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Multilocus Sequence Typing of Historical *Burkholderia pseudomallei* Isolates Collected in Southeast Asia from 1964 to 1967 Provides Insight into the Epidemiology of Melioidosis

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A collection of 207 historically relevant *Burkholderia pseudomallei* isolates was analyzed by multilocus sequence typing (MLST). The strain collection contains environmental isolates obtained from a geographical distribution survey of *B. pseudomallei* isolates in Thailand (1964 to 1967), as well as stock cultures and colony variants from the U.S. Army Medical Research Unit (Malaysia), the Walter Reed Army Institute for Research, and the Pasteur Institute (Vietnam). The 207 isolates of the collection were resolved into 80 sequence types (STs); 56 of these were novel. eBURST diagrams predict that the historical-collection STs segregate into three complexes when analyzed separately. When added to the 760 isolates and 365 STs of the *B. pseudomallei* MLST database, the historical-collection STs cluster significantly within the main complex of the eBURST diagram in an ancestral pattern and alter the *B. pseudomallei* "population snapshot." Differences in colony morphology among reference isolates were found not to affect the STs assigned, which were consistent with the original isolates. Australian ST84 is likely characteristic of *B. pseudomallei* isolates of Southeast Asia rather than Australia, since multiple environmental isolates from Thailand and Malaysia share this ST with the single Australian clinical isolate in the MLST database. Phylogenetic evidence is also provided suggesting that Australian isolates may not be distinct from those of Thailand, since ST60 is common to environmental isolates from both countries. MLST and eBURST are useful tools for the study of population biology and epidemiology, since they provide methods to elucidate new genetic relationships among bacterial isolates.

Burkholderia pseudomallei is a gram-negative organism endemic to Southeast Asia and Northern tropical Australia and is the causative agent of the disease melioidosis. The organism is an environmental saprophyte capable of long-term survival in soil or water (8). *B. pseudomallei* infection may be acquired due to inhalation, aspiration, or through direct contact of wounded or abraded skin with contaminated material (7). In areas to which it is endemic, individuals having frequent exposure to contaminated soil or stagnant waters are at the greatest risk of developing melioidosis; however, encountering *B. pseudomallei* in the environment can lead to one of three outcomes: no effect, asymptomatic seroconversion, or clinically apparent infection (2, 7, 30). The spectrum of disease ranges from asymptomatic infection to localized skin ulcers or abscesses, acute pulmonary infection, acute septicemic infection, or fulminant disease with abscesses throughout the body (3, 34). Many of the infections occur in individuals with preexisting compromising health conditions (3). Recrudescent melioidosis has been reported to occur as long as 62 years following initial exposure (22). Virulence factors of *B. pseudomallei* contributing to pathogenesis include capsular polysaccharide, type III secretion, and protease production (24, 25, 26, 32). *B. pseudomallei* is also able to survive in eukaryotic cell lines and professional phagocytic cells (18). A simple diagnostic technique to differentiate *B. pseudomallei* from the avirulent organism *Burkholderia thailandensis* is a lack of arabinose assimilation by *B. pseudomallei* (1, 20).

The epidemiology of melioidosis is complicated due to the environmental persistence of the organism and is subject to distinct differences in the organism's distribution in soil, disease presentation, and incidence rates among different areas of endemicity. Two important regions for comparison and contrast are Thailand and Australia. *B. pseudomallei* is commonly isolated from soil in animal paddocks or from water sources in Australia, which is in contrast to Southeast Asia, where in general *B. pseudomallei* is commonly isolated from cleared, cultivated, and irrigated agricultural sites (7). In both countries, melioidosis is primarily a rainy-season disease and is a well-recognized cause of community-acquired pneumonia and septicemia (2, 6, 30, 34). The incidence rates of melioidosis vary in the two regions. The annual incidence in the Northern Territories of Australia is 19.6 cases per 100,000 people, compared with 4.4 cases per 100,000 people in Thailand (5, 30). There are also unique disease presentations reported for each region. In the Northern Territories of Australia, genitourinary disease, prostatic disease, and encephalomyelitis are reported, whereas acute suppurative parotitis is more common in the pediatric melioidosis patients of Thailand (3, 4, 34). It is unknown why there are regional differences in melioidosis epidemiology. It was speculated that Australian isolates of *B. pseudomallei* are distinct from those of Thailand or Southeast Asia in general (4). The advent of multilocus sequence typing

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(MLST) provides a novel and beneficial scheme for the study of melioidosis epidemiology worldwide.

Maiden et al. developed MLST in 1998 as a relatively new typing scheme resulting in portable data easily shared and compared between laboratories worldwide via the Internet (19). The MLST scheme for *B. pseudomallei* was developed by Godoy et al. in 2003 in a similar manner using a strain collection of 147 isolates of *B. pseudomallei*, *Burkholderia mallei*, and *B. thailandensis* from wide geographical and temporal ranges (14). The typing method is based on sequence variation within seven housekeeping-gene fragments. Allele numbers are assigned to each of the seven housekeeping loci based on sequence differences and are then arranged into a string of seven integers to give the allele profile of an isolate. The *B. pseudomallei* MLST allele profile corresponds to the gene order *ace-gltB-gmhD-lepA-lipA-narK-ndh* (14). The sequence type (ST) of an isolate is defined specifically by the allele profile. The relationships among the STs may then be examined using various methods, such as eBURST (based upon related sequence types) (10, 15, 28). At the time of writing, the *B. pseudomallei* MLST database contained 760 isolates and 365 STs (http://bpseudomallei.mlst.net/). Approximately 63% of all *B. pseudomallei* isolates in the database are of clinical origin, and 18.6% are of environmental origin (including soil and water isolates). A larger proportion of environmental *B. pseudomallei* isolates in the MLST database would contribute to a greater understanding of melioidosis epidemiology.

In this study, we examined a significant historical *B. pseudomallei* strain collection of Southeast Asian isolates from predominantly environmental sources using MLST analysis. We then compared the MLST data with those of the *B. pseudomallei* MLST database using the eBURST method (10, 28) in order to determine the implications of the addition of the historical-strain data. The current study demonstrates the ancestral nature of the historical strains examined compared with the existing *B. pseudomallei* population data and highlights a potential epidemiological connection between environmental *B. pseudomallei* isolates of Thai and Australian origin. The addition of the historical-strain data to the *B. pseudomallei* MLST database also promotes a balance between the clinical and environmental isolates reported to date.

MATERIALS AND METHODS

Bacterial strains. A total of 207 *B. pseudomallei* isolates were analyzed by MLST and are listed in Table 1. The isolates examined belong to a historical collection of strains collected in a geographic distribution survey of *B. pseudomallei* (then *Pseudomonas pseudomallei*) in Thailand by Finkelstein et al. during the period of 1964 to 1967 (11, 12). The historical collection is comprised of environmental *B. pseudomallei* isolates recovered from soil and water samples across Thailand by the hamster isolation technique (9, 11, 12). The collection also includes reference and stock cultures as well as colony variants from sources such as the U.S. Army Medical Research Unit (USAMRU) in Malaysia, the Walter Reed Army Institute for Research (WRAIR), and the Pasteur Institute in Vietnam (9, 12, 29). *B. pseudomallei* isolates obtained from soldiers serving in Vietnam were also included (12). The isolates were confirmed as *B. pseudomallei* based upon colony morphology on selective media, serology, and additional biochemical testing (12). Confirmed *B. pseudomallei* isolates were then lyophilized to preserve their characteristics. Upon receipt of the historical strain collection, each isolate was tested for growth on arabinose to exclude potential *B. thailandensis* species (1, 20). The strains were maintained as suspensions in 10% skim milk or 20% glycerol at −70°C.

The Thai culture collection, as well as antigen preparations and high-titer rabbit sera against representative strains, are also stored at the Centers for Disease Control and Prevention (Mindy Glass), Atlanta, Ga., and are available, on request, to qualified investigators.

