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Use of a Variable Amplicon Typing Scheme Reveals Considerable Variation in the Accessory Genomes of Isolates of *Burkholderia pseudomallei*†

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Melioidosis, a disease caused by the bacterium *Burkholderia pseudomallei*, is endemic in southeast Asia and northern Australia. We used suppression subtractive hybridization (SSH) to identify sequences that varied between two *B. pseudomallei* isolates from Australia and determined the distribution of 45 SSH-derived sequences among a panel of *B. pseudomallei* and *B. thailandensis* isolates. Sequences exhibiting variable prevalence were included in a variable amplicon typing (VAT) scheme designed to score the presence or absence of 14 PCR amplicons. VAT analysis was carried out with 48 isolates from Thailand, which were typed by multilocus sequence typing (MLST), and 44 isolates from Australia of known MLST type. The VAT scheme could be used to divide the 48 isolates from Thailand into 23 VAT types and the 44 isolates from Australia into 36 VAT types. Some of the sequences included in the VAT scheme were more commonly PCR positive among isolates from Australia than among isolates from Thailand, and vice versa. No isolate from Australia was PCR positive for genomic island 11 or a putative transposase sequence, whereas four SSH-derived sequences were far more prevalent among the Australian isolates. Analysis based on the VAT scheme indicated that the isolates clustered into groups, some of which were mainly or exclusively from one geographical origin. One cluster included Australian isolates that were mostly associated with severe disease, including rare neurological melioidosis, suggesting that the content of the accessory genome may play an important role in determining the clinical manifestation of the disease.

The disease melioidosis, caused by the gram-negative bacterium *Burkholderia pseudomallei*, is endemic in southeast Asia and northern Australia (4). *B. pseudomallei*, a normal inhabitant of soil and surface water in regions of endemicity, infects via direct inoculation or inhalation and can cause severe sepsis or pneumonia. Not only can melioidosis affect many different sites in the body, but it also has a wide spectrum of severity, ranging from acute and often fatal sepsis to more chronic disease (8, 16, 28). Several years ago, *B. pseudomallei* was separated from an avirulent biotype lacking the ability to assimilate arabinose, now known as *B. thailandensis* (24). However, it is clear that the levels of virulence exhibited by different *B. pseudomallei* isolates can vary considerably in animal models (26). Such variations can occur between related strains and do not necessarily correlate with clinical outcome or the source of the isolate (26).

Various typing methods have been applied to the study of genetic variation among *B. pseudomallei* populations (14, 18), including molecular fingerprinting approaches such as ribotyping (13), random amplified polymorphic DNAs (RAPDs) (17, 26), and macrorestriction analysis coupled with pulsed-field gel electrophoresis (PFGE) (5, 15, 26). More recently, a multilocus sequence typing (MLST) scheme has been developed (11). Extensive typing of isolates by MLST has demonstrated that isolates from Australia differ from those isolated elsewhere (6), but there was no correlation between strain type and clinical presentation, a finding supported by analysis by PFGE (4).

Molecular typing methods often suffer from the lack of portability (RAPDs), the requirement for specialized equipment (PFGE), the length of the procedures (PFGE and MLST), or the cost (MLST). MLST has emerged as a preferred typing method for phylogenetic studies because of its portability and unequivocal output data. However, MLST typing specifically targets the conserved regions of bacterial genomes rather than the accessory genome, which may have an important role to play in virulence. The genome sequence of *B. pseudomallei* K96243 revealed the presence of 16 genomic islands (GIs) with variable distributions among *B. pseudomallei* isolates, suggesting that horizontal gene transfer has played an important role in the evolution of this pathogen (12). More recently, 16 regions of difference (RDs) in the genome of strain K96243, 13 of which corresponded to the GIs, were reported following a comparison with strain Bp15682 by the use of microarrays (22). Other studies have provided further evidence of considerable variations in the accessory genome of *B. pseudomallei* (9, 21).

Subtractive hybridization is a powerful technique for the identification of DNA sequences present in one strain (the tester) but absent from another (the driver or reference), and it has widely been applied to the study of bacterial pathogens (29), including *B. pseudomallei* and its close relative, *B. mallei* (9, 10, 20). In this study we describe the use of suppression

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TABLE 1. Strains used in this study[a]

