 30 min at 25°C. Excess fluid was removed, and the grid was placed on a drop of 1% phosphotungstic acid, pH 6.6, for 2 min at 25°C. Excess fluid was removed, and the specimen was examined on a Philips CM100 transmission electron microscope. Nickel grids were glow discharged on the day of use.

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 (Bio 101). Bacterial genomic DNA was prepared by using the Masterpure DNA Kit (Epicentre) for methylase dot blot assays and by a previously described protocol (75) for all other experiments. Plasmids were purified from overnight cultures using Wizard *Plus* SV Minipreps (Promega). The broad-host-range plasmids pBHR1 and pBHR1-*wbiE* were electroporated into *E. coli* S17-1λ*pir* (12.25 kV/cm) and conjugated to *B. mallei* NCTC 120 for 8 h as described elsewhere (22). Similarly, the suicide vector pSKM3.2 was electroporated into *E. coli* S17-1λ*pir* and conjugated to *B. thailandensis* E125 for 8 h as described elsewhere (22). The resulting strain, *B. thailandensis* DD5003, contained pSKM3.2 integrated into the φE125 genome at the 3.2-kb *Hin*dIII fragment. Chromosomal DNA was isolated from DD5003 and digested with the restriction endonuclease *Bam*HI, and the bacteriophage attachment site and flanking bacterial DNA were obtained by self-cloning (22).

Immunoblot analysis. Fifty microliters of a saturated broth culture of *B. mallei* was subjected to centrifugation, and the bacterial pellet was washed with phosphate-buffered saline, pH 7.4. The sample was resuspended in 50 μl of sample buffer (4% sodium dodecyl sulfate [SDS], 10% glycerol, 5% 2-mercaptoethanol,

0.005% bromphenol blue in Tris buffer, pH 6.8) and boiled for 10 min. The sample was treated with proteinase K (25 μg dissolved in 10 μl of sample buffer) and incubated at 37°C for 1 h. Forty microliters of sample was boiled for 5 min, loaded onto a 4% polyacrylamide stacking gel–12% polyacrylamide separating gel, and SDS-polyacrylamide gel electrophoresis (PAGE) was performed using 1× Tris-Glycine SDS Running buffer (Novex). The gel was blotted to Immuno-Blot PVDF Membrane (Bio-Rad) by using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) according to the manufacturer's instructions. The membrane was subjected to a blocking step (5% skim milk, 0.1% Tween 20) and was reacted with a 1:2,000 dilution of 3D11, a monoclonal mouse immunoglobulin G1 (IgG1) antibody that reacts with *B. mallei* lipopolysaccharide (LPS) O antigen (Research Diagnostics, Inc.). Following several washing steps with blocking buffer, the membrane was reacted with a 1:5,000 dilution of peroxidase-labeled goat antibody to mouse IgG (Kirkegaard & Perry Laboratories, Inc. [KPL]). Finally, it was washed three times with blocking buffer and once with PBS (pH 7.4) and then incubated with TMB Membrane Peroxidase Substrate (KPL).

DNA sequencing and analysis. DNA sequencing was performed at ACGT, Inc. (Northbrook, Ill.) and at LMT Sequencing Lab (Frederick, Md.). Most ϕE125 genes were identified by using GeneMark.hmm (43), whereas others were identified by visual inspection, guided by BLAST (4) results. DNA and protein sequences were analyzed with GeneJockeyII and MacVector 7.1 software for the Macintosh. The gapped BLASTX and BLASTP programs were used to search the nonredundant sequence database for homologous proteins (4). In order to determine the nucleotide sequence of the ϕE125 *cos* sites, we sequenced the ends of ϕE125 DNA directly by using the following primers: COS4, 5′-AATCC GGCTCGTCCTTATTC-3′ and COS10, 5′-GTTGCGGTGACGTGGTGGTG-3′. The nucleotide sequences obtained contained a gap relative to the ligated ϕE125 ends on pDW9.5, which corresponded to unsequenceable 3′ ends (64).

PCR amplifications. PCR products were sized by using agarose electrophoresis and cloned using the pCR2.1 TOPO TA cloning kit (Invitrogen) and chemically competent *E. coli* TOP10 (Invitrogen). PCR amplifications were performed with a final reaction volume of 100 μl and contained 1× *Taq* PCR Master Mix (Qiagen), 1 μM oligodeoxyribonucleotide primers, 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. The eight oligodeoxyribonucleotide primer pairs used in the PCR amplification of the LPS O-antigen gene cluster were as follows: 1-1, 5′-CGAGTTCACGGTATCACAA G-3′, and 1-2, 5′-GTTGTCGTAGAAGTACAGCC-3′; 2-1, 5′-GGCTGTACTT CTACGACAAC-3′, and 2-2, 5′-GCATCAGCAGCGGATTGAAG-3′; 3-1, 5′-CTTCAATCCGCTGCTGATGC-3′, and 3-2, 5′-GAATGCGACTTCAACAAC AC-3′; 4-1, 5′-GTGTTGTTGAAGTCGCATTC-3′, and 4-2, 5′-CATAAACGT TCTGCAGACGC-3′; 5-1, 5′-GCGTCTGCAGAACGTTTATG-3′, and 5-2, 5′-GATTTGCTGCAAATAGCGTG-3′; 6-1, 5′-CACGCTATTTGCAGCAAAT C-3′, and 6-2, 5′-CGAAGATATCGAGCCAGTGC-3′; 7-1, 5′-GCACTGGCTC GATATCTTCG-3′, and 7-2, 5′-CCGAAGCGGTTGAAGAAGTG-3′; 8-1A, 5′-CTGGAAATGGCTATGAGCAG-3′, and 8-2A, 5′-AAATGCTCGCGTCATG TTGC-3′.

In order to determine the order and orientation of the *Hin*dIII fragments in the intact ϕE125 genome, outward-oriented primers specific for the ends of each *Hin*dIII fragment (except the 1,068-bp fragment) were synthesized and PCR was performed with ϕE125 genomic DNA and all possible primer combinations. We reasoned that two *Hin*dIII fragments were adjacent if we obtained a PCR product with primer pairs specific for the corresponding ends of those fragments. All PCR products were cloned and sequenced to confirm the PCR results. For these PCRs, and all of the PCR experiments mentioned below, the conditions mentioned above were used, with the following exception: we used 72°C for 30 s instead of 72°C for 2 min in the three-temperature cycling protocol. The 14 oligodeoxyribonucleotide primers used in this analysis were as follows: 3.2F, 5′-AGACGATCAAGCAACACGAG-3′; 3.2R, 5′-TCGAAGCGCCAATAAA ACGC-3′; 4.4F, 5′-CAAGCTCTCTCAGCTTCTCG-3′; 4.4R, 5′-ACCAGCGG CCATACATTATG-3′; 5.5F, 5′-GGTCTCCGGATCGTAATTGT-3′; 5.5R, 5′-TCGTGCGTCAGTTCAAATGG-3′; 7.5F, 5′-CCAGATCCAGAATACGCAA C-3′; 7.5R, 5′-ATAACGCGCTTTGTCGATCG-3′; 9.5F, 5′-GAGTGAAGCCA TCGAAGATC-3′; 9.5R, 5′-ACGGAAAGGAGCATGTCATC-3′; 11F, 5′-TCA TCGACGAGGAACTTCAC-3′; 11R, 5′-AATGATGGTCAGCACGAACG-3′; 18F-2, 5′-TCAAGGTAGAACAGCGTGTG-3′; 18R, 5′-GCTCCTTGTCCAA GTAGATG-3′.

PCR was performed with genomic DNAs from *B. mallei* ATCC 23344, *B. mallei* BML10, ϕE125, and the primers Pro (5′-TATACCCGACCGAATTGG-3′) and Int (5′-TATGACGTGAAGGCACTC-3′) to determine if ϕE125 integrated into the proline tRNA (UGG) gene in *B. mallei*. We obtained a single PCR product of the expected size (550 bp) with *B. mallei* BML10 DNA. This product was cloned, and its nucleotide sequence was determined. No PCR products were obtained when genomic DNAs from *B. mallei* ATCC 23344 or ϕE125 were used in the PCR.