Bacterial growth conditions and isolation of genomic DNA. Live organisms were cultured, and genomic DNA was isolated in a category III biocontainment facility at the University of Calgary. Overnight bacterial cultures were inoculated from freezer stock and grown in Luria-Bertani (LB) broth (Invitrogen, Burlington, Ontario, Canada) at 37°C. Genomic DNA was isolated using the Wizard genomic DNA purification kit (Promega, Madison, Wis.) according to the manufacturer's instructions. Genomic DNAs were stored at −20°C.

Multilocus sequence typing. MLST was carried out according to the methods of Godoy et al. (14) with modifications to promote consistency of product amplification and to improve sequencing quality. The primers used in the PCR amplification and sequencing of the seven housekeeping gene fragments are listed in Table 2. The following primer pairs were used in the PCR amplification of seven housekeeping-gene fragments from all *B. pseudomallei* strains analyzed: ace-up and ace-dn, gltB-up and gltB-dn, gmhD-up and gmhD-dn(outer), lepA-up and lepA-dn, lipA-up and lipA-dn, narK-up(outer) and narK-dn, and ndh-up and ndh-dn (Table 2).

Fifty-microliter-volume PCRs were carried out in a 96-well PCR plate format, allowing for two *B. pseudomallei* K96243 (Sanger sequencing strain) genomic DNA-positive controls and two sterile water negative controls per plate. PCRs contained the following: 1 μg genomic DNA template, 1× PCR buffer, 1.5 mM MgCl2, 0.2 mM mixed deoxynucleoside triphosphates, 0.8 μM mixed primers, 0.5× Q solution (QIAGEN, Mississauga, Ontario, Canada), and 2.5 U *Taq* DNA polymerase. All standard PCR reagents were acquired from Promega (Madison, Wis.). Initial denaturation at 95°C for 4 min was followed by 35 cycles of 95°C for 30 s, 61°C for 30 s (except for the *gmhD* and *lepA* primers, which require 63°C, and *narK* primers, which require 62°C), and 72°C for 1 min. Final extension was carried out at 72°C for 5 min, and the samples were maintained at 4°C. The amplified housekeeping-gene fragments were purified with 20% polyethylene glycol-8000–2.5 M NaCl precipitation according to the methods of Godoy et al. (14). The purified PCR products were analyzed via 1.5% agarose gel electrophoresis for sufficient product, correct size, and product purity following both PCR and purification steps. PCRs lacking sufficient product or displaying multiple products were discarded and repeated.

The purified housekeeping-DNA fragments were submitted in a 96-well PCR plate format for sequencing to the University of Calgary DNA Sequencing Core Service Facility. Each DNA fragment was sequenced on both strands using the primers indicated in Table 2. Seminested sequencing reactions were carried out for *gmhD* and *narK* gene fragments using the primers gmhD-dn and narK-up in place of gmhD-dn(outer) and narK-up(outer), respectively, to improve sequencing quality. All other housekeeping-gene products were sequenced using the same primers as for amplification (Table 2).

Data analysis. For the sequence analysis of each DNA fragment, the forward and reverse sequences were aligned with a reference allele sequence obtained from the *B. pseudomallei* MLST website using the SeqManII module of Lasergene v. 6.0 software (DNAStar, Madison, Wis.). The resulting contig was trimmed and edited if required. Each sequence alignment was examined for sequencing quality. Sequences that were too short, of poor quality, or featured two nucleotide signals at the same position were discarded, and the samples were resequenced.

Allele numbers were assigned to each of the seven housekeeping loci by submission of the forward sequence of the alignment contig to the *B. pseudomallei* MLST website (http://bpseudomallei.mlst.net/). The allele numbers for all seven housekeeping gene fragments of each isolate were assembled into a string of seven integers corresponding to the gene order of *ace-gltB-gmhD-lepA-lipA-narK-ndh*, giving the allele profile for each isolate. The allele profiles were queried against the *B. pseudomallei* MLST website to obtain an ST number.

Novel allele sequences were confirmed with repeat PCR and sequencing reactions. Novel allele profiles were confirmed by repeated PCR and sequencing reactions for the locus that differed from an existing allele profile already cataloged in the *B. pseudomallei* MLST database. Novel allele sequences and novel allele profiles were then forwarded to the *B. pseudomallei* MLST website curator for allele number and ST assignment, respectively. The MLST analysis results for the 207 isolates of the historical *B. pseudomallei* collection were confirmed with repeat PCR and sequencing reactions for 10% of the remaining samples. The 5 novel allele sequences and 56 novel STs encountered during this study have been submitted to the *B. pseudomallei* MLST database, along with the strain information for all 207 isolates of the historical-strain collection.

In order to display the relatedness among the isolates of the historical *B. pseudomallei* collection, eBURST diagrams were generated. The eBURSTv3 Java application is available as a link from the *B. pseudomallei* MLST website. eBURST is based on a model of bacterial evolution whereby a single ancestral

TABLE 1. Southeast Asian isolates of the historical *B. pseudomallei* strain collection analyzed by multilocus sequence typing

Strain	Source	Country	Yr	ST	Allele profile						
					ace	*gltB*	*gmhD*	*lepA*	*lipA*	*narK*	*ndh*
K96243[a]	Human	Thailand	1996	10	1	1	13	1	1	1	1
Antigen 13 Smooth			1965	46	3	1	2	1	1	3	3
Antigen 25 Donut[b]		Malaysia	1965	46	3	1	2	1	1	3	3
Antigen 25 HP Trans-muc[b]		Malaysia	1965	46	3	1	2	1	1	3	3
Antigen 25 Medusa[b]		Malaysia	1965	46	3	1	2	1	1	3	3
Antigen 25 Smooth[b]		Malaysia	1965	46	3	1	2	1	1	3	3
Antigen 1188HP-R[c]	Human	Malaysia	1965	99	1	1	4	1	1	4	1
Antigen 1691[d]	Human	Malaysia	1965	46	3	1	2	1	1	3	3
Antigen 6066				35	1	6	14	2	8	8	4
Chumphon 76 W-2	Water	Thailand	1965	377	3	30	11	3	1	4	3
Hansen[e]	Human	Vietnam	1966	397	3	12	6	1	1	4	3
J77[e]	Human	Vietnam	1965	46	3	1	2	1	1	3	3
Lavict[e]	Human	Vietnam	1965	211	3	1	3	1	1	4	1
Loei KK-S2	Soil	Thailand	1965	10	1	1	13	1	1	1	1
Loei KK-S2(2)	Soil	Thailand	1965	10	1	1	13	1	1	1	1
Loei KK-W5(2)	Water	Thailand	1965	365	3	2	4	1	1	3	1
Nakhon Phanom 32-3	Environment	Thailand	1966	375	3	1	4	3	1	4	3
Phangna 64 W	Water	Thailand	1965	10	1	1	13	1	1	1	1
Pasteur Institute 6068		Vietnam	1964	169	1	1	2	3	8	4	3
Pasteur Institute 6606		Vietnam	1964	35	1	6	14	2	8	8	4
Pasteur Institute 52237		Vietnam	1964	411	1	4	4	3	1	3	1
Pasteur Institute 63503		Vietnam		370	4	2	3	1	1	22	1
Phattalung 49 W-1	Water	Thailand	1965	369	3	1	2	1	5	4	3
Phattalung 49 W-2	Water	Thailand	1965	289	3	4	11	4	5	4	6
Phattalung 51 W	Water	Thailand	1965	369	3	1	2	1	5	4	3
Phattalung 52 W-2	Water	Thailand	1965	164	3	1	2	3	5	4	3
Phuket 3 W-1	Water	Thailand	1965	54	3	1	3	3	1	2	1
Phuket 6 S-1	Soil	Thailand	1965	46	3	1	2	1	1	3	3
Ramos[e]	Human	Vietnam	1966	211	3	1	3	1	1	4	1
Ranong 8	Environment	Thailand	1965	409	3	1	36	1	1	4	1
Ranong 70 W	Water	Thailand	1965	38	1	12	2	1	1	1	1
Ranong 73 W-1	Water	Thailand	1965	399	3	1	4	1	1	22	1
Ranong 73 W-2	Water	Thailand	1965	227	1	1	2	1	5	4	1
Smith 373			1966	99	1	1	4	1	1	4	1
Smith 384			1966	211	3	1	3	1	1	4	1
Smith 541			1967	410	1	1	11	1	1	4	1
Smith 660			1966	211	3	1	3	1	1	4	1
Smith 001963			1966	403	1	4	3	1	6	1	1
Smith 002025			1966	84	3	1	11	4	5	4	6
Smith 002026			1966	418	1	4	37	3	1	1	1
Smith 002159			1966	419	3	4	37	3	1	1	1
Smith 002559			1966	403	1	4	3	1	6	1	1
Smith 22179			1967	387	1	12	6	1	1	1	1
Smith 22294			1966	387	1	12	6	1	1	1	1
Songkhla 11 W	Water	Thailand	1965	168	3	1	2	1	5	4	1
Songkhla 21 W-1	Water	Thailand	1965	289	3	4	11	4	5	4	6
Songkhla 21 W-2	Water	Thailand	1965	228	1	2	3	1	1	4	1
Songkhla 25 W-1	Water	Thailand	1965	382	1	1	3	4	5	1	1
Songkhla 25 W-2	Water	Thailand	1965	382	1	1	3	4	5	1	1
Songkhla 27 W-1	Water	Thailand	1965	369	3	1	2	1	5	4	3
Songkhla 27 W-2	Water	Thailand	1965	369	3	1	2	1	5	4	3
Songkhla 34 W-1	Water	Thailand	1965	414	3	1	2	1	8	4	3
Songkhla 34 W-2	Water	Thailand	1965	84	3	1	11	4	5	4	6
STW 1	Water	Thailand	1965	369	3	1	2	1	5	4	3
STW 3	Water	Thailand	1965	366	3	1	2	3	8	4	3
STW 4-2	Water	Thailand	1965	366	3	1	2	3	8	4	3
STW 5-1	Water	Thailand	1965	289	3	4	11	4	5	4	6
STW 7	Water	Thailand	1965	376	1	4	2	3	8	4	3
STW 7-2	Water	Thailand	1965	376	1	4	2	3	8	4	3
STW 10	Water	Thailand	1965	392	1	2	6	1	1	4	1
STW 11-3	Water	Thailand	1965	288	3	2	3	2	1	3	1
STW 22-1	Water	Thailand	1965	46	3	1	2	1	1	3	3
STW 25	Water	Thailand	1965	46	3	1	2	1	1	3	3
STW 26	Water	Thailand	1965	385	1	4	11	4	5	4	6
STW 27-2	Water	Thailand	1965	70	3	4	11	3	5	4	6
STW 28-2	Water	Thailand	1965	415	1	1	11	4	5	4	6