Isolate(s)	Country	Source; notes[b]	MLST group[c]	Allele no.						
				ace	*gltB*	*gmhD*	*lepA*	*lipA*	*narK*	*ndh*
B. pseudomallei isolates for subtraction and distribution analysis										
338[d]	Australia	Melioidosis (chronic)	243	1	2	13	4	15	12	1
520[d]	Australia	Melioidosis (fulminant)	ND	ND	ND	ND	ND	ND	ND	ND
146 (VE05)	Australia	Goat isolate; same ribotype as 511 (B); LD_{50} of 9.01×10^2	ND	ND	ND	ND	ND	ND	ND	ND
511 (VE02)	Australia	Goat isolate, same ribotype as 146 (B); LD_{50} of 6.32×10^4	ND	ND	ND	ND	ND	ND	ND	ND
157 (CL26)	Australia	Melioidosis (A); LD_{50} of 3.00×10^0	ND	ND	ND	ND	ND	ND	ND	ND
161 (VE06)	Australia	Sheep isolate (A); LD_{50} of 8.00×10^0	ND	ND	ND	ND	ND	ND	ND	ND
169 (EN11)	Australia	Soil isolate (A); LD_{50} of 5.00×10^0	ND	ND	ND	ND	ND	ND	ND	ND
244 (EN10)	Australia	Soil isolate (B); LD_{50} of 8.43×10^2	ND	ND	ND	ND	ND	ND	ND	ND
186 (VE03)	Australia	Soil isolate (B); LD_{50} of 7.82×10^3	ND	ND	ND	ND	ND	ND	ND	ND
265 (EN07)	Australia	Soil isolate (B); LD_{50} of 4.25×10^3	ND	ND	ND	ND	ND	ND	ND	ND
295 (EN08)	Australia	Soil isolate (B); LD_{50} of 3.13×10^3	ND	ND	ND	ND	ND	ND	ND	ND
1655	Australia	From patient with long-term carriage	ND	ND	ND	ND	ND	ND	ND	ND
E503	Malaysia	Melioidosis	ND	ND	ND	ND	ND	ND	ND	ND
E505	UK/Goa	Clinical isolate	ND	ND	ND	ND	ND	ND	ND	ND
E506	Malaysia	Melioidosis	ND	ND	ND	ND	ND	ND	ND	ND
E955 (204), E957 (576)	Thailand	Clinical isolates	ND	ND	ND	ND	ND	ND	ND	ND
E958, E8 (E960)	Thailand	Environmental isolates	ND	ND	ND	ND	ND	ND	ND	ND
G185 (K96243)	Thailand	Clinical isolate; genome sequence strain	ND	ND	ND	ND	ND	ND	ND	ND
B. thailandensis isolates for subtraction and distribution analysis										
E82 (E959), E32, E111, E125, E132, E135, E216, E251, E253, E254, E255, E260	Thailand	T. Pitt	ND	ND	ND	ND	ND	ND	ND	ND
B. pseudomallei isolates used for VAT analysis (excluding 338)										
303	Australia	Tracheotomy isolate	36	1	7	14	7	1	12	11
332	Australia	Human isolate	106	1	2	3	2	16	21	1
973	Australia	Human isolate	107	1	2	3	4	1	8	1
1152	Australia	Rectal swab isolate	108	1	2	3	2	6	22	1
64	Australia	Blood isolate	109	1	2	13	4	1	19	1
1080	Australia	Wound swab isolate	111	1	2	13	2	1	9	1
911	Australia	Sputum isolate	112	1	2	13	16	1	22	1
502	Australia	Soil isolate	114	1	3	3	4	1	24	1
875	Australia	Urine isolate	115	1	4	3	2	4	26	1
1164	Australia	Blood isolate	116	1	4	3	4	1	12	1
1153	Australia	CSF isolate	117	1	4	13	14	8	22	11
978	Australia	Blood isolate	118	1	4	14	2	1	8	1
789	Australia	Human isolate	120	1	4	22	2	5	23	1
114	Australia	Lesion nodule isolate	121	1	4	23	2	1	8	1
1357	Australia	Human isolate	122	1	6	13	2	1	8	11
449	Australia	Blood isolate	126	1	14	20	1	15	9	15
944	Australia	Blood isolate	127	1	15	3	2	6	27	1
634	Australia	Human isolate	128	1	15	13	2	8	12	1
668	Australia	Blood isolate	129	1	15	13	2	1	22	1
614	Australia	Prostate isolate	132	1	16	13	4	6	21	1
1128	Australia	Skin lesion isolate	133	1	16	13	4	15	21	1
130	Australia	Blood isolate	134	1	16	14	4	1	19	1
99	Australia	Human isolate	135	1	17	13	4	15	22	1
129	Australia	Right-foot wound isolate	138	4	2	14	4	1	6	1
239	Australia	Blood isolate	140	4	7	3	4	1	19	1
362	Australia	Urine isolate	141	4	16	3	4	1	9	6
983	Australia	Throat isolate	142	8	2	3	4	1	19	1
1123	Australia	Blood isolate	143	8	2	13	4	1	6	1
1168	Australia	Blood isolate	144	8	2	13	15	1	27	1
1161	Australia	Sputum isolate	146	10	2	3	4	3	2	1
1174	Australia	Blood isolate	147	10	2	3	4	15	2	1
62	Australia	Human isolate	148	10	15	3	4	3	22	1
356	Australia	Blood isolate	149	11	2	14	2	1	6	1
272	Australia	Sputum isolate	236	1	1	13	1	18	23	11
210	Australia	Blood isolate	238	1	2	3	21	15	9	1
112C	Australia	Human isolate	241	1	2	13	2	8	6	1
253	Australia	Right-foot ulcer	242	1	2	13	4	1	22	1
271	Australia	Blood isolate	247	1	2	23	2	1	31	1
222	Australia	Knee swab isolate	259	1	6	13	2	15	8	11

Continued on facing page

TABLE 1—*Continued*

Isolate(s)	Country	Source; notes[b]	MLST group[c]	Allele no.						
				ace	*gltB*	*gmhD*	*lepA*	*lipA*	*narK*	*ndh*
504	Australia	Soil isolate	268	1	15	31	4	6	19	1
527	Australia	Blood isolate	269	1	21	3	2	5	22	11
506	Australia	Soil isolate	281	12	4	6	2	3	33	1
157	Australia	Human isolate	284	13	15	13	4	6	6	11
139	PNG	Abscess isolate	246	1	2	22	18	1	22	11
141	PNG	Blood isolate	274	4	20	13	2	3	6	11
140	Fiji	Abscess isolate	280	12	1	3	1	1	22	1
314	Malaysia	Blood isolate	58	3	1	5	1	1	4	1
P1	Thailand	Blood isolate; northeastern Thailand	u	1	1	12	2	6	4	1
P2	Thailand	Pus isolate; northeastern Thailand	16	1	2	2	1	1	10	1
P3	Thailand	Pus isolate; south Thailand	u	1	1	11	1	6	22	1
P4	Thailand	Blood isolate; eastern Thailand	211	3	1	3	1	1	4	1
P5	Thailand	Pus isolate; northeastern Thailand	u	3	1	2	1	8	4	3
P6	Thailand	Blood isolate; south Thailand	93	1	1	2	1	1	4	1
P7	Thailand	Blood isolate; Bangkok, Thailand	307	1	2	3	1	1	3	1
P8	Thailand	Sinus isolate; Bangkok, Thailand	51	3	1	2	3	1	4	3
P9	Thailand	Pus isolate; south Thailand	u	3	1	2	3	5	2	1
P11	Thailand	Blood isolate; south Thailand	u	3	2	3	1	1	4	1
P12	Thailand	Urine isolate; Bangkok, Thailand	46	3	1	2	1	1	3	3
P14	Thailand	Pleural fluid; Chiang Mai, Thailand	u	1	4	3	4	n	n	3
P15	Thailand	Pleural fluid; Chiang Mai	56	3	1	4	1	1	4	1
P16, P22	Thailand	Blood isolate; Chiang Mai	290	3	4	11	3	5	4	1
P17, P21	Thailand	Blood isolate; Chiang Mai	u	1	1	4	17	1	4	1
P18, P19	Thailand	Blood isolate; Chiang Mai	167	1	1	4	1	1	3	1
P20	Thailand	Blood isolate; Chiang Mai	70	3	4	11	3	5	4	6
P23	Thailand	Blood isolate; Chiang Mai	u	1	4	3	3	5	3	3
P24	Thailand	Blood isolate; Chiang Mai	u	1	1	4	17	1	3	1
P25	Thailand	Pus isolate; Chiang Mai	17	1	2	3	1	1	1	1
P26	Thailand	Pus isolate; Chiang Mai	10	1	1	13	1	1	1	1
P27	Thailand	Pus isolate; Chiang Mai	u	1	2	4	1	1	22	1
P28, P31	Thailand	Pus isolate; Chiang Mai	u	1	1	4	17	1	4	1
P29	Thailand	Pus isolate; Chiang Mai	u	4	12	13	2	1	2	1
P30, P45	Thailand	Pus isolate; Chiang Mai	u	3	2	3	1	1	4	1
P32	Thailand	Pus isolate; Chiang Mai	u	1	2	4	3	6	3	1
P33, P35	Thailand	Pus isolate; Chiang Mai	u	1	4	6	1	6	3	1
P34, P44	Thailand	Pus isolate; Chiang Mai	70	3	4	11	3	5	4	6
P36	Thailand	Pus isolate; Chiang Mai	u	3	2	4	1	1	4	1
P37	Thailand	Pus isolate; Chiang Mai	u	4	1	2	2	6	4	1
P38	Thailand	Sputum isolate; Chiang Mai	56	3	1	4	1	1	4	1
P39	Thailand	Urine isolate; Chiang Mai	10	1	1	13	1	1	1	1
P40	Thailand	Urine isolate; Chiang Mai	u	4	1	2	2	6	4	1
P41	Thailand	Urine isolate; Chiang Mai	u	1	1	4	17	1	4	1
P42	Thailand	Pleural fluid; Chiang Mai	70	3	4	11	3	5	4	6
P43	Thailand	Blood isolate; Chiang Mai	10	1	1	13	1	1	1	1
P46	Thailand	Sputum isolate; Chiang Mai	10	1	1	13	1	1	1	1
P47	Thailand	Sputum isolate; Chiang Mai	u	3	1	2	3	5	2	1
P48	Thailand	Sputum isolate; Chiang Mai	46	3	1	2	1	1	3	3
P49	Thailand	Sputum isolate; Chiang Mai	70	3	4	11	3	5	4	6
P50	Thailand	Urine isolate; Chiang Mai	23	1	2	13	1	1	1	1
B. thailandensis isolate used in VAT analysis										
E52	Thailand	Water isolate; Chiang Mai	ND	ND	ND	ND	ND	ND	ND	ND