Genomic DNA from ϕE125 was used for PCR amplification of gene *27* with the following primers: AM-UP, 5′-CAAGTTTAAAAACGGCTTTCAC-3′, and AM-DOWN, 5′-CAGCCAATCGATCAGAACAG-3′. The resulting PCR product was cloned, sequenced, and designated pAM1 (Table 1). Similarly, gene *56* was amplified by PCR using ϕE125 genomic DNA and the following primers: CM-UP, 5′-CACAGGTGCTGTTCAATCTC-3′, and CM-DOWN, 5′-CTCAC ATGACCTCCAAAACG-3′. The resulting PCR product was cloned, sequenced, and designated pCM1 (Table 1).

Dot blot assay for DNA methyltransferase activity. The plasmids pCR2.1, pAM1, and pCM1 (Table 1) were electroporated into *E. coli* DB24, a strain that lacks all endogenous DNA methylation (36). The transformants were grown overnight in the presence of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and genomic DNA was isolated as described above. Genomic DNA preparations were diluted in Tris-EDTA buffer (10 mM Tris-Cl [pH 7.4], 1 mM EDTA [pH 8.0]) to yield stocks of 150, 50, and 15 ng/μl, and 3-μl aliquots of each were spotted onto a BA85 nitrocellulose filter (Schleicher & Schuell). Methylase activity was assessed by using rabbit primary antibodies that react specifically with DNA containing N6-methyladenine (m6A) or N4-methylcytosine (m4C) in a dot blot assay, as described previously (36). The secondary antibody was a peroxidase-labeled goat anti-rabbit IgG (H + L) conjugate (KPL). Primary and secondary antibodies were used at dilutions of 1:50,000 and 1:1,000, respectively. Detection was accomplished by using the luminol system (Amersham/Pharmacia), and exposures were made to hyperfilm-ECL (Amersham/Pharmacia). The film images were digitally captured using a UMAX flatbed scanner (S900) and Adobe Photodeluxe (version 1.1) software for the PowerMac.

GenBank and American Type Culture Collection (ATCC) accession numbers. The nucleotide sequences reported in this paper were deposited in the GenBank database under the accession numbers AF447491 (ϕE125 genome) and AY063741 (*B. thailandensis* bacteriophage attachment site). ϕE125 was deposited in the ATCC bacteriophage collection and was assigned the accession number ATCC 23344-B1.

RESULTS

***B. thailandensis* strains spontaneously produce bacteriophage that infect *B. mallei*.** Five strains of *B. thailandensis* (E125, E202, E251, E264, and E275) were examined for the production of *B. mallei*-specific bacteriophage. All of the strains, with the exception of E251, spontaneously produced bacteriophage that formed plaques with diameters of 1.5 to 2 mm on *B. mallei* ATCC 23344. Strain E264 produced two bacteriophages that formed distinct plaques, one turbid and one clear. Strains E125, E202, and E275 each produced a bacteriophage that formed turbid plaques. Bacteriophage production was increased 2-fold (E264 and E275), 6-fold (E125), and 55-fold (E202) by brief exposure to UV light. The clear plaque bacteriophage from E264 was not induced, and UV light did not induce bacteriophage production by E251. We examined the host range of all five *B. thailandensis* bacteriophages on 10 strains of *B. mallei* and 13 strains of *B. pseudomallei* and found that the temperate bacteriophages produced by E264, E202, and E275 formed plaques on 9 of 10 *B. mallei* strains and on 3 of 13 *B. pseudomallei* strains. Since these bacteriophages were not specific for *B. mallei*, they were not further characterized. The clear plaque bacteriophage produced by E264 (LPE264) and the temperate bacteriophage produced by E125 (ϕE125) formed plaques on 8 of 10 and 9 of 10 *B. mallei* strains, respectively. Neither bacteriophage formed plaques on *B. pseudomallei* or on *B. mallei* NCTC 120. Typical yields of plate lysate stocks of LPE264 were 10^5 PFU/ml, and yields of ϕE125 were 10^8 PFU/ml. Bacteriophage

LPE264 was not further characterized in this study due to its low yield and its inability to form plaques on *B. mallei* NCTC 3709. Taken together, these results indicate that lysogenic *B. thailandensis* strains exist in nature and that the bacteriophage they harbor are spontaneously produced and infect *B. mallei*.

Bacteriophage ϕE125 is *B. mallei* specific. The host range of ϕE125 was examined with 139 bacterial strains, including 13 strains of *B. mallei*, 50 strains of *B. pseudomallei*, and 32 strains of *B. thailandensis* (Table 2). Bacteriophage ϕE125 formed plaques on 9 of 10 *B. mallei* strains obtained from NCTC and ATCC. It also formed plaques on DD3008, a capsule-deficient mutant derived from ATCC 23344 (24). Three *B. mallei* strains were resistant to plaque formation by ϕE125, NCTC 120, DB110795 (a laboratory-passaged derivative of ATCC 15310), and BML10 (ATCC 23344 harboring the ϕE125 prophage).

ϕE125 did not form plaques on any of the *B. pseudomallei* or *B. thailandensis* strains used in this study (Table 2). It should be noted that the *B. pseudomallei* strains employed in this study were from a variety of sources; 15 clinical isolates, 30 Thai soil isolates, and 5 Australian soil isolates. Similarly, the *B. thailandensis* strains were isolated in northeastern Thailand (15 strains) and central Thailand (17 strains).

Finally, ϕE125 plaque formation was evaluated with 15 additional species of *Burkholderia*, 4 species of *Pandoraea*, 2 species of *Pseudomonas*, *Ralstonia solanacearum*, *Stenotrophomonas maltophilia*, *S. enterica* serovar Typhimurium, *Serratia marcescens*, and *E. coli*. None of these bacteria formed plaques with bacteriophage ϕE125 (Table 2). These results demonstrate that bacteriophage ϕE125 forms plaques only on *B. mallei* strains, that ϕE125-resistant *B. mallei* strains exist, and that the capsular polysaccharide (24) is not required for plaque formation by ϕE125.

ϕE125 is a new member of the family *Siphoviridae*. Bacteriophage may be tailed, cubic, filamentous, or pleomorphic and can be classified by morphotype and host genus (2). Numerous negatively stained bacteriophage were examined, and a representative image of ϕE125 is shown in Fig. 1. ϕE125 possessed an isometric head of 63 nm in diameter and a long noncontractile tail of 203 nm in length and 8 nm in diameter. Based on its B1 morphotype, ϕE125 can be classified as a member of the order *Caudovirales* and the family *Siphoviridae* (1, 2). To our knowledge, this is the first bacteriophage of the *Siphoviridae* family described as being harbored by the host genus *Burkholderia* (2).

LPS O antigen is required for plaque formation by ϕE125. Of the 10 *B. mallei* strains obtained from NCTC and ATCC, only NCTC 120 was resistant to plaque formation by ϕE125 (Table 2). We hypothesized that resistance was due to the absence of a surface receptor for ϕE125 on NCTC 120. The result obtained with DD3008 demonstrated that the capsular polysaccharide was not the ϕE125 receptor (Table 2). We next performed an immunoblot on whole-cell lysates of the NCTC and ATCC strains with a commercially available monoclonal antibody (3D11) that reacts with *B. mallei* LPS O antigen (Fig. 2A). All of the NCTC and ATCC *B. mallei* strains, with the exception of NCTC 120, demonstrated a typical ladder LPS appearance after immunostaining with 3D11 (Fig. 2A). The laboratory-passaged derivative of ATCC 15310, termed DB110795, also does not form plaques with ϕE125 (Table 2). We performed an immunoblot on a whole cell lysate of DB110795 with the monoclonal antibody 3D11 and found that it did not produce LPS O antigen (Fig. 2B). These results demonstrate that there is a correlation between the absence of LPS O antigen and resistance to plaque formation by ϕE125.