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TABLE 1—*Continued*

Strain	Source	Country	Yr	ST	Allele profile						
					ace	*gltB*	*gmhD*	*lepA*	*lipA*	*narK*	*ndh*
STW 28-5	Water	Thailand	1965	376	1	4	2	3	8	4	3
STW 32-1	Water	Thailand	1965	389	3	1	11	1	1	4	1
STW 32-3	Water	Thailand	1965	407	3	1	3	3	1	2	18
STW 33	Water	Thailand	1965	385	1	4	11	4	5	4	6
STW 34	Water	Thailand	1965	407	3	1	3	3	1	2	18
STW 35-1	Water	Thailand	1965	371	1	12	6	2	1	4	1
STW 36-1	Water	Thailand	1965	366	3	1	2	3	8	4	3
STW 38	Water	Thailand	1965	84	3	1	11	4	5	4	6
STW 39	Water	Thailand	1965	371	1	12	6	2	1	4	1
STW 42	Water	Thailand	1965	371	1	12	6	2	1	4	1
STW 43-1	Water	Thailand	1965	371	1	12	6	2	1	4	1
STW 44	Water	Thailand	1965	371	1	12	6	2	1	4	1
STW 45-1	Water	Thailand	1965	289	3	4	11	4	5	4	6
STW 55-2	Water	Thailand	1965	289	3	4	11	4	5	4	6
STW 58-1	Water	Thailand	1965	289	3	4	11	4	5	4	6
STW 61-2	Water	Thailand	1965	372	1	2	3	1	5	22	1
STW 62	Water	Thailand	1965	84	3	1	11	4	5	4	6
STW 64	Water	Thailand	1965	385	1	4	11	4	5	4	6
STW 66	Water	Thailand	1965	384	1	12	3	3	1	4	1
STW 67-1	Water	Thailand	1965	374	1	4	11	4	5	2	6
STW 94-1	Water	Thailand	1965	164	3	1	2	3	5	4	3
STW 95-1	Water	Thailand	1965	371	1	12	6	2	1	4	1
STW 96-2	Water	Thailand	1965	371	1	12	6	2	1	4	1
STW 97-1	Water	Thailand	1965	84	3	1	11	4	5	4	6
STW 98-1	Water	Thailand	1965	51	3	1	2	3	1	4	3
STW 99-2	Water	Thailand	1965	380	3	2	2	1	1	4	3
STW 100-1	Water	Thailand	1965	15	1	2	2	2	1	3	1
STW 101-1	Water	Thailand	1965	84	3	1	11	4	5	4	6
STW 102-3	Water	Thailand	1965	84	3	1	11	4	5	4	6
STW 104-1	Water	Thailand	1965	51	3	1	2	3	1	4	3
STW 105-1	Water	Thailand	1965	371	1	12	6	2	1	4	1
STW 106-1	Water	Thailand	1965	54	3	1	3	3	1	2	1
STW 107-1	Water	Thailand	1965	372	1	2	3	1	5	22	1
STW 110-1	Water	Thailand	1965	51	3	1	2	3	1	4	3
STW 111-2	Water	Thailand	1965	164	3	1	2	3	5	4	3
STW 114-1	Water	Thailand	1965	369	3	1	2	1	5	4	3
STW 115-2	Water	Thailand	1965	51	3	1	2	3	1	4	3
STW 116-2	Water	Thailand	1965	289	3	4	11	4	5	4	6
STW 117-4	Water	Thailand	1965	371	1	12	6	2	1	4	1
STW 120-1	Water	Thailand	1965	389	3	1	11	1	1	4	1
STW 122	Water	Thailand	1965	168	3	1	2	1	5	4	1
STW 152	Water	Thailand	1966	416	1	1	6	2	1	42	1
STW 154	Water	Thailand	1965	371	1	12	6	2	1	4	1
STW 157	Water	Thailand	1965	168	3	1	2	1	5	4	1
STW 162-1	Water	Thailand	1965	289	3	4	11	4	5	4	6
STW 168-3	Water	Thailand	1965	54	3	1	3	3	1	2	1
STW 174	Water	Thailand	1965	402	3	1	2	3	1	2	1
STW 175-1	Water	Thailand	1965	369	3	1	2	1	5	4	3
STW 176	Water	Thailand	1965	401	3	3	2	1	1	4	1
STW 181-1	Water	Thailand	1965	51	3	1	2	3	1	4	3
STW 185	Water	Thailand	1965	46	3	1	2	1	1	3	3
STW 185-1	Water	Thailand	1965	405	1	4	6	2	1	2	1
STW 186-2	Water	Thailand	1965	366	3	1	2	3	8	4	3
STW 187-3	Water	Thailand	1965	366	3	1	2	3	8	4	3
STW 189-2	Water	Thailand	1965	396	3	2	2	3	5	3	1
STW 197-1	Water	Thailand	1965	376	1	4	2	3	8	4	3
STW 199-2	Water	Thailand	1965	376	1	4	2	3	8	4	3
STW 200-1	Water	Thailand	1965	376	1	4	2	3	8	4	3
STW 202-3	Water	Thailand	1965	366	3	1	2	3	8	4	3
STW 204-2	Water	Thailand	1965	366	3	1	2	3	8	4	3
STW 205-1	Water	Thailand	1965	51	3	1	2	3	1	4	3
STW 208	Water	Thailand	1965	51	3	1	2	3	1	4	3
STW 208-1	Water	Thailand	1965	51	3	1	2	3	1	4	3
STW 214	Water	Thailand	1965	84	3	1	11	4	5	4	6
STW 215-1	Water	Thailand	1965	408	3	1	2	3	8	4	24
STW 216-2	Water	Thailand	1965	400	1	12	3	2	1	8	1

