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EPIDEMIOLOGICAL TYPING

Identification of a novel repetitive DNA element and its use as a molecular marker for strain typing and discrimination of ara$^-$ from ara$^+$ *Burkholderia pseudomallei* isolates

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A novel 10-bp repeat (5′-CGACGCAGGC-3′)$_{34}$ was identified in a strain of *Burkholderia pseudomallei*, the first repetitive element found in this species. A pair of primers, based on the flanking sequences of the repetitive region, was used in PCR and DNA sequence analysis to determine the genomic structure and distribution of the repetitive element in 76 arabinose$^-$ (ara$^-$) and 7 ara$^+$ *B. pseudomallei* isolates. DNA fragments of 400–700 bp were amplified in all ara$^-$ isolates. Ara$^+$ isolates were characterised by a uniform fragment of 402 bp. Nucleotide sequence analysis of these fragments revealed broad heterogeneity of the variable-number tandem repeats with 26 distinct alleles ranging between (5′-CGACGCAGGC-3′)$_{13}$ and (5′-CGACGCAGGC-3′)$_{45}$ identified in the ara$^-$ isolates. In contrast, a novel non-repetitive sequence was identified in each of the ara$^+$ isolates. This was confirmed by Southern blot analysis. Such biotype-specific variable-number tandem repeats may be useful as genetic markers for rapid strain differentiation of ara$^-$ isolates.

Introduction

Burkholderia pseudomallei is the causative agent of melioidosis, an often fatal infection of man and animals, which is endemic in south-east Asia and northern Australia [1]. In Singapore, a city state with a predominantly urban environment, melioidosis usually occurs among older adults with underlying illness such as diabetes mellitus, but even fit and healthy young servicemen of the Singapore Armed Forces have succumbed after acquiring the infection while training in the field [2]. *B. pseudomallei* can be isolated from soil, stagnant water and especially rice paddy fields in endemic regions. The organism enters the host through skin abrasions or wounds or by inhalation and ingestion. Melioidosis presents with various clinical manifestations, ranging from acute septicaemia with a high mortality to chronic infection with localised lesions in multiple organs. Treatment is difficult because the organism is resistant to a wide range of antibiotics [3].

There has been much interest recently in the contribution of environmental reservoirs to clinical melioidosis. Molecular typing methods including ribotyping [4–7], pulsed-field gel electrophoresis (PFGE) [7, 8], random amplified polymorphic DNA (RAPD) [9, 10] and multiplex PCR amplification [11] have been developed to discriminate between *B. pseudomallei* isolates. These methods vary greatly in their discriminatory power and, generally, generate multiple banding patterns which may be difficult to interpret.

Two distinct biotypes of *B. pseudomallei* strains have been defined recently by their differential ability to assimilate L-arabinose, their virulence in animal models and nucleotide variations in the 16S rRNA gene [12–15]. The arabinose-positive biotype (ara$^+$) is avirulent and has been found almost exclusively in environmental samples. In contrast, isolates from clinical disease fail to utilise arabinose (ara$^-$), are highly virulent and are also found in the environment [13]. Recently it has been proposed that ara$^+$ isolates should be grouped together to form a new species, *Burkholderia thailandensis*, on the basis of differences in 16S rRNA sequence [16].

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The present study describes the characterisation of a novel repetitive DNA element, 5′-CGACGCAGGC-3′, which could be used as a stable genetic marker for the epidemiological study of *B. pseudomallei*.

Materials and methods

Bacterial isolates and culture conditions

A total of 83 isolates comprising 76 unrelated ara$^-$ and 7 ara$^+$ (including the proposed type strain of *B. thailandensis*) isolates of *B. pseudomallei* was studied (Table 1). Isolates were identified by Gram's stain and colonial morphology on Ashdown's Medium (BioMedia Laboratories, Malacca, Malaysia). Biochemical identification and arabinose utilisation status of the isolates were confirmed by API 20NE (bioMèrieux, Marcy, France).

Strains of other bacterial species examined for comparison were *B. mallei* ATCC23344 and ATCC10399, *B. cepacia* ATCC17616 and ATCC25416, *B. gladioli* NC010073, *Pseudomonas aeruginosa* ATCC27853 and *Ralstonia pickettii* ATCC27511 and NC011149. All isolates were routinely cultured on LB agar at 37°C.