[a] Abbreviations: u, MLST type not present in the database; ND, not determined; n, novel sequence for this locus; UK, United Kingdom; PNG, Papua New Guinea; CSF, cerebrospinal fluid.

[b] The 50% lethal doses (LD_{50}) were determined by using a BALB/c mouse model (26). RAPD subtypes are denoted by letter designations in parentheses.

[c] Where known, the MLST group is indicated.

[d] Isolates in SSH.

subtractive hybridization (SSH) to identify sequences that vary between two *B. pseudomallei* isolates from Australia. We further study the distribution of such sequences among a panel of *B. pseudomallei* isolates. Using this information and data from previous studies, we describe the development of a portable multiplex PCR (M-PCR)-based method to screen for the presence or absence of 14 PCR amplicons. Finally, we describe the use of this variable amplicon typing (VAT) scheme for its ability to discriminate between isolates from Australia and Thailand.

MATERIALS AND METHODS

Bacterial strains. The isolates used in this study are listed in Table 1. The Australian isolates chosen for SSH were isolate 338 and isolate 520. Isolate 338 was isolated from the sputum of a 50-year-old man with chronic lung disease who had a mild clinical infection and intermittently positive sputum cultures over several years, despite specific therapy for melioidosis. Isolate 520 was isolated from a 62-year-old woman on corticosteroids for chronic lung disease who died of progressive septicemic melioidosis pneumonia. Uniquely among 49 isolates tested, strain 338 has been found in a mouse model to induce a strong immunoprotective capacity against subsequent infection with another strain (27).

The strain panel used to analyze the distribution of subtracted sequences

included *B. pseudomallei* isolates from Australia (26) and *B. pseudomallei* and *B. thailandensis* isolates described previously (30). Further collections of 48 uncharacterized isolates from Thailand and 47 isolates mainly from Australia, each of which represented a different MLST group, were used to test sequence distributions by use of the VAT scheme (Table 1). Isolate 338 was included in this analysis. All isolates used in the VAT analysis were isolated from different patients.

Extraction of DNA. DNA was isolated from strains 338 and 520 for use in SSH by the guanidium thiocyanate method, as described previously (25). Small-scale isolation of DNA from the collection of Thai isolates was carried out with the Wizard Genomic DNA Purification kit (Promega). DNA from the larger collection of mainly Australian isolates was extracted by using the QIAamp DNA mini kit (QIAGEN).

MLST typing. MLST typing of 48 *B. pseudomallei* isolates from Thailand was carried out by PCR amplification and DNA sequencing of the seven loci (*ace*, *gltB*, *gmhD*, *lepA*, *lipA*, *narK*, and *ndh*) used in the published MLST typing scheme (11). The loci were amplified by using the oligonucleotide primers and conditions recommended at the website http://bpseudomallei.mlst.net/ and were sequenced with the same primers. The search facility at http://bpseudomallei.mlst.net/ was used to assign the sequences obtained to allele types and to screen for previously reported MLST types. The MLST types for the 47 isolates mainly from Australia were determined previously (6).

Construction and screening of subtraction libraries. SSH was carried out with the CLONTECH PCR-Select bacterial genome subtraction kit (Clontech) as recommended by the supplier, but with a hybridization temperature of 73°C to take account of the high G+C content of the organism. PCR products obtained following SSH were cloned into pGEM-T (Invitrogen) to produce a subtracted DNA library of RsaI fragments. Plasmid DNA from individual clones was extracted and sequenced with vector primers by Lark Technologies. Nucleotide sequences were analyzed for their presence in genome sequence strains by using the BLASTN facilities at the websites http://www.sanger.ac.uk/Projects/B_pseudomallei (*B. pseudomallei* K96243) and http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi (*B. mallei* ATCC 23344 and *B. thailandensis* E264). BLASTX searches of the general database were carried out by using the website http://www.ncbi.nlm.nih.gov.

PCR and M-PCR amplification. The oligonucleotide primers (Sigma-Genosys) used in PCR assays and for the labeling of probes are listed in Table 2 and Table S1 in the supplemental material, along with the annealing temperatures used. Amplifications were carried out in an Eppendorf MasterCycler thermal cycler for 30 cycles consisting of 95°C (1 min), the annealing temperature (1 min), and 72°C (2 min), with an additional extension time at 72°C (10 min) following completion of the 30 cycles.

Dot blot hybridization. Dot blot hybridization of genomic DNA was carried out as described previously (30) with a digoxigenin labeling and detection system (Roche). Posthybridization washes were carried out by using stringent conditions.

RESULTS

SSH between two Australian isolates. Two rounds of SSH were carried out between strains 338 and 520, using each strain in turn as the tester strain. In excess of 50 clones were sequenced for each of the subtractions. The presence or absence of each of the nonduplicated subtracted sequences in the tester and the driver strains was assessed by PCR assay. The results of the SSH experiments are summarized in Table 3. We identified 20 sequences that were PCR positive for strain 338 but PCR negative for strain 520 and 19 sequences that were PCR positive for strain 520 but PCR negative for strain 338.

Distribution of subtracted sequences among a panel of strains. Using PCR amplification assays, we studied the distribution of 45 sequences from the subtractions between strain 338 and strain 520 among a panel of 19 *B. pseudomallei* isolates and 14 *B. thailandensis* isolates (Table 1). The sequences screened included 10 sequences that were PCR positive for both strain 338 and strain 520 (Table 3), 5 of which were absent from the genome sequence strain (K96243). Among *B. pseudomallei* isolates, only strain 338 was PCR positive for sequences 338-B1, 338-B3, 338-B8, 338-B20, 338-2A7, 338-2B2, 338-2B4, 338-2B10, 338-2C3, and 338-2D9, whereas only strain 520 was PCR positive for sequences 520-E12, 520-2E7, 520-2F2, and 520-E18. The distribution of the remaining sequences among the *B. pseudomallei* isolates, based on PCR assays, is shown in Fig. 1. Interestingly, a single strain of *B. thailandensis* was PCR positive for the sequences 338-B3 and 338-B8, both of which had been detected only in a single strain of *B. pseudomallei*. *B. thailandensis* isolates were also PCR positive for sequence 520-E35 (all 14 isolates), sequence 520-2H3 (12 isolates), sequence 520-2E10 (11 isolates), sequence 338-2C5 (10 isolates), sequence 338-2D10 (8 isolates), sequence 520-2F6 (3 isolates), sequence 520-E16 (1 isolate), and sequence 520-E19 (1 isolate). All other PCR amplification tests conducted with the *B. thailandensis* isolates were negative.