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TABLE 1—*Continued*

Strain	Source	Country	Yr	ST	Allele profile						
					ace	*gltB*	*gmhD*	*lepA*	*lipA*	*narK*	*ndh*
STW 217-2	Water	Thailand	1965	383	1	4	6	2	3	1	1
STW 219	Water	Thailand	1965	368	3	1	2	3	8	4	1
STW 220-2	Water	Thailand	1965	289	3	4	11	4	5	4	6
STW 221-1	Water	Thailand	1965	371	1	12	6	2	1	4	1
STW 222-2	Water	Thailand	1965	378	18	12	3	2	1	2	1
STW 224-1	Water	Thailand	1965	289	3	4	11	4	5	4	6
STW 225-3	Water	Thailand	1965	84	3	1	11	4	5	4	6
STW 230-1	Water	Thailand	1965	366	3	1	2	3	8	4	3
STW 233-1	Water	Thailand	1965	371	1	12	6	2	1	4	1
STW 235-1	Water	Thailand	1965	376	1	4	2	3	8	4	3
STW 244-1	Water	Thailand	1965	378	18	12	3	2	1	2	1
STW 305	Water	Thailand	1965	300	1	1	3	1	1	4	1
STW 307-2	Water	Thailand	1965	395	1	3	36	1	1	4	3
STW 312-1	Water	Thailand	1965	300	1	1	3	1	1	4	1
STW 358-2	Water	Thailand	1965	312	1	4	2	1	1	3	1
STW 359-1	Water	Thailand	1965	364	1	3	4	3	1	4	3
STW 362	Water	Thailand	1965	364	1	3	4	3	1	4	3
STW 364	Water	Thailand	1965	364	1	3	4	3	1	4	3
STW 368-3	Water	Thailand	1965	364	1	3	4	3	1	4	3
STW 402	Water	Thailand	1965	379	1	2	2	3	5	4	1
STW 406	Water	Thailand	1965	164	3	1	2	3	5	4	3
STW 414-1	Water	Thailand	1965	390	1	1	14	2	1	22	1
STW 415	Water	Thailand	1965	290	3	4	11	3	5	4	1
STW 420-2	Water	Thailand	1965	417	18	4	6	2	5	1	3
STW 422	Water	Thailand	1965	391	1	2	10	2	1	8	1
STW 424-1	Water	Thailand	1965	417	18	4	6	2	5	1	3
STW 426-2	Water	Thailand	1965	409	3	1	36	1	1	4	1
STW 429	Water	Thailand	1965	413	3	2	6	1	1	3	3
STW 430	Water	Thailand	1965	51	3	1	2	3	1	4	3
STW 447	Water	Thailand	1965	366	3	1	2	3	8	4	3
STW 487-1	Water	Thailand	1965	3	1	1	2	2	5	3	1
STW 539-1	Water	Thailand	1965	392	1	2	6	1	1	4	1
STW 551	Water	Thailand	1965	385	1	4	11	4	5	4	6
STW 561-1	Water	Thailand	1966	393	3	2	3	1	1	4	1
STW 638-1	Water	Thailand	1966	398	1	2	3	1	1	22	1
STW 640	Water	Thailand	1966	381	1	2	2	1	1	3	1
STW 723	Water	Thailand	1966	388	1	1	4	1	1	22	1
STW 729	Water	Thailand	1966	394	3	4	11	3	1	4	3
STW 730-1	Water	Thailand	1966	404	1	1	4	3	1	4	3
STW 753	Water	Thailand	1966	412	1	4	10	1	1	2	1
STW 754	Water	Thailand	1966	386	3	1	3	1	1	2	1
STW 760-2	Water	Thailand	1966	376	1	4	2	3	8	4	3
STW 765	Water	Thailand	1966	70	3	4	11	3	5	4	6
UB-8-Soil 1	Soil	Thailand	1965	167	1	1	4	1	1	3	1
UB-8-Soil 2	Soil	Thailand	1965	60	3	1	12	1	1	3	1
Ubol 6-1	Environment	Thailand	1965	373	3	1	11	2	5	4	1
USAMRU Malaysia 1		Malaysia	1964	46	3	1	2	1	1	3	3
USAMRU Malaysia 3		Malaysia	1964	46	3	1	2	1	1	3	3
USAMRU Malaysia 4		Malaysia	1964	51	3	1	2	3	1	4	3
USAMRU Malaysia 5		Malaysia	1964	46	3	1	2	1	1	3	3
USAMRU Malaysia 7		Malaysia	1964	46	3	1	2	1	1	3	3
USAMRU Malaysia 8		Malaysia	1964	46	3	1	2	1	1	3	3
USAMRU Malaysia 10		Malaysia	1964	51	3	1	2	3	1	4	3
USAMRU Malaysia 11		Malaysia	1964	46	3	1	2	1	1	3	3
USAMRU Malaysia 12		Malaysia	1964	54	3	1	3	3	1	2	1
USAMRU Malaysia 13		Malaysia	1964	46	3	1	2	1	1	3	3
USAMRU Malaysia 14		Malaysia	1964	84	3	1	11	4	5	4	6
USAMRU Malaysia 15		Malaysia	1964	84	3	1	11	4	5	4	6
USAMRU Malaysia 16		Malaysia	1964	84	3	1	11	4	5	4	6
USAMRU Malaysia 17		Malaysia	1964	84	3	1	11	4	5	4	6
USAMRU Malaysia 18		Malaysia	1964	406	1	4	13	2	1	1	1
USAMRU Malaysia 19		Malaysia	1964	406	1	4	13	2	1	1	1
USAMRU Malaysia 22		Malaysia	1964	46	3	1	2	1	1	3	3
USAMRU Malaysia 23		Malaysia	1964	46	3	1	2	1	1	3	3
USAMRU Malaysia 24		Malaysia	1964	46	3	1	2	1	1	3	3
USAMRU Malaysia 25		Malaysia	1964	46	3	1	2	1	1	3	3

Continued on following page

TABLE 1—*Continued*

Strain	Source	Country	Yr	ST	Allele profile						
					ace	*gltB*	*gmhD*	*lepA*	*lipA*	*narK*	*ndh*
USAMRU Malaysia 26		Malaysia	1964	51	3	1	2	3	1	4	3
USAMRU Malaysia 27		Malaysia	1964	51	3	1	2	3	1	4	3
USAMRU Malaysia 28		Malaysia	1964	289	3	4	11	4	5	4	6
USAMRU Malaysia 29		Malaysia	1964	46	3	1	2	1	1	3	3
USAMRU Malaysia 30		Malaysia	1964	46	3	1	2	1	1	3	3
USAMRU Malaysia 31		Malaysia	1964	51	3	1	2	3	1	4	3
USAMRU Malaysia 32		Malaysia	1964	46	3	1	2	1	1	3	3
VW from infected rat	Rat	Malaysia?	1967	367	3	1	11	1	1	3	1
WRAIR 294		Malaysia	1964	51	3	1	2	3	1	4	3
WRAIR 1188	Human	Malaysia	1964	99	1	1	4	1	1	4	1

[a] K96243 is the Sanger sequencing *B. pseudomallei* strain used as a positive control in this study (16).
[b] Antigen 25 strains are colonial variants of USAMRU 25.
[c] Antigen 1188HP-R is a rough colonial variant of WRAIR 1188.
[d] Antigen 1691 is from a human isolate in Kuala Lumpur, 1921 (33).
[e] Hansen, J77, Lavict and Ramos strains were isolated from soldiers in Vietnam.

founding ST undergoes diversification to produce a subset of closely related STs (10, 28). Descendants of the founding ST diversify by accumulating point mutations or undergo recombination that eventually generates diversity among the MLST housekeeping-allele sequences, resulting in closely related but variant STs of the founding ST. Single locus variants (SLVs) of the group founding ST differ in their allele profiles by 1/7 housekeeping allele sequences. The SLVs diversify further into double locus variants where 2/7 housekeeping alleles differ from the original founding ST. A single spot on the eBURST diagram represents each individual ST, and the size of the spot is proportional to the number of isolates in the population that share that ST. SLVs are joined to the founding ST by a line, double locus variants are joined to the SLVs, and so on until a bacterial population is represented as a series of clonal complexes with the founding ST (defined as the ST with the most SLVs) located centrally with a series of variant STs radiating outward. Only the STs with similar allele profiles are grouped together with the default group definition of 6/7 shared alleles (10, 28).