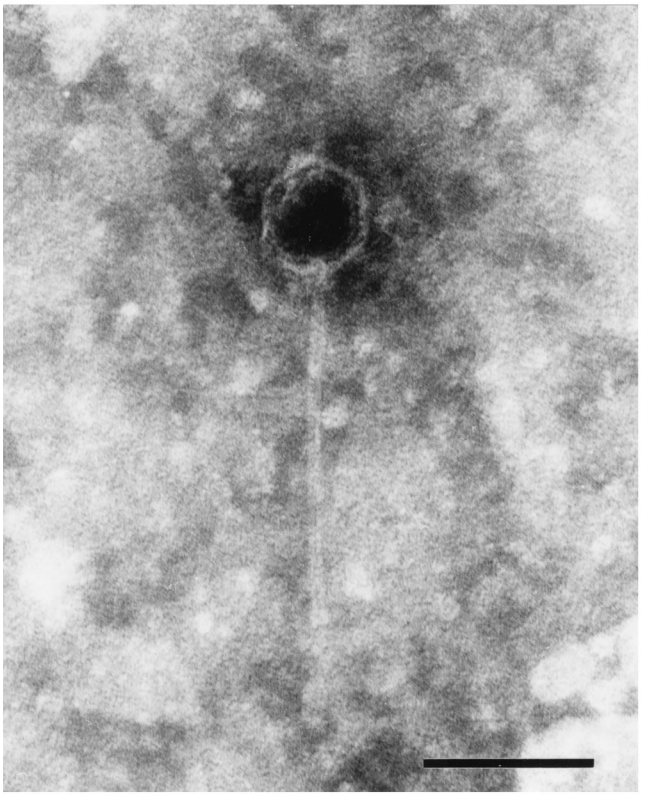

FIG. 1. Transmission electron micrograph of bacteriophage ϕE125 negatively stained with 1% phosphotungstic acid. Scale bar, 100 nm.

A previous study demonstrated that IS*407*A is active in *B. mallei* during serial subculture in vitro. IS*407*A integrated into the capsule gene cluster in *B. mallei* DD420 and resulted in a capsule-deficient strain (24). The LPS O-antigen gene clusters of NCTC 120 and DB110795 were analyzed to determine if this 1.2-kb insertion element (IS) was responsible for the lack of LPS O-antigen production by these strains. The nucleotide sequence of the *B. pseudomallei* LPS O-antigen gene cluster is known (23), and it was used to design eight PCR primer pairs that would result in 2-kb amplicons spanning the LPS O-antigen locus in *B. mallei*. Eight 2-kb amplicons were generated when PCR assays were performed with these primer pairs and genomic DNA from *B. pseudomallei* 1026b and *B. mallei* ATCC 23344 (data not shown). When the PCR assays were performed with genomic DNA from NCTC 120 and DB110795, seven 2-kb amplicons and one 3.2-kb amplicon were produced (data not shown). The 3.2-kb amplicons generated using primer pairs 7-1–7-2 (NCTC 120) and 8-1A–8-2A (DB110795) were cloned and sequenced. The sequencing results demonstrate that NCTC 120 and DB110795 harbor IS*407*A insertions in *wbiE* and *wbiG*, respectively. There was a 4-bp duplication of the sequence 5′-CTGC-3′ flanking the insertion site in NCTC 120 and a 4-bp duplication of the sequence 5′-GCAG-3′ flanking the insertion site in DB110795.

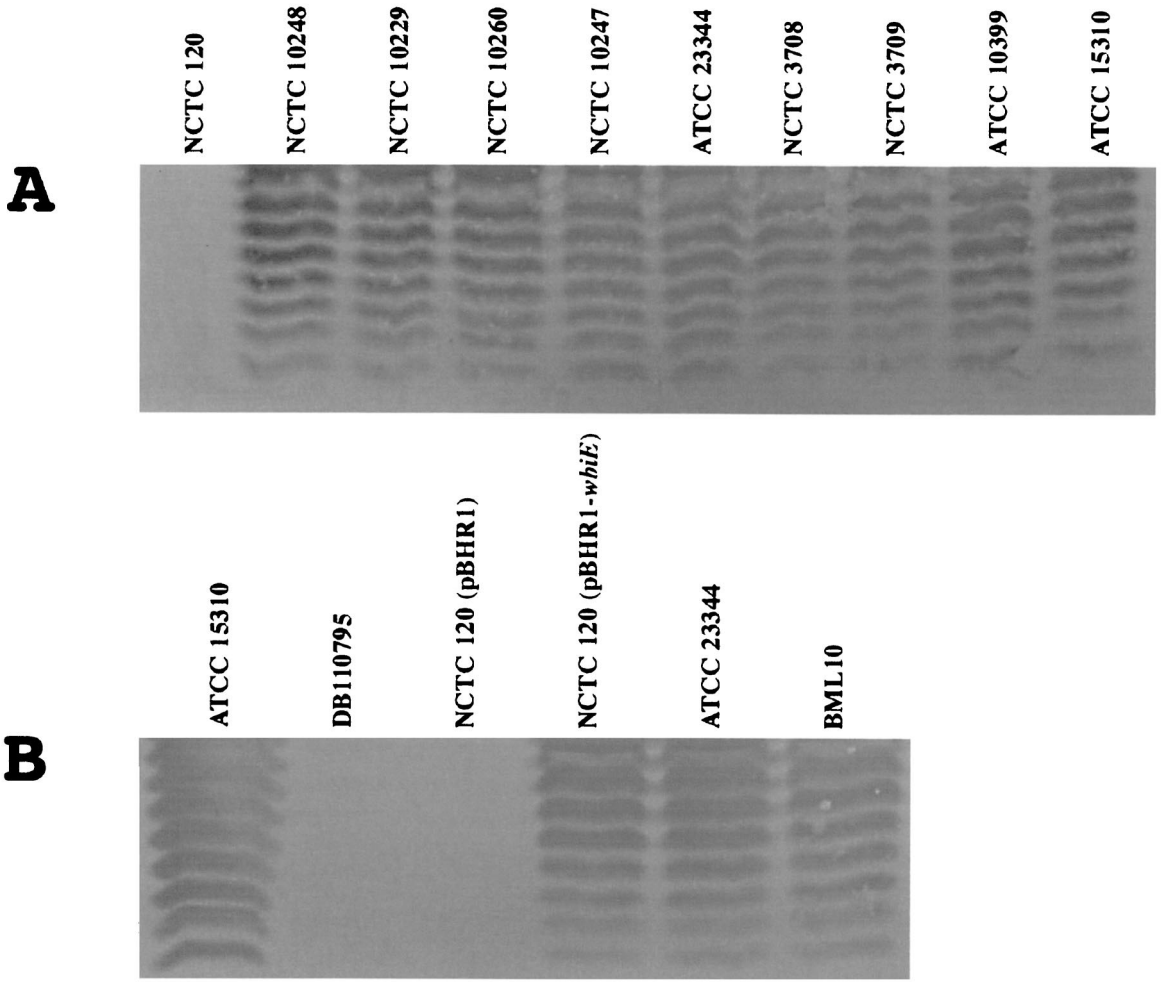

FIG. 2. Immunoblot analysis of *B. mallei* LPS O antigens. Bacteria were washed, resuspended in SDS-PAGE sample buffer, boiled, treated with proteinase K, and subjected to SDS-PAGE. The LPS O antigens were blotted to a polyvinylidene difluoride membrane and reacted with the monoclonal antibody 3D11. (A) LPS O-antigen profiles of NCTC and ATCC *B. mallei* strains. (B) Comparative LPS O-antigen profiles of ϕE125-resistant and ϕE125-susceptible *B. mallei* strains. All strains form plaques with bacteriophage ϕE125 except NCTC 120, DB110795, and NCTC 120 (pBHR1).

Interestingly, the *B. mallei* capsule mutant DD420 harbors an IS*407*A insertion in *wcbF* that is also flanked by a duplication of the sequence 5′-GCAG-3′ (24).