Sequences 338-B4 and 338-B7 shared common distribution profiles and matched putative genes located in close proximity to each other, upstream from the previously reported genomic island GI5 (12), and within RD6 (22) in strain K96243 (Table 3; Fig. 1). Sequence 338-B18, included in the distribution analysis, matched a sequence located in the previously reported genomic island GI14 (12). All but three of the isolates tested were PCR positive for this sequence (Fig. 1). All but one of the isolates tested were PCR positive for sequence 520-E42, located within GI7 (12).

Development of a VAT scheme. Using the information derived from the distribution analysis we chose several sequences that exhibited variation between strains of *B. pseudomallei* and tested various primer set combinations with a view to developing M-PCR assays designed to give variable amplicon profiles. As a positive control we included a PCR assay for a capsule gene (*gmhA*; Table 2). In addition, we included a PCR assay for a putative transposase gene originally identified in strain E503 (23) and known to have a variable prevalence among *B. pseudomallei* strains (unpublished data) and PCR assays for some of the genomic islands identified previously (12). After testing numerous combinations of primers, we developed a strategy involving four separate M-PCRs (Fig. 2). M-PCR1 is designed to assay for the *gmhA*-positive control and the variable sequences 338-B7, 520-E42, and 520-E33. M-PCR2 assays for the putative transposase from strain E503 and the variable sequences 338-2C5, 520-E35, and 520-2G9. M-PCR3 assays for GI11 and the variable sequences 338-2D10 and 520-E36. Finally, M-PCR4 assays for GI12 and the variable sequences 338-B3 and 520-2E10. Amplicon sizes are given in Table 2. Overall, four sequences matched within or near genomic islands, four sequences matched transposases, one sequence was bacteriophage related, one sequence was DNA helicase related, two sequences matched hypothetical proteins of unknown function, and one sequence had no significant match (Table 3).

Application of VAT to isolates from Australia and Thailand. The 48 isolates of *B. pseudomallei* from Thailand were assigned to an allele type for each of the loci *ace*, *gltB*, *gmhD*, *lepA*, *lipA*, *narK*, and *ndh* (Table 1). With the exception of the *lipA* and *narK* loci of one isolate (P14), all alleles matched a sequence already deposited in the MLST database. The allele profiles were used to assign 24 of the isolates to previously reported MLST groups (Table 1). The 48 isolates could be subdivided into 29 MLST groups, 11 of which contained more than one

TABLE 2. Oligonucleotide primers used for M-PCR amplification

M-PCR no. and primer	Sequence (5′ to 3′)	Amplicon size (bp)	Target	A.T. (°C)[a]	Reference or source
M-PCR1				58	
146-5F	ATCTGATCAGGACGCTTG	666	*gmhA* (BPSL2795[b]) (capsule)		This study
146-5R	CACTGCTTCCCGAAAATG				
338-B7F	ACTGGAATCGGGAAAAAC	482	338-B7 (near GI5)		This study
338-B7R	ACGATATTTTTCCGCTGC				
520-E42F2	ATGCCGGCAGCGTCATAGA	257	520-E42 (GI7)		This study
520-E42R2	ACAACGCATGCTTACAGTA				
520-E33F	GATCCATGACCACGGCCA	135	520-E33		This study
520-E33R	AGGCCGAGAGTCTGATTG				
M-PCR2				58	
TRANSF	TTTACCGAAGTCATGAGC	657	Transposase		This study
TRANSR	TTTGAAGTGCTGGTCGAC				
338-2C5F	AGCAATAAGCGGGCAAAA	403	338-2C5		This study
338-2C5R	ATCACAAGCTATCCGCAG				
520-E35F	CTACTAGCCACTGATTCC	290	520-E35		This study
520-E35R	ATAGATCATTCGTCCGAG				
520-2G9F	ACCTCGATTTTGCGTCTG	145	520-2G9		This study
520-2G9R	AGAATGGCGTGGAGATTG				
M-PCR3				58	
338-2D10F2	ATGTCGTGCCTCCGTTCA	320	338-2D10		This study
338-2D10R2	ATGAGTCGGATCGGATCA				
GI11BF	TGTCGTGGCCCGGGGATTTGTA	238	BPSL3260[b] (GI11)		12
GI11BR	TATTCGTTGCTTTCGCGTGTGGTC				
520-E36F	GTAATGACGCAAGACGCCG	132	520-E36		This study
520-E36R	ACGGCCGAACACAAGAAC				
M-PCR4				50	
GI12F	GCAATGGAATCGACGCAACATTG	788	BPSL3349[b] (GI12)		12
GI12R	GACGCTGGCGGGTATGGGTAAG				
338-B3F	AATCAGACACTCGAGGAC	605	338-B3		This study
338-B3R	ATAACCTGCTCGATTTTCC				
520-2E10F	CTCCACCGTGACGCTAAG	392	520-2E10		This study
520-2E10R	GAGCACTTCACGCGTCTG				

[a] A.T., annealing temperature.
[b] The GenBank accession number is given.

strain. Eighteen isolates had MLST profiles that were unique among the collection of Thai isolates. The largest MLST groups (sequence type 70 [ST70] and one MLST type previously unreported) comprised five isolates (isolates P20, P34, P44, P42, and P49 and isolates P17, P21, P28, P31, and P41, respectively). Four isolates (isolates P26, P39, P43, and P46) shared MLST type ST10. None of the isolates from Thailand shared an MLST type with any of the isolates from Australia.

The M-PCR assays for VAT were applied to DNA extracted from strains of the two collections. Positive control DNA comprising individual or mixed DNA samples known to contain the relevant sequences were included in each of the PCR amplifications, and each DNA sample was tested on a minimum of two occasions. An amplicon was obtained from all DNA preparations for the positive control capsule gene (M-PCR1) with the exception of the preparation for *B. thailandensis* E52. The full VAT profiles are available in Table S2 in the supplemental material. The 95 *B. pseudomallei* isolates could be separated into a total of 57 VAT types. The 48 isolates from Thailand, comprising 29 different MLST groups, could be separated into 23 VAT types. The 44 isolates from Australia, comprising 44 different MLST groups, could be separated into 36 VAT types.