The 207 isolates of the historical collection were analyzed separately by generating a "population snapshot" of all 80 STs encountered during the analysis by setting the group definition from 6/7 to 0/7 shared alleles. The 56 novel sequence types encountered were differentially highlighted by using the comparative function of eBURST. The existing STs encountered during the analysis (Reference) were compared to the novel STs encountered (Query). Since the novel STs do not appear in the Query data set, they are highlighted green. The comparative function of eBURST was then used to display the changes to the full-size (i.e., MLST database) *B. pseudomallei* "population snapshot" due to the addition of the historical-isolate MLST data. The database information excluding the novel STs encountered was loaded as the initial data set (Reference). The second data set (Query) included all STs pertaining to the historical-strain collection, including the 56 novel STs and the 24 existing STs that were already present in the database, which allowed differentiation of the specific historical-isolate STs encountered and all other STs of the *B. pseudomallei* MLST database. The novel STs do not appear in the Reference data set and are highlighted green. The existing STs do appear in the Reference data set and are highlighted magenta.

RESULTS

The historical collection of Southeast Asian *B. pseudomallei* isolates exhibits sequence type diversity. The 207 isolates of the historical *B. pseudomallei* collection were analyzed by MLST (Table 1). Five new allele sequences were encountered: one (each) for *ace* (allele 18), *gltB* (allele 30), and *ndh* (allele 24) and two for *gmhD* (alleles 36 and 37). The strain collection was resolved into 80 different STs, 24 which were already present in the *B. pseudomallei* MLST database and 56 novel STs, which were designated ST364 through ST419 upon submission of the unique allele profiles to the database curator. Approximately half (102/207) of the isolates in the strain collection were assigned existing STs and are representative of *B. pseudomallei* MLST database isolates sourced from diverse

TABLE 2. Primers used in amplification and sequencing of seven housekeeping loci for multilocus sequence typing analysis of the historical *B. pseudomallei* strain collection

Locus	Primer name	Primer sequence (5′→3′)	Annealing temp (°C)	Application[a]
ace	ace-up	GCT CGG CGC TTC TCA AAA CG	61	Amp & Seq
	ace-dn	CAT GTC CGT GCC GAT GTA GC		
gltB	gltB-up	GGC GGC AAG TCG AAC ACG G	61	Amp & Seq
	gltB-dn	GCA GGC GGT TCA GCA CGA G		
gmhD	gmhD-up	CTC GCG CAG GGC ACG CAG T	63	Amp & Seq
	gmhD-dn	GTC AGG AAC GGC GCG TCG TA		Seq
	gmhD-dn(outer)	GGC TGC CGA CCG TGA GAC C		Amp
lepA	lepA-up	CGC TTG ATC GGC ACT GAA TGG	63	Amp & Seq
	lepA-dn	CGA ACC ACG AAT CGA TGA TGA G		
lipA	lipA-up	CAT ACG GTG TGC GAG GAA GC	61	Amp & Seq
	lipA-dn	CAG GAT CTC GTC GGT CGT CT		
narK	narK-up(outer)	GCC GCG CAC GAC CAG CGC	62	Amp
	narK-up	CGG ATT CGA TCA TGT CCA CTT C		Seq
	narK-dn	CGG CAC CCA CAC GAA GCC C		Amp & Seq
ndh	ndh-up	GCA GTT CGT CGC GGA CTA TC	61	Amp & Seq
	ndh-dn	GGC GCG GCA TGA AGC TCC A		

[a] Amp, amplification; Seq, sequencing; Amp & Seq, both.

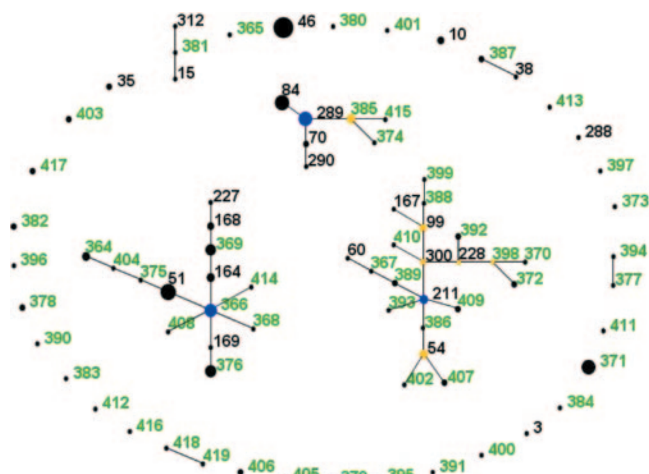

FIG. 1. eBURST diagram displaying the relatedness of the 207 isolates and 80 sequence types of the historical *B. pseudomallei* strain collection. Predicted group founders are indicated in blue, and subgroup founders are indicated in yellow. The predicted group founder of all STs is ST366. The 56 novel STs encountered during the study are displayed in green text. The diagram was edited manually for clarity.

geographical locations, including Thailand, Laos, Malaysia, Indonesia, Bangladesh, Singapore, China, Hong Kong, the Philippines, Ecuador, The United States, Fiji, and Australia (http://bpseudomallei.mlst.net/). The 56 novel STs are most similar to *B. pseudomallei* MLST database isolates sourced from Thailand, Malaysia, Vietnam, Cambodia, Bangladesh, Singapore, China, Hong Kong, the Philippines, the United States, Australia, and Burkina Faso in that they share five or six out of seven housekeeping alleles in common (i.e., single or double locus variants, respectively).

The historical-strain collection of *B. pseudomallei* isolates is biased towards the southern regions of Thailand. Isolates recovered from Phangna, Phattalung, Phuket, Ranong, Songkhla, and south Thailand water (STW) sources account for 69% (143/207) of the collection (Table 1). The greatest amount of ST diversity occurs among these isolates. Sixty-two percent (89/143) of the south Thailand isolates were assigned novel STs.

An eBURST diagram was generated to explore the relationships among the 207 isolates and 80 STs of the historical *B. pseudomallei* collection. A "population snapshot" was generated by setting the group definition in the "Analysis" panel from the default 6/7 alleles to 0/7 shared alleles and is shown in Fig. 1. The comparative function of eBURST was used to allow for the differential identification of the 56 novel STs encountered (shown in green text) from the 24 existing STs already present in the *B. pseudomallei* MLST database. The STs of the historical collection form three small, discontinuous complexes with ST366 as the predicted group founder for all 80 STs indicated in the figure. The majority of the STs appearing in the three complexes were isolated from the south of Thailand (i.e., STW isolates, Songkhla, Ranong, and Phuket), with Malaysian isolates among others interspersed. Multiple outlier STs surround the three complexes, since they are distinct from the predicted group founding ST and remain unlinked.

The STs of the historical collection of Southeast Asian *B. pseudomallei* isolates are ancestral compared to the *B. pseudomallei* population. In order to determine the phylogenetic nature of the historical *B. pseudomallei* isolates, an eBURST diagram was generated to display the changes to the *B. pseudomallei* MLST database "population snapshot" upon the addition of the historical-isolate ST data. Figure 2A is the current "population snapshot" of all 760 isolates and 365 STs in the *B. pseudomallei* MLST database, excluding the historical-isolate data and novel sequence types submitted (ST364 to ST419). Figure 2B is the *B. pseudomallei* "population snapshot" upon the addition of the historical-strain collection MLST data. The comparative function of eBURST was used again to allow the differential identification of the 56 novel STs encountered (shown with green halos) and the 24 existing STs encountered (shown with magenta halos) from the remaining STs of the *B. pseudomallei* MLST database (Fig. 2B).