The *wbiE*::IS*407*A mutation in NCTC 120 was complemented by providing the *wbiE* gene from ATCC 23344 in *trans* on the broad-host-range plasmid pBHR1 (Table 1). Figure 2B shows that NCTC 120 (pBHR1) does not produce LPS O antigen but that NCTC 120 (pBHR1-*wbiE*) does. Furthermore, NCTC 120 (pBHR1-*wbiE*) formed plaques with ϕE125, but NCTC 120 (pBHR1) did not. These results demonstrate that the lack of LPS O-antigen production by NCTC 120 is due to an IS*407*A mutation in *wbiE* and that the LPS O antigen is required for plaque formation by ϕE125.

BML10 is immune to ϕE125 superinfection and produces LPS O antigen. Lysogenic bacteria are resistant to superinfection by the temperate bacteriophage that they harbor. Following infection, the ϕE125 genome integrates in the *B. mallei* chromosome at a specific site and becomes a prophage (see below). ATCC 23344 was infected with ϕE125, and a lysogenic derivative was isolated and designated BML10. *B. mallei* BML10 spontaneously produced approximately 500 ϕE125 per ml of broth culture. In comparison, *B. thailandensis* E125 spontaneously produced approximately 1,100 ϕE125 per ml of broth culture. As shown in Table 2, ϕE125 does not form plaques on BML10. Whole-cell lysates of ATCC 23344 and BML10 were analyzed by immunoblot analysis with the monoclonal antibody 3D11, and both strains produced a typical LPS O-antigen banding pattern (Fig. 2B). As shown above, NCTC 120 and DB110795 are resistant to infection with ϕE125 because they do not produce LPS O antigen. BML10, on the other hand, produces LPS O antigen but is still resistant (immune) to ϕE125 superinfection, probably via a prophage-encoded gene product(s). It should be noted that *B. thailandensis* E125 also harbors the ϕE125 prophage and is also immune to superinfection with ϕE125 (Table 2).

Molecular characterization of the bacteriophage ϕE125 genome. The ϕE125 genome was digested with *Hin*dIII, and eight fragments were generated of the following sizes: 1.0, 3.2,

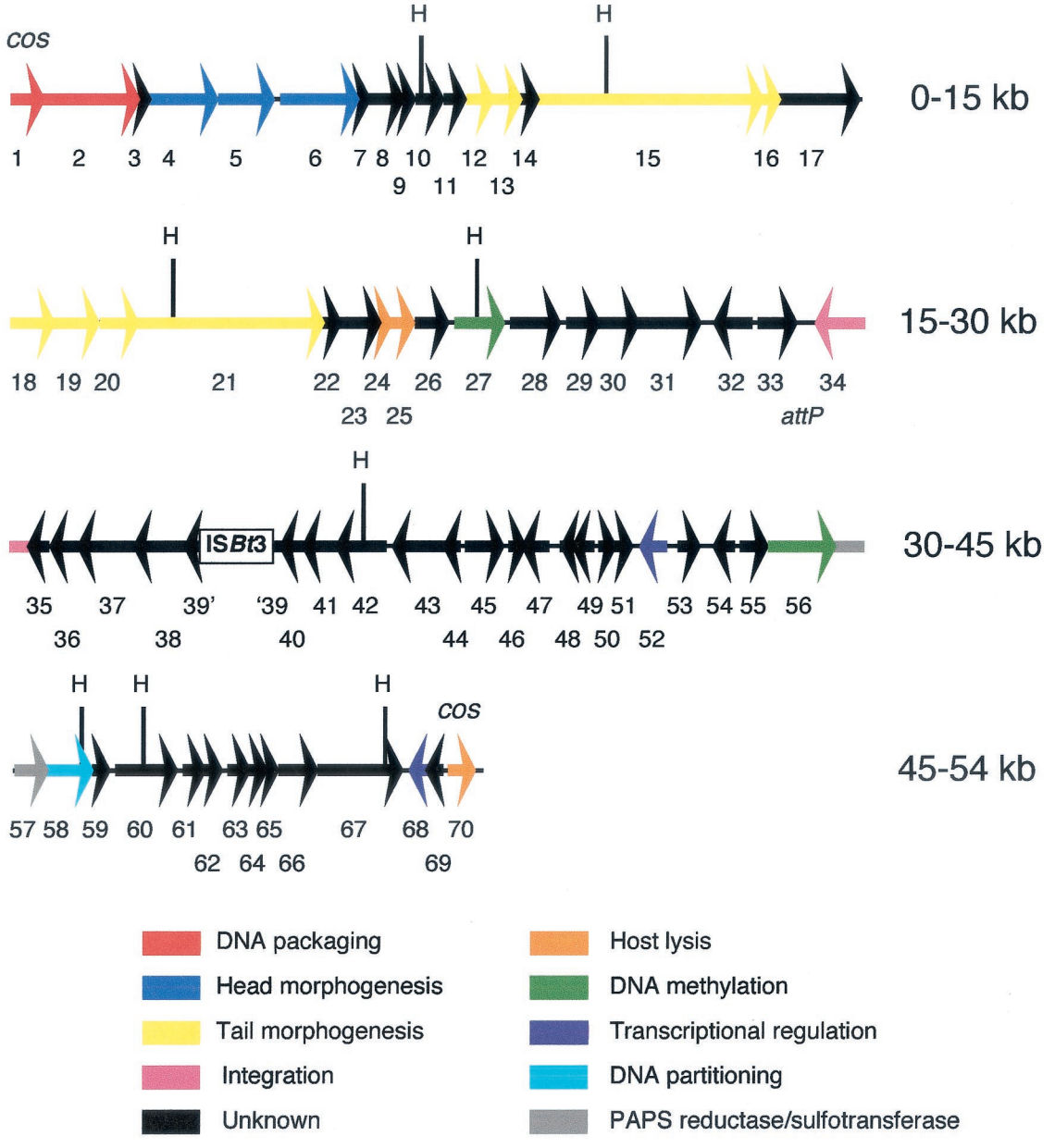

FIG. 3. Physical and genetic map of the bacteriophage ϕE125 genome. The locations and directions of transcription of genes are represented by arrows, and the gene names are shown below. The locations of *Hin*dIII endonuclease restriction sites are shown (H), and the insertion sequence IS*Bt3* is represented as a rectangle. The locations of the cohesive (*cos*) and bacteriophage attachment (*attP*) sites are shown above and below the ϕE125 genome, respectively. The putative functions of proteins encoded by ϕE125 genes are color coded.

4.4, 5.5, 7.3, 9.0, 9.9, and 13.0 kb. The fragments were heated to 80°C, and the 9.0-kb fragment dissociated into two fragments (1.7 and 7.3 kb), suggesting the presence of a cohesive (*cos*) site on this fragment (data not shown). The eight *Hin*dIII fragments were cloned, and their nucleotide sequences were determined. The nucleotide-sequencing results are depicted schematically in Fig. 3, and pertinent features of ϕE125 genes and gene products are shown in Table 3.

The ϕE125 genome is a linear molecule of 53,373 bp in length, and it contains 10-base 3′ single-stranded extensions on the left (3′-GCGGGCGAAG-5′) and right (5′-CGCCCGCTTC-3′), as depicted in Fig. 3. The G + C content of the ϕE125 genome is 61.2%, which is lower than the 69.3% G + C content of the *B. thailandensis* genome (77). The ϕE125 genome encodes 70 proteins, and 44% of them show no homology to proteins in the GenBank databases using the BLASTP search algorithm (Table 3 and Fig. 3). The bacteriophage genome also harbors a novel IS*3* family insertion sequence (45), designated IS*Bt3* (Table 3 and Fig. 3). IS*Bt3* is 1,318 bp in length, and it has 27-bp terminal inverted repeats flanked by a 3-bp direct duplication. IS*Bt3* integrated into ϕE125 gene*39*, suggesting that the encoded protein (gp39) is not essential for a productive lysogenic infection.