The five isolates from Thailand of ST70 could be subdivided by their VAT profiles into three groups. Only one isolate (isolate P42) was PCR positive for GI11, and only isolates P20 and P44 were PCR negative for sequence 338-2D10. Of isolates P17, P21, P28, P31, and P41, which shared an MLST type, isolates P17, P28, and P31 also shared common VAT profiles. However, isolate P21 differed by being PCR positive for GI11, and isolate P41 differed by being PCR positive for sequence 338-B7, located upstream of GI5. Of the four isolates sharing ST10, one isolate (isolate P39) differed from the others in that it was PCR negative for GI11. Another pair of isolates (isolates P33 and P35) that shared a common MLST type also differed in their VAT profiles. However, four other pairs of isolates (isolates P12 and P48, P9 and P47, P30 and P45, and P37 and P40) shared a common MLST type and identical VAT profiles.

The prevalences of the VAT PCR amplicons are summarized in Table 4. Some of the sequences included in the VAT scheme were more commonly PCR positive among isolates from one of the main geographical locations than among isolates from the other. In particular, no isolate from Australia was PCR positive for the putative transposase sequence (TRANS) or GI11; a far higher proportion of Australian isolates were PCR positive for sequences 520-E33, 520-2G9, and 520-E36 (all transposase related) and sequence 338-B3 (DNA helicase related); a higher proportion of the isolates from Thailand were PCR positive for sequences 338-B7 (near GI5)

TABLE 3. Summary of subtractive hybridization

SSH[a] sequence and sequence type	Length (bp)	G+C content (%)[b]	Presence of sequenced genome[c]			Best BLASTX match; comments (GenBank accession no.)	% Identity	Length (no. of amino acids)	*E* value
			Bpm	Bt	Bm				
Sequences present in strain 338 but not 520									
Recombination related									
338-B3 (DQ351720)	1,097	55.7	–	–	–	DNA helicase-related protein (*Xanthomonas campestris*) (NP_637459)	32	364	$2e^{-45}$
338-2D1	760	57.8	–	–	–	DNA helicase-related (*Xanthomonas campestris*) (NP637459); different region of same protein as 338-B3	72	248	$7e^{-83}$
338-B20	333	54.1	–	–	–	Uncharacterized protein (*Rubrivivax gelatinosus*) (ZP_00241526); Membrane proteins, DNA recombination protein RmuC (*Salmonella* and others) (NP_457782)	80 54	110 110	$4e^{-43}$ $5e^{-26}$
Bacteriophage related									
338-2C9	509	51.3	–	p	–	Putative transmembrane protein (*Ralstonia solanacearum*) (NP_520413); DNA methylase of bacteriophage Φ E125 (*B. thailandensis*) (AAL47559)	74 96	50 28	$1e^{-15}$ $3e^{-8}$
Transcriptional regulators									
338-B7	482	54.8	+1	–	–	DeoR family transcriptional regulator in RD6/GI5 (BPSL0939)	99	160	$4.5e^{-82}$
338-2C3	305	57.4	–	–	–	Transcriptional regulator (*Ralstonia eutropha*) (ZP_00169018)	47	59	$2e^{-13}$
Enzymes									
338-2A12	283	57.6	–	–	–	Maleylacetate reductase (*Ralstonia* sp.) (AAS87585)	55	68	$4e^{-16}$
338-2D9	480	59.2	–	–	–	Alcohol dehydrogenase (*Polaromonas* sp.) (ZP_00364129)	72	159	$5e^{-60}$
Hypothetical proteins									
338-B8	425	50.6	–	–	–	Hypothetical protein (*Escherichia coli* O157:H7) (NP_313283)	32	145	$3e^{-13}$
338-2C4	616	52.6	–	–	–	Hypothetical protein (*Rhodopseudomonas palustris*) (NP_949350)	31	217	$5e^{-16}$
338-B4	282	53.9	+1	–	–	Hypothetical protein in RD6/GI5 (BPSL0942)	100	88	$2e^{-42}$
338-2D7	>624	48.9	–	–	–	Hypothetical protein (*Chromobacterium violaceum*) (AAQ61798)	39	94	$3e^{-10}$
No significant BLASTX matches									
338-B1	190	50.0	–	–	–				
338-B16	374	48.1	–	–	–				
338-2A7	292	44.5	–	–	–				
338-2B2	429	50.4	–	–	–				
338-2B4	333	51.1	+2	+	+				
338-2B7	426	57.5	–	–	–				
338-2B10	282	43.3	–	–	–				
338-2D3	331	52.3	–	–	–				
Sequences present in strain 520 but not 338									
Mobile elements									
520-E15	337	54.0	–	–	–	Putative transposase (*Burkholderia fungoram*) (ZP_00283626)	75	29	$1e^{-7}$
520-E18	335	59.4	+2p	–	p	Putative transposase (BPSS2148)	97	49	$8e^{-21}$
520-E33	158	58.2	+2	–	–	Putative transposase (BPSS2148); different region but same protein as 520-E18	100	52	$8.7e^{-23}$
520-2F1	420	59.5	+2	–	–	Putative transposase (BPSS2148); different region but same protein as 520-E18 and 520-E33	99	128	$2e^{-66}$
Secretion related									
520-E12	765	53.9	–	–	–	Hypothetical SecA-related protein (*Photobacterium profundum*) (YP_133346)	48	218	$2e^{-53}$
Lipoprotein									
520-E44	202	56.4	+1	+	–	Putative lipoprotein (BPSL2045)	97	45	$5e^{-19}$
Enzymes									
520-E19	759	53.9	–	–	–	Appr-1-p processing enzyme family (*Nitrosomonas europaea*) (NP_841411); conserved hypothetical protein (*Synechocystis* spp.) (NP_942395)	77 65	127 119	$5e^{-53}$ $3e^{-43}$