The *B. pseudomallei* "population snapshot" contains a large complex at the center of the eBURST diagram (complex 1), as well as two smaller complexes in the bottom left (complex 2) and top right (complex 3) corners (Fig. 2A). Many outlying STs surround the main complex. The outlier STs are predominantly isolates of Australian origin as well as *B. mallei* and *B. thailandensis* spp. (http://bpseudomallei.mlst.net/). The predicted group founder for all STs in the diagram is ST48 (of Thai origin), shown in blue on the left-hand side of complex 1 (Fig. 2A). The addition of the historical *B. pseudomallei* strain collection MLST data to the "population snapshot" does not alter the predicted group founder (ST48) or the number of complexes present but alters the pattern of ST descent within complexes 1 and 2 but not 3. The majority (62/80) of the historical *B. pseudomallei* strain collection STs cluster within the main clonal complex in the center of the eBURST diagram, as well as within complex 2 (Fig. 2B). The novel STs, in particular, provide "branch points" or "linker" STs for descendant STs that are already cataloged in the MLST database and contribute to branch rearrangement within the main complex, suggesting an ancestral nature for the historical-strain collection STs.

Three isolates of the historical collection of *B. pseudomallei* isolates share ST10 with K96243. *B. pseudomallei* K96243 is the Sanger sequencing strain and was previously assigned the MLST allele profile 1-1-13-1-1-1-1, which corresponds to ST10 (14, 16). There are currently two other clinical isolates in the *B. pseudomallei* MLST database with this ST. They are SID4350 and 2687. Three of the historical *B. pseudomallei* isolates sourced from environmental sampling in Thailand were found to share this ST. They are Loei KK-S2 (soil isolate), Loei KK-S2 (2) (soil isolate), and Phangna 64W (water isolate); all three were isolated in 1965. These are the first environmental *B. pseudomallei* isolates reported to be assigned ST10 according to the MLST database (http://bpseduomallei.mlst.net/). The Loei isolates were recovered in a geographical origin similar to that of the K96243 sequencing strain, which was isolated in 1996 from a female patient in Khon Kaen hospital in Northeast Thailand; however, the 31-year difference in the dates of isolation is considerable (16). The assignment of ST10 to the Phangna 64W strain indicates that ST10 may not be restricted to this region of Thailand alone and is also present in the southern regions of Thailand.

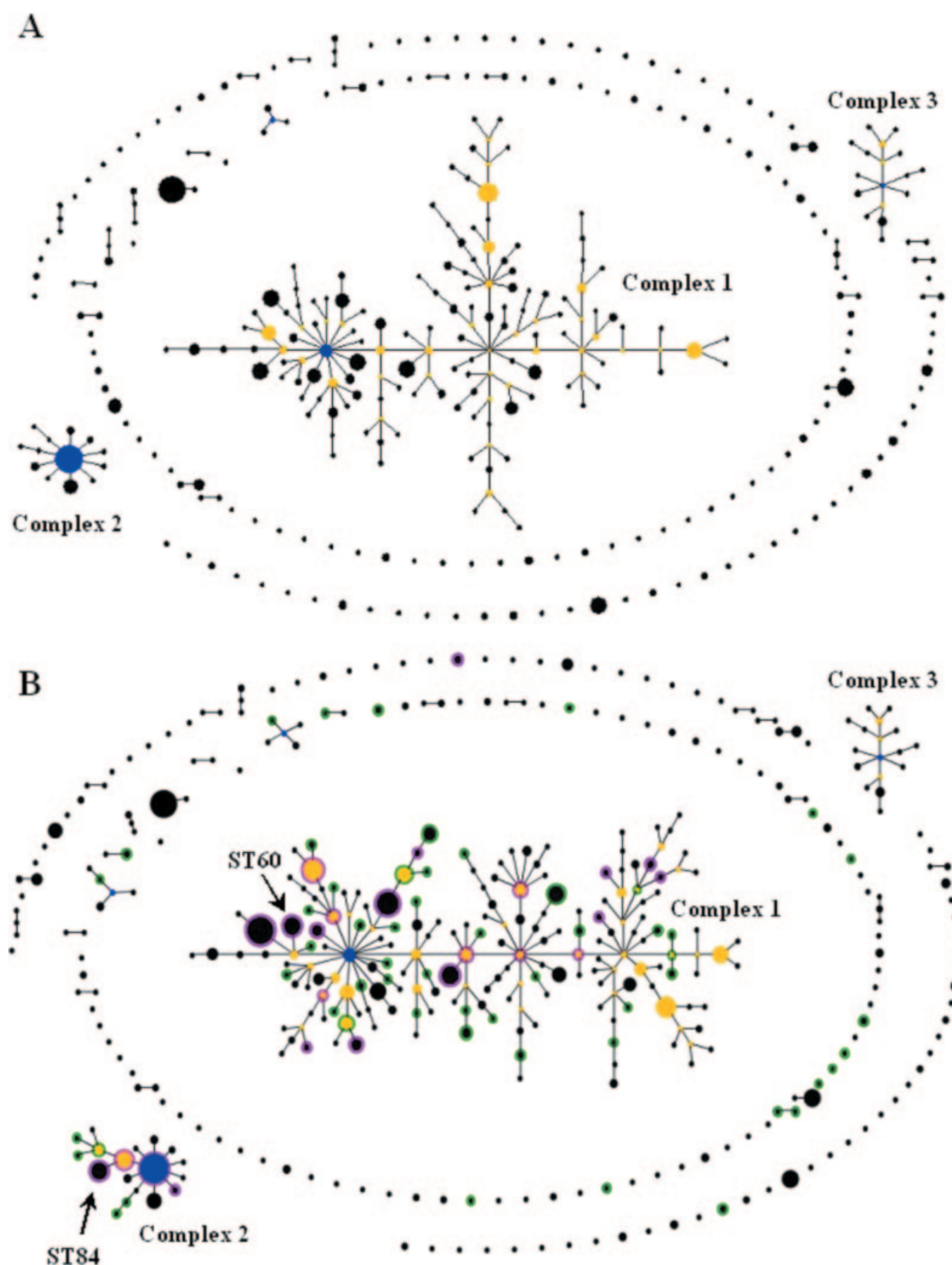

FIG. 2. eBURST diagram displaying the "population snapshot" of the *B. pseudomallei* isolates of the MLST database before (A) and after (B) the addition of the MLST data of the historical *B. pseudomallei* collection. The predicted group founder of all isolates is ST48 in both panels. Panel A is an eBURST diagram demonstrating the relatedness of the 760 isolates and 365 STs of the *B. pseudomallei* MLST database. Panel B is a comparative eBURST diagram demonstrating the alteration to the original "population snapshot" upon the addition of the 207 isolates and 80 STs of the historical *B. pseudomallei* strain collection to the isolates of the *B. pseudomallei* MLST database. The 56 novel STs encountered during the study are indicated with green halos, and the 24 existing STs encountered during the study are indicated with magenta halos. The diagrams were edited manually, and the ST numbers were omitted for clarity. The clonal complex numbers are indicated. ST60 and ST84 are indicated with arrows.