Twenty-eight proteins encoded by ϕE125 are similar to pro-

TABLE 3. Characteristics of bacteriophage ϕE125 genes and gene products

Gene	Orientation[a]	Start (position)	End (position)	Size of protein (kDa)	Protein function and homologs
1	R	46	531	17.2	Terminase (small subunit); phage GMSE-1 Orf16; 1e-12; AF311659; phage 7201 Orf21; 2e-09; AF145054
2	R	541	2253	64.2	Terminase (large subunit); *E. coli* YmfN; 1e-162; NP_415667; phage D3 terminase; 1e-125; NP_061498
3	R	2250	2435	6.3	
4	R	2440	3699	46.4	Portal protein; *H. influenzae* Orf25-like protein; 1e-40; AAF27362; CP-933C Z1849; 1e-40; NP_287334
5	R	3759	4664	31.7	Capsid assembly protein/protease; phage WO Orf7; 8e-44; AB036665; prophage Gifsy-1 STM2605; 2e-34; AE008818
6	R	4767	6074	46.3	Major capsid protein; CP-933N Z1804; 2e-09; NP_287292; CP-933M Z1360; 2e-09; NP_286882
7	R	6134	6319	6.4	
8	R	6326	6892	20.6	
9	R	6892	7218	12.1	Phage HK022 gp9; 3e-08; AF069308
10	R	7211	7633	15.4	Phage HK97 gp10; 4e-20; AF069529 CP-933M Z1368; 1e-19; NP_286890
11	R	7630	7977	12.1	
12	R	8039	8497	16.4	Major tail subunit protein; phage HK97 gp12; 2e-20; NP_037706; *E. coli* ECs1800; 7e-17; NP_309827
13	R	8519	8989	17.4	Tail assembly chaperone protein; phage HK97 gp13; 9e-06; NP_037708; phage HK97 gp14; 1e-05; NP_037707
14	R	8989	9273	10.0	Phage HK97 gp14; 9e-07; NP_037707
15	R	9287	13351	143.0	Tail length tape measure protein; CP-933P Z6034; 2e-25; NP_287971; *E. coli* ECs2240; 2e-25; NP_310267
16	R	13348	13686	12.5	Minor tail protein; phage HK97 gp17; 1e-18; NP_037711; phage HK022 gp17; 3e-18; NP_037677
17	R	13695	15083	50.1	
18	R	15080	15763	25.2	Minor tail protein; *P. aeruginosa* PA0638; 2e-62; G83565; phage N15 gp18; 3e-51; AF064539
19	R	15783	16565	28.6	Tail component protein; *P. aeruginosa* PA0639; 1e-51; AE004499; phage HK97 gp19; 1e-43; AF069529
20	R	16562	17146	20.1	Tail component protein; *Y. pestis* YPO2129; 3e-33; AJ414151; phage N15 gp20; 3e-28; AF064539
21	R	17143	20448	118.3	Tail tip fiber protein; *Y. pestis* YPO2131; < 1e-119; AJ414151; phage N15 gp21; < 1e-119; AF064539
22	R	20445	20759	11.6	
23	R	20759	21493	27.6	
24	R	21536	21748	7.6	Class II holin; phage PS119 gp13; 8e-04; AJ011581; phage PS34 gp13; 8e-04; AJ011580
25	R	21826	22230	14.7	Lysozyme; *X. fastidiosa* XF0513; 2e-13; AE003900; phage PS119 gp19; 2e-11; AJ011581
26	R	22227	22775	18.6	
27	R	22918	23706	30.2	DNA adenine methylase; phage GMSE-1 Orf10; 1e-40; AF311653; *A. lwoffii Alw*I methylase; 5e-19; AF431889
28	R	23815	24693	30.9	
29	R	24875	25435	19.1	*P. aeruginosa* PA1508; 7e-07; AE004579; *Y. pestis* YPO0866; 8e-07; AE004579
30	R	25432	26166	26.3	*P. aeruginosa* PA0822; 5e-23; AE004517; *P. aeruginosa* PA0823; 4e-09; AE004517
31	R	26194	27285	39.4	*P. aeruginosa* PA0821; 1e-48; AE004517
32	L	28059	27418	25.0	
33	R	28145	28816	25.3	Plasmid pFKN Orf11; 2e-17; AF359557; plasmid pNL1 Orf520; 3e-04; AF079317
		29062	29014		*attP* (3′ end of tRNA ProUGG)
34	L	30292	29192	42.2	Site-specific integrase; prophage XfP2 XF2530; 6e-09; AE004060; CP-933M Z1323; 1e-08; NP_286846
35	L	30609	30292	11.8	
36	L	31142	30642	19.3	
37	L	32131	31139	37.2	*M. tuberculosis* Rv2734; 8e-32; NP_217250; *N. punctiforme* hypothetical protein; 4e-28; AAK68643
38	L	33180	32128	37.9	
39′	L	33314	33195		
tnpB	L	34204	33350	32.6	Transposase (IS*3* family); IS*868* ORF4; 1e-101; X55075; IS*401* transposase subunit; 4e-99; L09108
tnpA	L	34539	34261	10.7	Transposase (IS*3* family); IS*401* transposase subunit; 6e-29; L09108; IS*868* Orf1; 9e-20; X55075
′39	L	34794	34636		

Continued on following page

TABLE 3—*Continued*

Gene	Orientation[a]	Start (position)	End (position)	Size of protein (kDa)	Protein function and homologs
40	L	35195	34794	14.6	Phage M×8 p77; 0.1; AF396866
41	L	35863	35192	25.1	CP-9330 Z2097; 4e-05; AE005346; CP-933U Z3120; 2e-04; AE005422
42	L	36671	35877	29.8	
43	L	37670	36858	29.7	*Y. pestis* YPMT1.49c; 1e-38; NC_003134; *S. enterica* HCM2.0006c; 2e-38; AL513384
44	L	37816	37667	5.5	
45	R	38065	38718	28.6	
46	R	38919	39173	9.1	
47	L	39586	39170	15.5	
48	L	39993	39862	4.7	
49	L	40278	40003	10.4	
50	R	40519	40662	5.0	
51	R	40747	40935	7.0	
52	L	41555	41163	14.4	Repressor protein; *X. fastidiosa* XF0499; 9e-14; AE003899; prophage e14 protein b1145; 9e-04; F64859
53	R	42006	42338	11.8	
54	L	42821	42567	9.4	
55	R	43007	43447	15.8	Phage phi CTX Orf33; 0.24; BAA36261
56	R	43452	44588	42.1	DNA cytosine methylase; *C. freundii Cfr*9I methylase; 8e-45; X17022; *P. alcaligenes Pac*25I methylase; 1e-44; U88088
57	R	44585	45583	36.8	PAPS reductase/sulfotransferase; phage 186 Orf84; 6e-55; U32222; *A. pernix* APE2075; 8e-07; F72512
58	R	45618	46439	29.9	DNA partitioning protein; *R. equi* ParA; 1e-06; NP_066815; *L. lactis* ParA; 2e-05; NP_266252
59	R	46436	46696	9.5	
60	R	46850	47842	36.3	CP-933R Z2397; 7e-06; NP_287824
61	R	48035	48388	13.9	Prophage pi3 protein 45; 4e-05; AL596172; *L. monocytogenes* lmo2306; 6e-05; CAD00384
62	R	48385	48741	13.2	
63	R	48843	49181	12.6	
64	R	49172	49516	13.1	
65	R	49492	49752	9.8	
66	R	49761	50408	24.1	
67	R	50482	51876	51.3	*S. meliloti* SMa0594; 6e-11; NP_435557
68	L	52603	52217	14.1	Helix-turn-helix transcriptional regulator; *S. meliloti* SMc00089; 4e-10; NP_385047; *A. tumefaciens* AGR_C_1081p; 2e-04; NP_353634
69	L	52857	52600	9.3	*P. horikoshii* PHS013; 1e-04; NP_142388; *P. jensenii* Orf10; 0.006; CAC38044
70	R	52916	53272	13.1	Class I holin; *X. nematophila* prophage Orf7; 1e-16; CAB58450; *H. influenzae* holin-like protein; 4e-08; AF198256

[a] R, right; L, left.

teins encoded by other bacteriophage, prophage, or prophage-like elements (Table 3). Interestingly, there are numerous similarities to HK022 and HK97 (34) and to λ-like cryptic prophages in *E. coli* O157 Sakai (52) and *E. coli* O157 EDL933 (53). 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 (11, 28, 34, 37). The ϕE125 genome contains a unique combination of multigene modules involved in DNA packaging, head morphogenesis, tail morphogenesis, and host lysis (Fig. 3 and Table 3). The relative order of these modules in the ϕE125 genome is similar to that of other *Siphovirida*e genomes (11, 34, 37, 42). Since ϕE125 possesses both structural and genetic similarities to the λ supergroup group of *Siphoviridae*, it probably should be included with λ, N15, HK97, HK022, and D3 in the λ-like genus (11).