Continued on facing page

TABLE 3—*Continued*

SSH[a] sequence and sequence type	Length (bp)	G+C content (%)[b]	Presence of sequenced genome[c]			Best BLASTX match, comments (GenBank accession no.)	% Identity	Length (no. of amino acids)	*E* value
			Bpm	Bt	Bm				
520-E1	374	50.0	+1p	−	p	Conserved hypothetical protein (*Synechocystis* spp.) (NP_942395); Appr-1-p processing enzyme family (*Nitrosomonas europia*) (NP841411.1)	68 56	29 30	$4e^{-5}$ 0.008
520-2F8	314	56.7	+1	p	+	Molybdopterin oxidoreductase (BPSL2207)	100	54	$3e^{-25}$
Hypothetical or uncharacterized proteins									
520-2E7	314	60.2	−	−	−	Uncharacterized protein (*Microbulbifer degradans*) (ZP_00318360)	55	103	$7e^{-25}$
520-2F2	773	54.1	+1	−	−	Hypothetical protein in RD7/GI6 (BPSL1146); variation in the C terminus	80	111	$7e^{-40}$
520-2E10 (DQ351721)	519	54.0	+1p	+	−	Hypothetical protein (BPSL2048)	49	123	$6e^{-26}$
520-E35 (DQ351716)	308	52.9	−	+	−	Hypothetical protein (BPSL2048A)	59	101	$3e^{-24}$
520-2G6	373	53.9	−	−	−	Hypothetical protein (*B. mallei*) (YP_105718)	47	113	$1e^{-23}$
No significant BLASTX matches									
520-E16	134	57.0	−	−	−				
520-E10	529	54.6	−	−	−				
520-2E1	233	52.8	−	−	−				
520-2F11	602	54.5	−	−	−				
520-2F6	814	50.7	−	−	−				
Sequences present in strains 338 and 520									
338-2D10 (DQ351718)	370	56.2	−	+	−	Bacteriophage protein from Φ1026b (*B. pseudomallei* 1026b) (NP_945078); bacteriophage protein from ΦE125 (*B. thailandensis*) (NP_536399)	87 84	100 100	$3e^{-47}$ $6e^{-45}$
338-2B9	663	47.1	+2	−	−	Putative exported protein (BPSS0658)	100	162	$4e^{-78}$
520-E36 (DQ351719)	150	58.0	−	−	−	Putative transposase (*Streptomyces avermitilis*) (NP_821845)	48	45	0.023
520-2G9 (DQ351717)	159	57.2	−	−	−	ISRSO16 transposase ORFB (*R. solanacearum*) (NP_523187)	80	51	$4e^{-18}$
338-B14	181	54.1	−	−	−	Hypothetical protein (*Methylococcus capsulatus*) (YP_115042)	90	31	$6e^{-9}$
338-B18	206	60.2	+2	−	−	Hypothetical protein in GI14 (BPSS0655)	98	68	$4e^{-34}$
338-2A1	>630	47.0	+2	p	+	Hypothetical proteins (BPSS1753)	100	68	$2e^{-33}$
520-E42	303	54.1	+1	−	−	Hypothetical protein in GI7 (BPSL1385)	100	71	$7e^{-34}$
No significant BLASTX matches									
338-B12	692	55.6	−	−	−				
338-2C5	429	51.3	+1	−	+	Overlaps BPSL2558 by 10 bp but lies mainly in the gap between BPSL2558 and BPSL2559			

[a] GenBank accession numbers are indicated in parentheses for those novel sequences used in the VAT analysis.
[b] G+C content for the subtracted sequence.
[c] The presence (+) or absence (−) of the subtracted sequence, based on >90% sequence identity by using BLASTN, is indicated for the genome-sequenced strains of *B. pseudomallei* (Bpm), *B. thailandensis* (Bt), and *B. mallei* (Bm). For *B. pseudomallei* the number of the matching chromosomes is indicated. p, a partial match, where the match does not extend over the entire length of the subtracted sequence.

and 520-2E10. Of the known genomic islands, GI11 was present in only five isolates from Thailand and GI12 was present in five isolates from Thailand and nine isolates from Australia. In contrast, the distribution of GI7, as inferred from the PCR assay results for sequence 520-E42, was more widespread (Table 4).

The distribution of the sequences among the isolates from Thailand indicated by PCR assays was confirmed by dot blot hybridizations with digoxigenin-labeled probes for the sequences *gmhA*, 520-E33, 338-B7, and 520-E42 (from M-PCR1); TRANS, 520-E35, and 520-2G9 (from M-PCR2); and 338-B3 and 520-2E10 (from M-PCR4) (data not shown). The PCR assays for GI11 and GI12 had been validated previously (12). From M-PCR3, the sequence 520-E36 dot blots were less clear due to background hybridization. We tested this sequence with a second primer set and obtained the same distribution as before. Because the PCR assay and dot blot data based on our initial primer set for 338-2D10 did not agree, new primers (primers 338-2D10F2 and 338-2D10R2) were designed and tested with the Thai isolates. The distribution results corresponded to those obtained by using dot blots; therefore, the new primer set was incorporated into M-PCR3.

DISCUSSION

SSH between the two Australian isolates 338 and 520 identified 39 sequences that varied between the two isolates. The subtracted sequences varied between 134 bp and 1,097 bp in

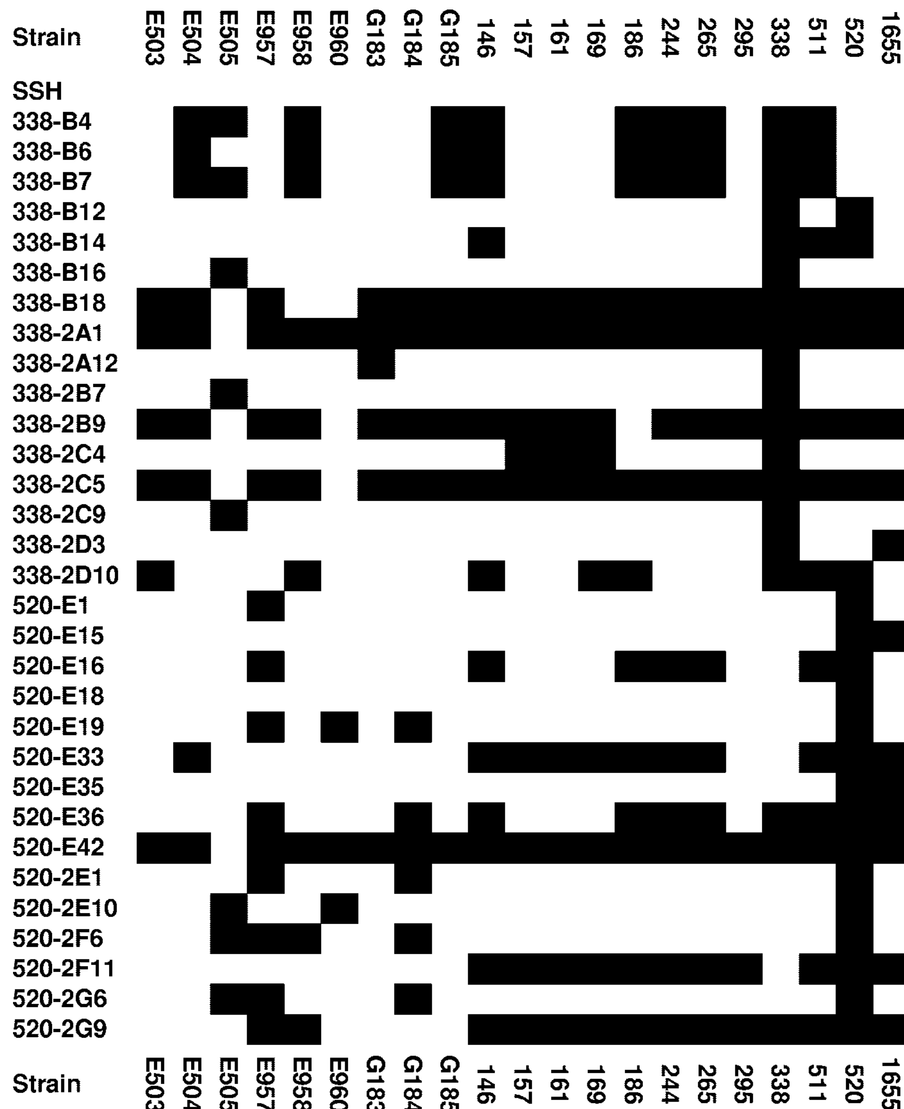

FIG. 1. PCR-based distribution analysis of SSH sequences. Filled boxes indicate PCR-positive results.