Variation in colony morphology does not affect the ST of *B. pseudomallei* isolates. The reference strains included in the historical collection of *B. pseudomallei* isolates contain colonial variants. The four Antigen 25 strains in the collection (Antigen 25 Donut, HP Trans-muc, Smooth, and Medusa) are colonial variants of USAMRU Malaysia 25 (Table 1). All five isolates were assigned the allele profile 3-1-2-1-1-3-3, which corresponds to ST46. Additionally, Antigen 1188HP-R is a rough colonial variant of WRAIR 1188 (Table 1). Both isolates were assigned ST99 (allele profile 1-1-4-1-1-4-1). These results sug-

TABLE 3. *B. pseudomallei* isolates of the historical strain collection that share ST60 and ST84 in common with *B. pseudomallei* isolates of the MLST database

Strain	ST	Source	Country	Yr	Reference
UB-8-Soil 2	60	Soil	Thailand	1965	This study
1244	60	Human	Thailand	Unknown	http://bpseudomallei.mlst.net/
1248	60	Human	Thailand	Unknown	http://bpseudomallei.mlst.net/
2708	60	Human	Thailand	Unknown	http://bpseudomallei.mlst.net/
2820	60	Human	Thailand	Unknown	http://bpseudomallei.mlst.net/
E0013	60	Environment	Thailand	Unknown	http://bpseudomallei.mlst.net/
E0031	60	Environment	Thailand	Unknown	http://bpseudomallei.mlst.net/
E0378	60	Environment	Thailand	Unknown	http://bpseudomallei.mlst.net/
E0383	60	Environment	Thailand	Unknown	http://bpseudomallei.mlst.net/
5892/339[a]	60	Human	Fiji	1992	http://bpseudomallei.mlst.net/
D228[a]	60	Environment	Australia	Unknown	http://bpseudomallei.mlst.net/
D260:53/30[a]	60	Environment	Australia	Unknown	http://bpseudomallei.mlst.net/
D304:S3/40[a]	60	Environment	Australia	Unknown	http://bpseudomallei.mlst.net/
2002721631[b]	60	Environment	Australia	Unknown	http://bpseudomallei.mlst.net/
2002721632[b]	60	Environment	Australia	Unknown	http://bpseudomallei.mlst.net/
Smith 002025	84	Unknown	Unknown	1966	This study
Songkhla 34W-2	84	Water	Thailand	1965	This study
STW 38	84	Water	Thailand	1965	This study
STW 62	84	Water	Thailand	1965	This study
STW 97-1	84	Water	Thailand	1965	This study
STW 101-1	84	Water	Thailand	1965	This study
STW 102-3	84	Water	Thailand	1965	This study
STW 214	84	Water	Thailand	1965	This study
STW 225-3	84	Water	Thailand	1965	This study
USAMRU Malaysia 14	84	Unknown	Malaysia	1964	This study
USAMRU Malaysia 15	84	Unknown	Malaysia	1964	This study
USAMRU Malaysia 16	84	Unknown	Malaysia	1964	This study
USAMRU Malaysia 17	84	Unknown	Malaysia	1964	This study
2002721162[b]	84	Human	Australia	1970	http://bpseudomallei.mlst.net/

[a] Australian strains previously characterized by MLST (14).
[b] Australian strains previously characterized by 16S rRNA gene sequencing (13).

gest that changes in colony morphology do not affect MLST typing of *B. pseudomallei* isolates.

The STs of the historical collection of Southeast Asian *B. pseudomallei* isolates indicate phylogenetic links between Thai and Australian STs. A single isolate of the historical strain collection, UB-8-Soil 2, was assigned the allele profile 3-1-12-1-1-3-1, which corresponds to ST60. Compared with the isolates of the MLST database, it was noted that ST60 represents eight clinical and environmental isolates from Thailand, a clinical isolate from Fiji (isolated in 1992), and five environmental isolates from Australia (Table 3) (http://bpseudomallei.mlst.net/) (13, 14). This finding indicates that multiple environmental isolates from Thailand and Australia are very closely related as they share the same ST. ST60 also shares five out of seven alleles in common with ST48 (allele profile 3-1-2-1-1-4-1), the predicted group founder of Thai origin in the eBURST "population snapshot" of all *B. pseudomallei* isolates in the MLST database (Fig. 2).

Thirteen isolates of the historical-strain collection were assigned the allele profile 3-1-11-4-5-4-6, which corresponds to ST84 (Table 3). The 13 isolates were recovered from the southern region of Thailand and Malaysia between the years 1964 and 1966. ST84 represents a single clinical isolate from Australia in the MLST database, isolated in 1970 from a female patient with empyema whose travel history was unknown (13; J. Gee, personal communication) (Table 3). Within the eBURST "population snapshot," of all *B. pseudomallei* isolates in the MLST database, ST84 clusters within complex 2 among other STs characteristic of isolates from Southeast Asia (Fig. 3). ST84 is a single locus variant of ST289 (allele profile 3-4-11-4-5-4-6), which is represented by a single clinical isolate from Thailand isolated in 1993. ST289 was also encountered in this study and was assigned to 11 isolates of the historical strain collection. These isolates were recovered in the southern region of Thailand (i.e., STW, Songkhla, and Phattalung) and

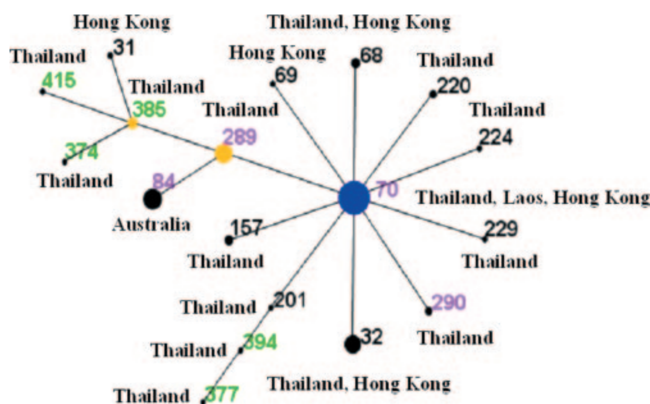

FIG. 3. Clonal complex two of the altered eBURST "population snapshot" from panel B of Fig. 2. The group founder for the complex is ST70 and is indicated in blue. The novel STs encountered during the study are indicated with green text, and the existing STs encountered during the study are indicated with magenta text. The diagram was edited manually, and the origins of the STs were added.

Malaysia between 1964 and 1965 (Table 1). According to eBURST, ST84 is likely representative of *B. pseudomallei* isolates originating from Southeast Asia.

DISCUSSION

MLST is an excellent tool for the study of bacterial populations and global epidemiology when paired with a phylogenetic analysis algorithm, such as eBURST, to display the relatedness of bacterial isolates as a phylogenetic network. MLST results in digital data that are easily shared between laboratories via the internet. Multiple methods for MLST data analysis are available. The unweighted-pair group method with arithmetic averages is the most commonly used but has many shortcomings. The unweighted-pair group method with arithmetic averages cannot detect zero-length branches and usually places the root of the tree based on an assumption that is usually false (15). Neighbor-joining dendrograms and minimum-evolution trees using concatenated allele sequences are also common in MLST analysis; however, eBURST is a more appropriate choice for phylogenetic analysis of MLST data, since it was designed specifically for that purpose (15). eBURST allows the researcher to view the bacterial population as a whole by generating a "population snapshot" and makes no attempt to display the relatedness between very different multilocus genotypes in a population (28). The eBURST method of phylogenetic analysis also provides predicted pathways of evolution and ancestry that are often absent from dendrograms (28).