Early bacteriophage gene functions (lysogeny and replication) are typically located on the right half of *Siphoviriae* genomes, as depicted in Fig. 3 (11). However, the putative lysogeny and replication modules of ϕE125 appear to be unique relative to other members of the *Siphoviridae*. Some of the unusual proteins encoded by the right half of the ϕE125 genome include a DNA adenine methylase (gp27), a DNA cytosine methylase (gp56), a 3′-phosphoadenosine-5′-phosphosulfate (PAPS) reductase or PAPS sulfotransferase (gp57), and a chromosome partitioning protein (gp58) (Fig. 3 and Table 3). The ϕE125 genome also contains two putative holins, gp70 (class I) and gp24 (class II), to coordinate the programmed release of lysozyme (gp25) from the cytoplasm prior to bacteriophage release (72). It is currently unknown if gp70, gp24, or both gp70 and gp24 are required for membrane permeabilization during the ϕE125 life cycle. Finally, several recently sequenced bacterial genomes also encode proteins with similarities to gp29, gp30, gp31, gp33, gp37, gp43, gp61, gp67, gp68, and gp69 (Table 3), suggesting the presence of prophages or

prophage remnants in these bacterial genomes. Alternatively, ϕE125 may have acquired these genes via horizontal transfer from a bacterial host, and they may provide a selective advantage to a lysogen harboring this bacteriophage.

ϕE125 integrates into a proline tRNA (UGG) gene in *B. thailandensis* and *B. mallei*. As with other lambdoid bacteriophages, ϕE125 DNA probably circularizes at the *cos* sites after it is injected into the bacterial cell and follows one of two possible pathways (14). The circularized genome may replicate and produce bacteriophage progeny (lytic response), or it may integrate into the bacterial chromosome and be maintained in a quiescent state (lysogenic response). Temperate bacteriophage genomes often contain an attachment site (*attP*) that they utilize to integrate into a homologous region on the bacterial genome (*attB*) via site-specific recombination (18). Since ϕE125 encodes a site-specific integrase (gp34), we were interested in identifying where the ϕE125 genome was integrated in *B. thailandensis* E125 and *B. mallei* BML10 and in determining the nucleotide sequences of *attP* and *attB*.

Chromosomal DNA flanking one side of the ϕE125 attachment site in *B. thailandensis* E125 was cloned and sequenced (see Materials and Methods). The nucleotide sequence of this region contained a 49-bp sequence that was identical for the ϕE125 genome and the *B. thailandensis* E125 chromosome. This sequence corresponded to the 3′ end of a 77-bp proline tRNA (UGG) gene on the *B. thailandensis* chromosome (Fig. 4A). tRNA genes often serve as target sequences for site-specific integration of temperate bacteriophages, plasmids, and pathogenicity islands (27, 63). Immediately upstream of the proline tRNA (UGG) gene on the *B. thailandensis* chromosome was a divergently transcribed gene designated *orfB* (Fig. 4B). BLASTP results demonstrated that OrfB was 52% identical to RSc1539, a probable hydrolase protein from *R. solanacearum*. The *B. thailandensis* proline tRNA (UGG) gene and *orfB* were also present in the *B. mallei* ATCC 23344 genome (http://www.tigr.org/), and they were 100 and 91% identical at the nucleotide level, respectively. Downstream of the proline tRNA (UGG) gene in *B. mallei* ATCC 23344 was *orfA*, a gene that encoded a protein with 40% identity to RSc2888, a hypothetical protein from *R. solanacearum* (Fig. 4B). In order to determine if ϕE125 integrates in the 3′ end of the tRNA proline (UGG) gene in *B. mallei*, we designed PCR primers specific for *B. mallei orfA* and ϕE125 gene*34* (Fig. 4B). *B. mallei* ATCC 23344 and ϕE125 DNA did not yield a PCR product with these primers, but *B. mallei* BML10 did (data not shown). These results, represented schematically in Fig. 4B, demonstrate that bacteriophage ϕE125 integrates into the 3′ end of the proline tRNA (UGG) gene in *B. mallei* and *B. thailandensis*. It should also be noted that attachment at this site leaves the proline tRNA (UGG) gene intact on the right side, as depicted in Fig. 4B.

Survey of *B. thailandensis* strains for the presence of ϕE125-like prophages. As mentioned above, lysogenic bacteria are immune to superinfection with the same (or similar) bacteriophage that they harbor. The results presented in Table 2 demonstrate that all thirty-two *B. thailandensis* strains in our collection, including E125, are resistant to infection with ϕE125. To determine if the strains were resistant to infection because they harbored ϕE125-like prophages, genomic DNA was isolated from all strains and PCR was performed with primer pairs specific for four distinct regions of the ϕE125 genome. The primer pairs used were 9.5R and 3.2R (gene*9* and gene*10*), 7.5F and 5.5F (gene*21*), 18R and 11F (gene*42*), and 4.4R and 9.5F (gene*67*). Only ten of the thirty-two *B. thailandensis* strains yielded positive PCR results with these primer pairs (E96, E100, E125, E253, E254, E256, E263, E264, E286, and E293). As expected, E125 was positive for all of the PCR primer pairs. The only other strain that was positive for all four primer pairs was E286. Strains E253 and E264 yielded positive PCR results for two primer pairs, and all of the other strains were positive for three primer pairs. All 10 *B. thailandensis* strains spontaneously produced bacteriophage that formed plaques on *B. mallei* ATCC 23344. Thus, it appears that E96, E100, E253, E254, E256, E263, E264, E286, and E293 all harbor ϕE125-like prophage and may be immune to superinfection with ϕE125. On the other hand, 22 *B. thailandensis* strains did not yield a positive PCR product with any of the primer pairs and probably do not harbor a ϕE125-like prophage. These observations suggest that the molecular mechanism of ϕE125 resistance in these strains is probably not due to superinfection immunity.

Functional analysis of the putative DNA methyltransferases of ϕE125. ϕE125 encodes two proteins, gp27 and gp56, that contain similarities to Type II DNA methyltransferases (Table 3). Site-specific DNA methylation usually leads to the formation of three different products: N6-methyladenine (m6A), 5-methylcytosine (m5C), and N4-methylcytosine (m4C). Some tailed bacteriophage genomes contain unusual or modified DNA bases that may be important in protecting the infecting bacteriophage DNA from host restriction endonucleases (1). gp27 is a putative DNA adenine methylase, and gp56 is a putative DNA cytosine methylase. We were interested in determining if gp27 and gp56 were functional DNA methyltransferases.

The plasmids pCR2.1, pAM1, and pCM1 (Table 1) were transformed into *E. coli* DB24, a strain that is deficient in all of the *E. coli* DNA methylases (36), and DNA methylase dot blot assays were performed with rabbit primary antibodies specific for m6A and m4C. Figure 5A shows that the m6A antibody reacted with genomic DNA samples from λ (positive control) and DB24 (pAM1) but did not react with DB24 (pCR2.1) or DB24 (pCM1). The m6A antibody also reacted with genomic DNA samples from *B. mallei* BML10 and bacteriophage ϕE125 (Fig. 5A). On the other hand, there was only background reactivity of the m6A antibody with genomic DNA from *B. thailandensis* E125 and *B. mallei* ATCC 23344 (Fig. 5A). It appears that the ϕE125 m6A methylase has little or no activity in the *B. thailandensis* lysogen but is very active in the *B. mallei* lysogen (Fig. 5A, compare E125 and BML10). It is currently unclear if the *B. mallei* BML10 genome contains m6A or if the positive signal obtained with the m6A antibody is due to the ϕE125 genome, which also contains m6A (Fig. 5A). Taken together, these results clearly demonstrate that gene*27* is expressed in DB24, that gp27 is a functional m6A methylase, and that the ϕE125 genome contains m6A.