length, and all had a G+C content (<60.3%) below the average for the organism (68.1%). Among the 39 sequences were 7 matching sequences in chromosome 1 and 4 matching sequences in chromosome 2 of strain K96243. Analysis of the genome sequence of *B. pseudomallei* K96243 suggests that chromosome 1 contains a higher proportion of genes involved in core functions, while chromosome 2 contains a higher proportion of genes encoding accessory functions (12). However, we found no bias toward chromosome 2 among those subtracted sequences that matched the sequences of strain K96243. In common with previous SSH analysis between non-Australian isolates of *B. pseudomallei*, we identified several variable sequences that matched transposases and bacteriophages (9). Prophages make a significant contribution to genetic diversity in pathogenic bacteria (1–3). The temperate bacteriophage ΦE125 was originally identified in *B. thailandensis* as specific for *B. mallei* (31). More recently, bacteriophage Φ1026b was identified in *B. pseudomallei* and carries genes for DNA packaging, tail morphogenesis, host lysis, integration, and DNA replication nearly identical to those of ΦE125, while those genes involved in head morphogenesis differ from those of ΦE125 (9). Two sequences exhibiting variable prevalence among *B. pseudomallei* and *B. thailandensis* shared similarity but not 100% identity with sequences from these bacteriophages, suggesting that strain 338 and other isolates of *B. pseudomallei* may carry related bacteriophages. Both sequences matched a region shared by Φ1026b and ΦE125 at a nucleotide sequence identity of 94% (9). Interestingly, the two subtracted sequences (338-2C9 and 338-2D10) did not share the same distribution among the panel strains (Fig. 1), and 338-2D10 was present in both strain 338 and strain 520 (Table 3).

We identified transposase-related sequences from the subtraction only using strain 520 as the tester (Table 3). Interestingly, because of the approach to the initial screening of the subtracted libraries that we chose, we identified a number of

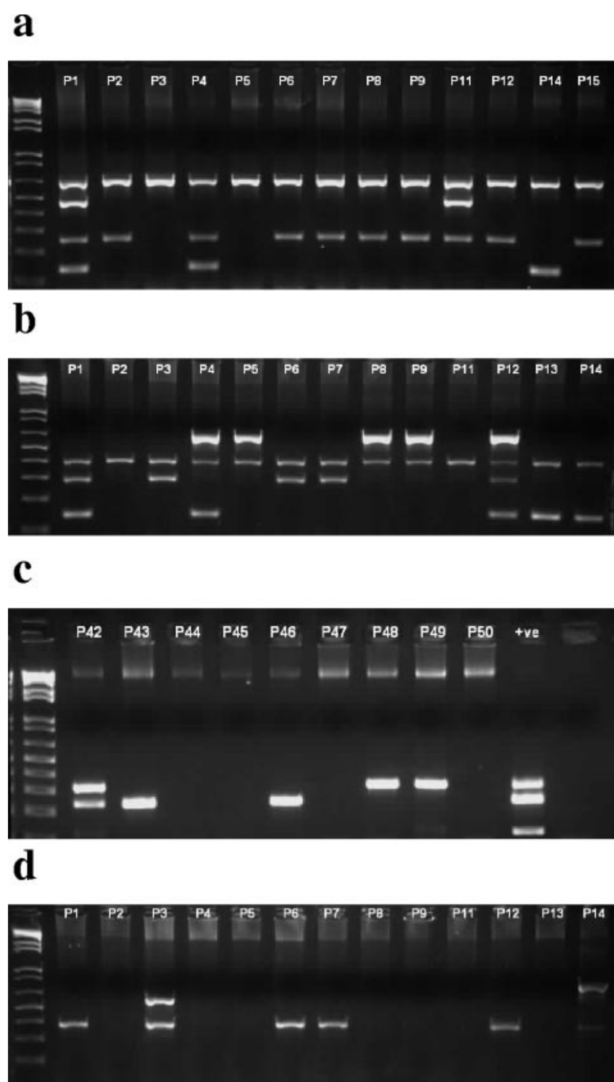

FIG. 2. M-PCR analysis of *B. pseudomallei* isolates. The figure shows agarose gels of example PCR amplicons derived from Thai isolates (indicated above individual lanes) by M-PCR1 (a), M-PCR2 (b), M-PCR3 (c), and M-PCR4 (d). The first lane on each gel contains a 1 kb-plus DNA ladder (Invitrogen). +ve, positive control.

variable sequences, including two putative transposase-related sequences, that were not genuinely subtracted. Often, libraries generated following SSH are prescreened to identify tester-specific sequences. Initially, for convenience, we took an approach whereby subtracted sequences were first sequenced and then used to screen the genome sequence strain (*B. pseudomallei* K96243) prior to the design of oligonucleotide primers for PCR screening of tester and driver DNA. Our observations suggest that the SSH procedure may enrich for regions with low G+C contents in a G+C-rich genome among those sequences not genuinely subtracted. Hence, we were able to identify some interesting sequences that were absent from the genome sequence strain or that were variable among the panel of isolates but that were not true subtracted sequences.

Our SSH analysis identified some sequences carried by the genomic islands previously identified in strain K96243 (12). Two additional islands were included in the VAT scheme. The prophage-like islands GI7 (sequence 520-E42) and GI12 differed considerably in overall prevalence, with GI12 sharing a similarly low prevalence with GI11, a putative integrated plasmid, or a conjugative element (12). However, our findings and those of others (9) suggest that there may be other genomic islands that are not present in strain K96243 but that exhibit a variable prevalence between isolates. The contribution of such islands and variable sequences to the variations in virulence or clinical manifestations exhibited by different strains remains unclear. However, our observations lend support to the notion that horizontal gene transfer has played an important role in the evolution of this pathogen.

Our VAT scheme is designed to give some indication of the mobilomes of isolates while also providing a cheap, reproducible, and portable method for strain discrimination. We chose to test the scheme with collections of isolates from Australia and Thailand. The isolates from Australia, all of which were different by MLST typing, have previously been used to demonstrate a difference between *B. pseudomallei* isolates from Australia and isolates from other regions of endemicity (6). The isolates from Thailand were first characterized by MLST typing in this study. Thus, some isolates that share the same MLST types were included among the isolates from Thailand. However, although some of these isolates also shared VAT profiles, some had different VAT profiles, suggesting that identical molecular strain types may vary in their mobilomes. Similarly, in some cases isolates of different strain types shared VAT profiles.