Typing bacterial isolates by MLST has the potential to identify multiple different alleles, allele profiles, and therefore STs among strain collections, since variation between housekeeping-locus alleles is detected by sequencing internal PCR fragments directly. The population structure of *B. pseudomallei* is clonal at the rRNA level, although clinical isolates of *B. pseudomallei* exhibit genetic diversity and are thus excellent candidates for MLST (23). It was therefore expected that historical isolates of the *B. pseudomallei* strain collection would exhibit tremendous diversity according to MLST analysis. Significant ST diversity is evident among the 207 historical isolates. Five novel alleles were encountered during the study, along with 56 novel STs and 24 STs already cataloged in the MLST database. The majority of the isolates from the southern region of Thailand were assigned novel STs, indicating a great amount of genotypic diversity among environmental *B. pseudomallei* isolates from this region. The ST diversity encountered for southern Thai isolates in this study contrasts with previous research, where ribotyping analysis demonstrated a lack of genetic diversity among *B. pseudomallei* isolates from southern Thailand (23). eBURST analysis of the MLST data predicts that the isolates of the historical collection form three groups of related STs with many outlying, or unlinked, STs when analyzed separately as a population. This is a discontinuous pattern among isolates of a relatively clonal species as demonstrated by ribotyping (23). The epidemiological significance of the historical isolates is demonstrated upon the addition of the MLST data generated during this study to the current MLST data of the *B. pseudomallei* MLST database. Although a greater proportion of the STs assigned in this analysis were novel, the majority of the historical-isolate STs clustered within the main complex as well as a smaller complex of the eBURST "population snapshot" and contributed to a rearrangement of branches within the main complex. The historical-isolate MLST data also support the eBURST assignment of ST48 of Thai origin as the group founder for all STs in the diagram. Many of the historical-isolate STs serve as branch points or linker STs in the phylogenetic eBURST network. The placement of the historical isolate STs within the eBURST diagram suggests an ancestral nature of the *B. pseudomallei* isolates obtained in an environmental survey of Thailand 40 years ago compared with the more recently obtained isolates of the MLST database.

The historical collection of *B. pseudomallei* isolates is heavily biased towards the southern region of Thailand, and therefore, isolates from this region are overrepresented in the collection. In the original geographic distribution study of *B. pseudomallei* isolates in Thailand carried out by Finkelstein, the southern region was sampled more extensively than the central, northern, and northeastern regions and resulted in the highest isolation rates of *B. pseudomallei* from water samples using the hamster isolation technique (11, 12). An early Malaysian study also reported the highest isolation rate of *B. pseudomallei* from water samples (29). Isolates were also recovered from sampling in Ubon, Nakhon Phanom, and Loei in the survey, but the high isolation rate for *B. pseudomallei* from the south of Thailand is in disagreement with clinical melioidosis data (11, 12). Other studies involving environmental sampling for *B. pseudomallei* isolation have reported contradictory isolation rates for *B. pseudomallei* from soil, which have been found to be the highest in the northeast region, where clinical cases of melioidosis are prevalent, as well as in the south, where there are fewer clinical cases reported (21, 27, 30, 31). The annual incidence of melioidosis in the northeast region of Thailand is 137.9 cases per 100,000 hospital inpatients, whereas the annual incidence in the southern region is 14.4 cases per 100,000 hospital inpatients (31). The number of CFU per ml of soil/water suspensions was found to be highest in the northeast region of Thailand and is probably related to the risk of disease in that region (27, 31). *B. pseudomallei* is reportedly unevenly distributed in soil, and the differences in isolation rates may be explained by regional variations in temperature, rainfall, sunlight, soil composition, and relative humidity (7, 27, 31). Although the collection itself is regionally biased towards the south, the addition of the historical-isolate data to the *B. pseudomallei* MLST database will provide balance, representing the geographical distribution and population biology of *B. pseudomallei* isolates in Thailand, and will shift the ratio of clinical isolates to environmental isolates, promoting increased support for environmental strains of this soil saprophyte and human pathogen in the MLST database.

MLST analysis of the historical collection of *B. pseudomallei* isolates revealed an epidemiological connection between the first environmental isolates reported to be assigned the same ST as Sanger sequencing strain K96243 (14, 16). Two of the three isolates were recovered from soil samples in Loei, which is in close geographical proximity to Khon Kaen, where strain K96243 was isolated from a female diabetic patient in 1996 (16). The 31-year difference in the dates of isolation is considerable and provides support for persistence of closely related *B. pseudomallei* isolates in the environment. ST10 is not restricted to the northeast region of Thailand. The third histor-

ical isolate assigned ST10 was isolated from a water sample obtained in Phangna in the southern region of Thailand. The occurrence of ST10 in the northeast and southern regions of Thailand suggests there is a lack of regional specificity for *B. pseudomallei* STs.

The colonial variants of the historical collection of *B. pseudomallei* isolates were found to retain the same allele profile and thus ST as their parent isolates. The Antigen 25 colonial variants Donut, HP Trans-muc, Medusa, and Smooth of USAMRU 25 and the Antigen 1188 HP-R rough colonial variant of WRAIR 1188 are genetically similar to their parent isolates by MLST definition despite phenotypic variation. MLST allows an organism to be typed in a manner representative of the genome and does not account for genomic rearrangement or discrete changes outside of the chosen housekeeping loci. Since MLST analysis cannot detect a correlation between ST and disease presentation, it would not be expected that a change in phenotype would affect the allele profile of an isolate (4).

The epidemiology of melioidosis in Australia is unique compared with that of other areas of endemicity. It has been speculated that Australian *B. pseudomallei* isolates are unique and distinct from those of Southeast Asia based on the geographical separation of Australia as well as epidemiological differences noted for each area (4). There have been longstanding reports in the literature regarding the different aspects of epidemiology specific to Australia and Thailand, including differences in *B. pseudomallei* distribution in soil, disease presentation, and annual incidence rates; however, there are aspects of epidemiology that are shared between the two regions, such as disease correlation with rainfall and *B. pseudomallei* infection as an important cause of community-acquired pneumonia and septicemia (2, 5, 6, 17, 27, 30, 31, 34). Interestingly, two Australian STs were encountered during the study, ST60 and ST84. ST84 was encountered among 13 strains of the historical collection and was assigned to isolates from the southern region of Thailand and Malaysia. According to the MLST database, ST84 is represented by a single clinical isolate from Australia obtained in 1970 from an Australian woman with empyema and an unknown travel history (13; J. Gee, personal communication). eBURST analysis places ST84 within complex 2, which includes STs representative of Southeast Asia. ST84 is also a single locus variant of ST289, an ST that is representative of *B. pseudomallei* isolates from Thailand and Malaysia. It is most likely that the patient traveled to Thailand or Malaysia and acquired *B. pseudomallei* abroad, suggesting that ST84 is representative of isolates from Southeast Asia rather than Australia.

A single isolate of the historical collection, UB-8-Soil 2, was isolated in 1965 from the northeast region of Thailand and was assigned ST60 in this study. According to the *B. pseudomallei* MLST database, ST60 is shared by four clinical isolates and four environmental isolates from Thailand, a single clinical isolate from Fiji, and five environmental isolates from Australia (http://bpseudomallei.mlst.net/) (13). Further investigation with eBURST revealed that ST60 shares five out of seven alleles in common with ST48, the predicted group founder of Thai origin in the "population snapshot" of all *B. pseudomallei* isolates in the MLST database, and clusters within the main clonal complex among other Thai and Southeast Asian STs. This finding is profoundly significant to melioidosis epidemiology and research, since it suggests an environmental association between Thai and Australian *B. pseudomallei* isolates specifically. Australian *B. pseudomallei* isolates were recently reported as being distinct from other isolates sourced elsewhere in the world based on MLST analysis (4). This is no longer the case. MLST analysis of the historical strain collection has highlighted an important epidemiological link between environmental isolates of Thai and Australian origin.

The development of MLST by Maiden et al. in 1998 provided a significant contribution of an effective typing scheme with great resolving power to the study of population biology and epidemiological study of pathogens. MLST databases for many different human pathogens are currently available for international access (http://www.mlst.net/ and http://pubmlst.org/). The *B. pseudomallei* MLST database is no exception. Although relatively young, the *B. pseudomallei* MLST database continues to grow at a rapid pace, facilitating the study of melioidosis epidemiology. MLST has allowed the epidemiological investigation of historical *B. pseudomallei* isolates collected in a nationwide geographical survey in Thailand 40 years prior to this study. The collection was found to be genetically diverse, and the historical isolates were ancestral in nature compared to the *B. pseudomallei* population even though isolates from the southern region of Thailand were overrepresented. The first environmental isolates possessing ST10, the same ST as K96243, were reported. Finally, an environmental epidemiological link was discovered between *B. pseudomallei* isolates originating from Thailand and Australia, which has important implications for future studies in melioidosis epidemiology.

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