The m4C antibody did not react with genomic DNA from DB24 (pCR2.1), DB24 (pAM1), DB24 (pCM1), or *B. thailandensis* E125, but it did react with DB24 genomic DNA methylated with M.*Rsa*I as a positive control (Fig. 5B). This indicates that gene*56* is not expressed or is inactive in DB24 (pCM1) and *B. thailandensis* E125. On the other hand, positive

A

5′ CGGAGCGTAGCGCAGCCTGGTAGCGCATCTGATT**TGG**GAT

CAGAGGGTCGTAGGTTCGAATCCTATCGCTCCGACCA 3′

B

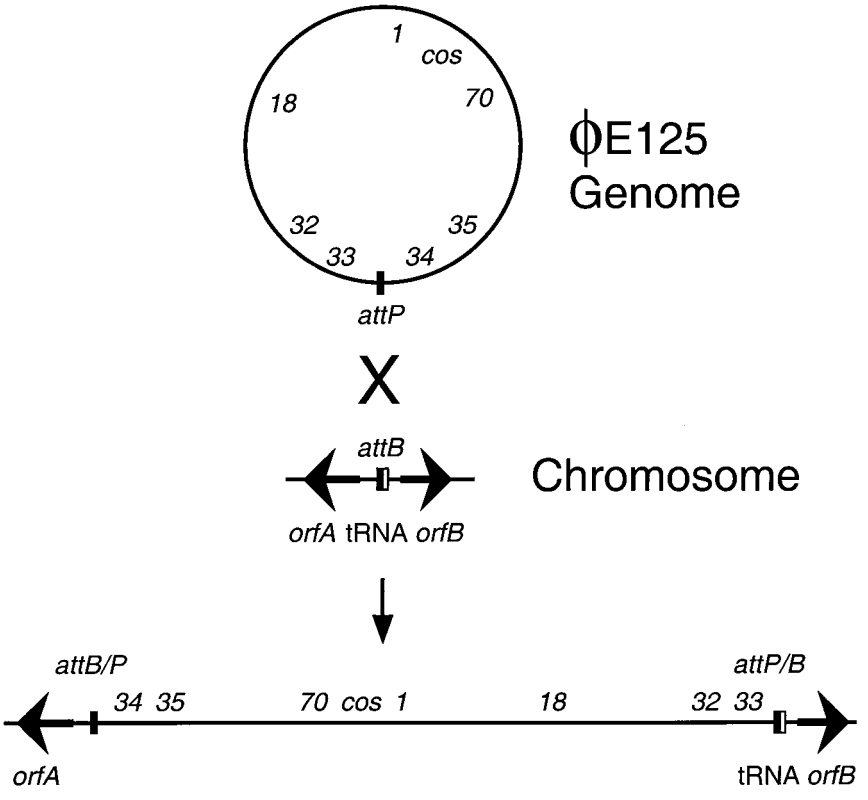

FIG. 4. Bacteriophage ϕE125 integrates into the proline tRNA (UGG) gene in *B. mallei* and *B. thailandensis*. (A) The nucleotide sequence of the proline tRNA (UGG) gene of *B. mallei* ATCC 23344 and *B. thailandensis* E125. The underlined sequence represents the 49-bp attachment site that is identical in the ϕE125 genome (*attP*), the *B. mallei* chromosome (*attB*), and the *B. thailandensis* chromosome (*attB*). The location of the anticodon in the proline tRNA gene is shown in bold. (B) Schematic representation of integration of the ϕE125 genome into the proline tRNA (UGG) gene of *B. mallei* and *B. thailandensis*. The ϕE125 genome is depicted as a circle, and the approximate locations of gene*1*, gene*18*, gene*32*, gene*33*, gene*34*, gene*35*, gene*70*, and the *cos* site are shown. The *B. mallei* and *B. thailandensis* chromosomes are represented as a line, and the location and direction of transcription of *orfA* and *orfB* are represented by arrows. The 5′ end of the proline tRNA (UGG) gene is shown as a thin white rectangle, and the 3′ end (the attachment site) is shown as a thin black rectangle. Following site-specific recombination (X), the *orfA* and *orfB* genes are separated by the integrated ϕE125 prophage.

signals were obtained when the m4C antibody was reacted with genomic DNA from *B. mallei* BML10 and ϕE125 (Fig. 5B). It is likely that gp56 is an m4C methylase because genomic DNA from *B. mallei* BML10 reacts with the m4C antibody, but *B. mallei* ATCC 23344 genomic DNA does not (Fig. 5B). Alternatively, ϕE125 infection may activate a cryptic *B. mallei* m4C methylase or a ϕE125 protein other than gp56 may be responsible for the m4C methylase activity in *B. mallei* BML10. It is not clear if the *B. mallei* BML10 genome contains m4C or if the positive signal obtained with the m4C antibody is strictly due to m4C methylation of the ϕE125 genome (Fig. 5B). Further studies will be required to determine the DNA specificities of gp27 and gp56.

DISCUSSION

In this study, we isolated and characterized ϕE125, a tailed bacteriophage specific for *B. mallei*. The host range of ϕE125 was examined by using bacteria from three genera of β-*Proteobacteria* (*Burkholderia*, *Pandoraea*, and *Ralstonia*) and five

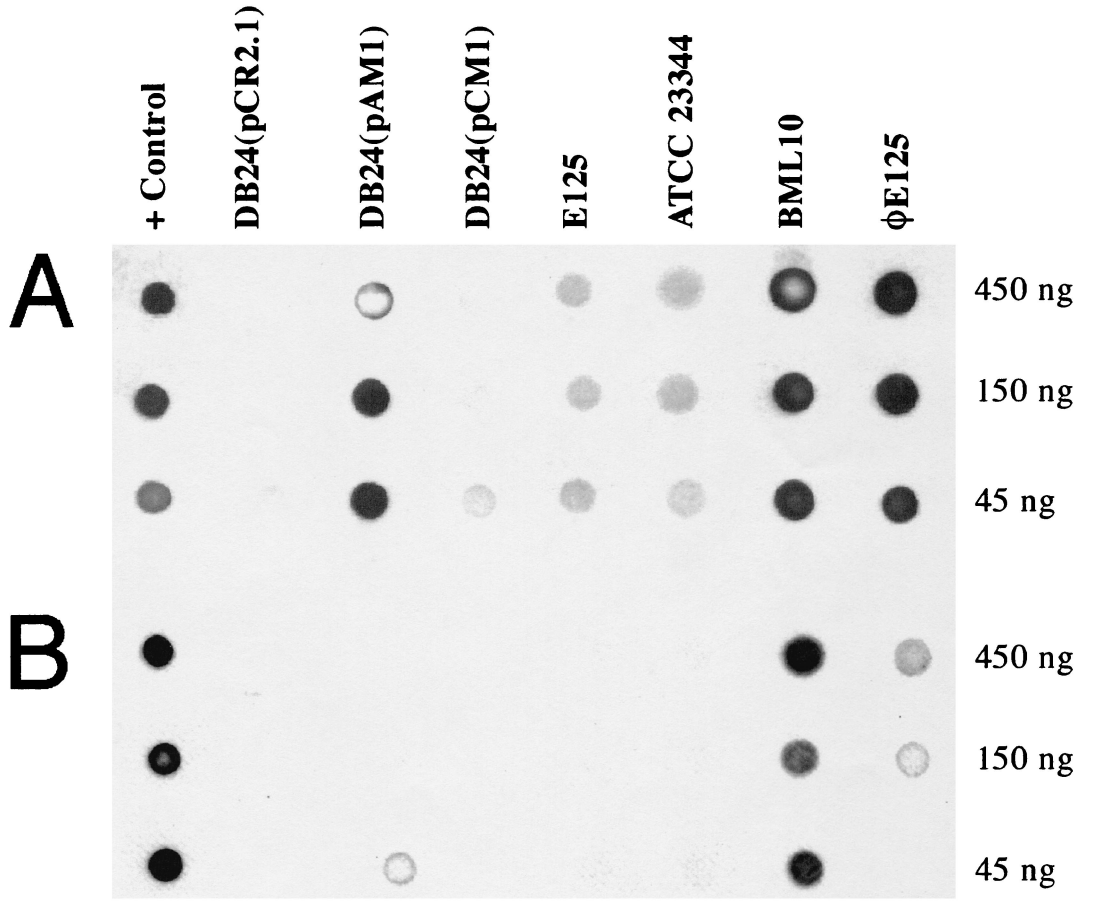

FIG. 5. Dot blot assay to detect genomic DNA methylation using rabbit primary antibodies specific for m6A or m4C. (A) Methylase dot blot assay using polyclonal antibodies specific for m6A. Bacteriophage 2 genomic DNA was used as a positive (+) control. (B) Methylase dot blot assay using polyclonal antibodies specific for m4C. *E. coli* DB24 genomic DNA methylated by M.*Rsa*I served as a positive (+) control. The quantities of genomic DNAs spotted on each panel are shown.