Cluster analysis was used to gain an insight into the relationships between strains based on VAT profiles (Fig. 3). The isolates cluster into groups, some of which are mainly or exclusively from one of the main geographical origins and some of which are mixed. Overall, there was a tendency for isolates to cluster with those isolates from the same geographical lo-

TABLE 4. Distribution of sequences among *B. pseudomallei* isolates

Isolate	% PCR positive													
	gmhA	338-B7	520-E42	520-E33	TRANS	338-2C5	520-E35	520-2G9	338-2D10	GI11	520-E36	GI12	338-B3	520-2E10
Original panel of *B. pseudomallei*	100	48	95	11	ND[a]	90	10	67	38	ND	48	ND	5	14
B. thailandensis (including E52)	0	0	0	0	ND	73	100	0	50	ND	0	ND	7	80
Australian isolates ($n = 44$)	100	7	68	64	0	95	34	91	27	0	39	20	27	23
Thai isolates ($n = 48$)	100	21	94	27	31	100	31	42	23	10	4	10	4	50
Other isolates ($n = 4$)	100	25	75	50	0	100	25	100	0	0	75	0	0	25
Combined ($n = 96$)	100	15	82	45	16	98	41	67	24	5	23	15	15	36

[a] ND, not determined.

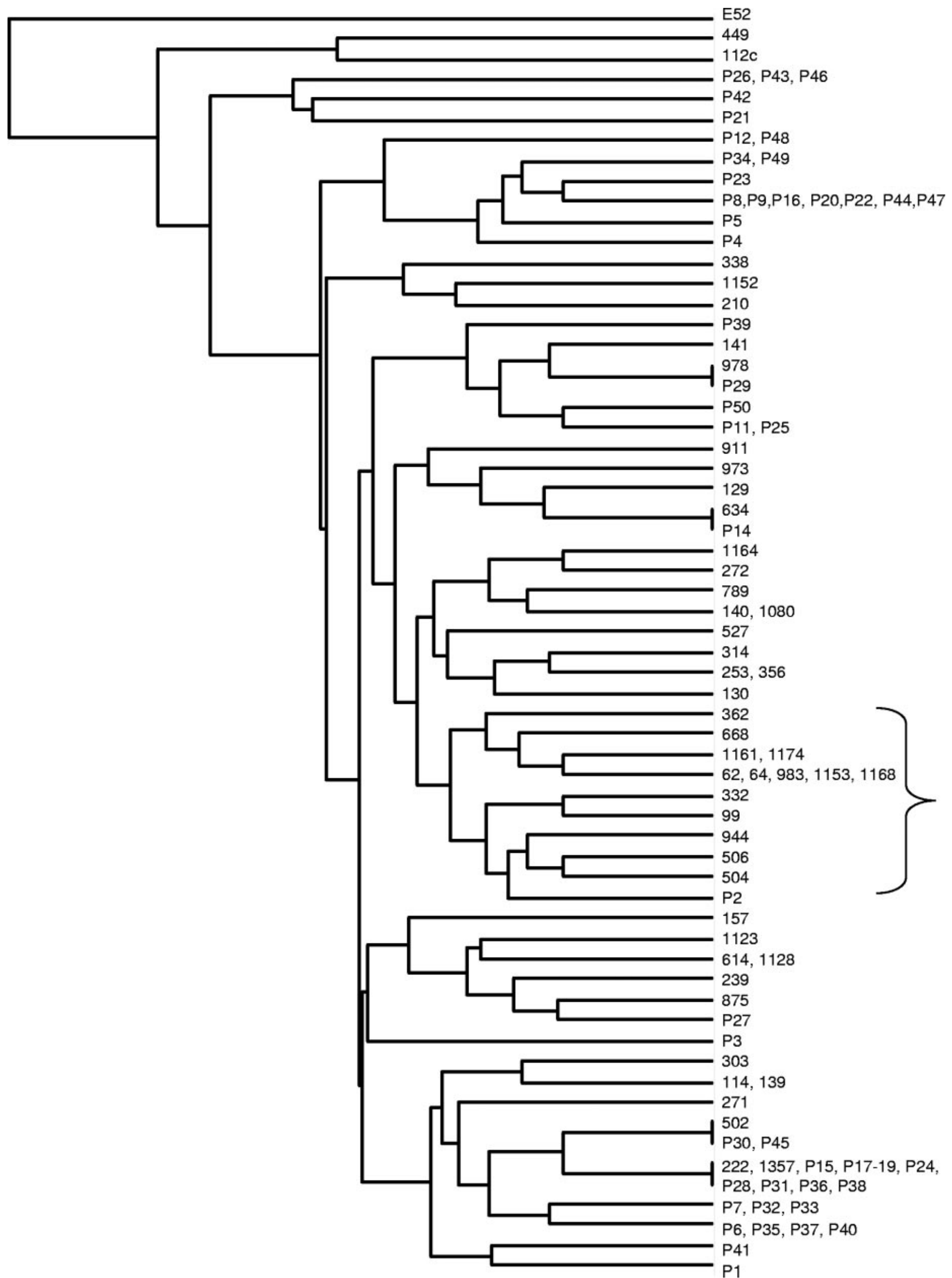

FIG. 3. Cluster analysis of VAT profiles. The VAT profiles were clustered by using a multivariate analysis for clustering of observations in the MINITAB software package. A dendrogram was constructed by using average linkage and Pearson distance. One isolate of *B. thailandensis* (isolate E52) was included in the analysis as an outlier. *B. pseudomallei* isolates P1 to P50 were from Thailand. All other isolates with the exception of isolates 139 to 141 and 314 were from Australia. The cluster that includes the neurotropic isolates is bracketed.

cation, suggesting a divergence in the mobilomes between isolates from Australia and Thailand that is in agreement with the apparently distinctive nature of isolates from Australia compared to the nature of the isolates from southeast Asia (6). However, the presence of minority isolates within clusters dominated by one geographical origin and the existence of some mixed clusters suggest that the picture is more complex. In order to resolve this, it will be necessary to sequence more *B. pseudomallei* genomes, especially those of Australian isolates, and conduct comprehensive microarray surveys of collections of isolates from different geographical locations.

Interestingly, one cluster included five isolates (isolates 668, 62, 983, 1153, and 332) from patients with the rare neurological melioidosis presentation, which is a specific entity that can occur in patients without risk factors (neurotropic isolates) (7). Of these, three isolates (isolates 62, 983, 1153) had identical VAT profiles, yet these three isolates and the other two neurological melioidosis-related isolates all had different MLST profiles (MLST groups ST129, ST148, ST142, ST117, and ST106, respectively), with no two isolates sharing more than three of the seven MLST alleles. To date, correlations between isolates associated with melioidosis encephalomyelitis have not been found by MLST or PFGE typing (4, 6). ST129, ST148, ST142, ST117, and ST106 were widely distributed in a dendrogram showing the results of cluster analysis based on MLST allele profiles and, apart from ST117 and ST129, were also widely distributed on a tree constructed from concatenated sequences (6). Furthermore, other isolates within the VAT cluster containing the five neurotropic isolates were mostly associated with more severe disease, including three isolates (isolates 1161, 64, and 944) that were associated with bacteremic pneumonia with septic shock. This suggests that the content of the accessory genome may play an important role in determining the clinical manifestations of some forms of melioidosis and that this can be independent of the conserved genome. Although the data obtained in this study are insufficient to identify specific genes or activities that might contribute to the success of the isolates causing disease of the central nervous system, we can discount the need for genomic islands GI11 and GI12, which were absent from these isolates.

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