genera of γ-*Proteobacteria* (*Pseudomonas*, *Stenotrophomonas*, *Salmonella*, *Serratia*, and *Escherichia*). In fact, eighteen different *Burkholderia* species were tested, and only *B. mallei* strains were sensitive to φE125 (Table 2). The most-impressive host specificity results were obtained with *B. pseudomallei* and *B. thailandensis*, two species closely related to *B. mallei*. Bacteriophage φE125 did not form plaques on any of the 50 strains of *B. pseudomallei* or 32 strains of *B. thailandensis* tested in this study. Glanders was eradicated from North America in the 1930s and we were able to test only 13 strains of *B. mallei* due to the difficulty of obtaining unique isolates of this species. Nonetheless, the results clearly demonstrate that φE125 specifically forms plaques on *B. mallei,* and we hope to use it, in conjunction with other methods, as a diagnostic tool for *B. mallei*.

The LPS O antigen was required for infection with φE125, suggesting that this molecule is the bacteriophage receptor. This is similar to the λ-like bacteriophage D3, which utilizes the LPS O antigen of *P. aeruginosa* for infection (37, 38). It is surprising that φE125 did not infect *B. pseudomallei* or *B. thailandensis* because the chemical structure of the *B. mallei* LPS O antigen, a heteropolymer of repeating D-glucose and L-talose, is similar to that previously described for these closely related species (10, 12, 35, 54). In fact, the gene clusters encoding the *B. mallei* and *B. pseudomallei* LPS O antigens are 99% identical at the nucleotide level (12, 23). However, unlike *B. pseudomallei* and *B. thailandensis*, the *B. mallei* LPS O antigen is devoid of an *O*-acetyl group at the 4′ position of the L-talose residue. The chemical structure of the *B. mallei* LPS O antigen is as follows: (3)-β-D-glucopyranose-(1,3)-6-deoxy-α-L-talopyranose-(1-, in which the talose residue contains 2-*O*-methyl or 2-*O*-acetyl substituents (12). Our present hypothesis is that *B. pseudomallei* and *B. thailandensis* are resistant to infection with 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. *B. pseudomallei* and *B. thailandensis* possess an *O*-acetyltransferase that is responsible for transferring the *O*-acetyl group to the 4′ position of the L-talose residue. This *O*-acetyltransferase gene is not present, is not expressed, or is mutated in *B. mallei*. We are currently attempting to identify the *B. pseudomallei* *O*-acetyltransferase gene and provide it in *trans* to *B. mallei* to see if it *O*-acetylates the 4′ position of L-talose and confers resistance to φE125. Alternatively, inactivation of the *O*-acetyltransferase gene should make *B. pseudomallei* sensitive to φE125.

It is also possible that *B. pseudomallei* and *B. thailandensis* are immune to superinfection with ϕE125 because they harbor a ϕE125-like prophage. The nucleotide sequence of a 1,068-bp *Hin*dIII fragment from a *B. mallei*-specific bacteriophage produced by *B. pseudomallei* 1026b (ϕ1026b) was recently obtained and was found to be 98% identical to the 1,068-bp *Hin*dIII fragment from ϕE125 (D. DeShazer, unpublished data). However, the nucleotide sequences of other *Hin*dIII fragments from ϕ1026b displayed no similarities to ϕE125, indicating that ϕ1026b and ϕE125 are distinct bacteriophages that share regions (modules) of genetic similarity. We found that 10 of the 32 *B. thailandensis* strains in our collection harbor a ϕE125-like prophage, and the genomic sequence of *B. pseudomallei* K96243 also contains several genes that are nearly identical to ϕE125 genes (http://www.sanger.ac.uk/). Thus, it is clear that some *B. pseudomallei* and *B. thailandensis* strains are lysogenic for a ϕE125-like bacteriophage and may be immune to superinfection with ϕE125. It is also important to note that 22 *B. thailandensis* strains in our collection did not possess an ϕE125-like prophage, suggesting that superinfection immunity alone is not responsible for their resistance to infection with ϕE125.

In this study, we found that *B. mallei* NCTC 120 and *B. mallei* DB110795 do not produce LPS O antigens due to IS*407*A insertions in *wbiE* and *wbiG*, respectively. Burtnick et al. (12) have recently obtained identical results with *B. mallei* NCTC 120 and *B. mallei* ATCC 15310, the parental strain of *B. mallei* DB110795. We found that *B. mallei* ATCC 15310 does produce LPS O antigen (Fig. 2A) and does not contain the *wbiG*::IS*407*A mutation. In fact, the ATCC stock cultures (1964 and 1974) of *B. mallei* ATCC 15310 do not harbor IS*407*A insertions in *wbiG* (Jason Bannan, personal communication). *B. mallei* DB110795 was obtained by routine laboratory passage of *B. mallei* ATCC 15310 at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID). The strain used in the study of Burtnick et al. (12) was obtained from USAMRIID and was probably *B. mallei* DB110795, not *B. mallei* ATCC 15310. It was previously reported by members of our group that IS*407*A integrated into a capsular polysaccharide gene during repeated laboratory passage of *B. mallei* ATCC 23344 (24). Taken together, these results suggest that IS*407*A transposition may be relatively common during routine laboratory passage of this microorganism. Serial subculture of *B. mallei* on laboratory media results in a loss of virulence for animals (48, 49, 51, 57), and it is tempting to speculate that IS*407*A transposition is responsible, directly or indirectly, for this phenomenon.

Finally, we found that ϕE125 genomic DNA contained the methylated bases m6A and m4C (Fig. 5). DNA methylation may protect ϕE125 DNA from host restriction endonucleases (1), or it may be involved in some other aspect of the ϕE125 life cycle. We cloned and expressed ϕE125 gene*27* in *E. coli* and found that gp27 was a functional m6A methylase. We were unable to provide direct evidence that gp56 was a m4C methylase, but it was intriguing that ϕE125 DNA and genomic DNA from a *B. mallei* lysogen contained m4C. It was surprising that genomic DNA from a *B. mallei* lysogen contained m6A and m4C, but genomic DNA from a *B. thailandensis* lysogen did not. We are currently examining the possibility that gp27 and gp56 require host factors for production and/or activity that are present in *B. mallei* but not in *B. thailandensis*. Type II DNA methylases specifically bind and methylate recognition sequences on a DNA substrate (58). The DNA sequence specificities of gp27 and gp56 are currently unknown, but BLASTP results show that gp56 is similar to cytosine methylases that recognize and methylate the sequence 5′-CCCGGG-3′, which occurs nine times in the ϕE125 genome. ϕE125 DNA was treated with five restriction endonucleases that recognize this sequence (*Sma*I, *Xma*I, *Cfr*9I, *Psp*AI, and *Xma*CI), and they all cleaved the DNA into nine fragments of the predicted sizes (D. DeShazer and J. A. Jeddeloh, unpublished data). The fact that cleavage was not blocked strongly suggests that this site is not methylated. Further studies are required to determine the specificity of gp27 and gp56 and to understand their role(s) in the ϕE125 life cycle.

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