Preparation of bacterial DNA and analysis of cloned fragments

A single colony from LB agar was inoculated into 10 ml of LB broth and incubated overnight at 37°C. Total chromosomal DNA was extracted and purified with a Qiagen Genomic DNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A genomic library of *B. pseudomallei* strain ATCC 23343 was constructed into Bluescript (SK+) phagemid (Stratagene, La Jolla, CA, USA) as described previously [17]. A set of 5′-end nested deletions from three cloned recombinant plasmids – YL1, YL3 and YL5 – were constructed, respectively, with the Erase-A-Base System (Promega Corporation, Madison, USA). Both strands of the inserts from individual subclones were sequenced.

Southern blot hybridisation

About 1.5 μg of genomic DNA from individual isolates was digested with *Eco*RI and *Pst*I for 2 h and subjected to electrophoresis in an agarose 0.7% gel (45 mM Tris-borate, 1 mM EDTA, pH 8.0). The fragments were transferred to a positively charged nylon membrane (Amersham Life Science, Buckinghamshire). A synthetic oligonucleotide designated SSR30 containing three copies of 5′-CGACGCAGGC-3′ was labelled with digoxigenin with the DIG Oligonucleotide 3′-End Labeling Kit (Roche Diagnostics GmbH, Mannheim, Germany). The fragments on the membrane were hybridised with the SSR30 probe at a concentration of 10 pmol/ml at 70°C overnight in DIG Easy Hyb solution (Roche) and then washed twice for 5 min at room temperature with 2× SSC containing SDS 1% (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate),

Table 1. Sources and biotypes of *B. pseudomallei*

Ref. no.	Biotype	Source	Country/origin	Year obtained
4–11, 18, 26, 28, 33–35, 54	ara$^-$	Human	Singapore	1988
1, 2, 12, 14, 15, 17, 19–24, 30, 31, 36, 37, 39, 40, 42, 43, 48, 52, 53	ara$^-$	Human	Singapore	1989
38, 56, 57	ara$^-$	Human	Singapore	1990
59, 62	ara$^-$	Human	Singapore	1991
Jumari	ara$^-$	Human	Singapore	1996
9-D38465, 0-D10468, 9-A57203, 3-D85239, 358, 4-589580, 4-D82316, 4-D84602	ara$^-$	Human	Malaysia	1997
23343	ara$^-$	Human	ATCC	1993
S95019	ara$^+$	Human	Thailand	2000
488	ara$^-$	Gorilla	Singapore	1984
153	ara$^-$	Mueller's gibbon	Singapore	1989
115	ara$^-$	Chimpanzee	Singapore	1990
413	ara$^-$	Cassowary	Singapore	1985
490	ara$^-$	Bird	Singapore	1986
612	ara$^-$	Crown pigeon	Singapore	1987
147	ara$^-$	Palm cockatoo	Singapore	1990
216408	ara$^-$	Monkey	Malaysia	1997
363679	ara$^-$	Guinea-pig	Malaysia	1997
15682	ara$^-$	Monkey	ATCC	1996
3/96, 6/96, 22/96, 28/96, 21/96	ara$^-$	Pig	NK	1996
DB, DC	ara$^-$	Water/soil	Singapore	1989
78/96, 109/96, 79/96, 77/96	ara$^-$	Soil	Singapore	1996
GD	ara$^-$	Soil	Singapore	1990
15/10	ara$^-$	Soil	Singapore	2000
700388	ara$^+$	Soil	ATCC	1999
TRF681, TRF682, TRF683, TRF686, TRF666	ara$^+$	Soil	Thailand	2000

ATCC, American Type Culture Collection, Rockville, MD, USA; NK, not known; TRF, Thailand Research Fund.

twice for 15 min at 65°C with 0.5× SSC-SDS 1% and once for 10 min at 65°C with 0.1× SSC-SDS 1%. The digoxigenin-labelled probe was detected according to the manufacturer's instructions.

PCR amplification

PCR primers SR1 (5′-ACCGCGTATGAAGGGATGTC-3′) and SR5 (5′-ACGCGCACGCACCTGCTGCAAC-3′) were designed to amplify the flanking sequences 79 bp upstream and 134 bp downstream of the repetitive region in the DNA fragments cloned from the genomic library. PCR amplification was performed in a total volume of 50 μl containing 100 ng of bacterial genomic DNA, 200 μM of each dNTP, 0.2 μM of each primer and the enzyme mix and buffers of the GC-Rich PCR Kit (Roche). The reaction mix was denatured initially at 95°C for 3 min followed by 35 cycles of 95°C for 0.5 min, 55°C for 0.5 min and 72°C for 1 min, and a final extension at 72°C for 7 min. PCR products were separated on an agarose 1.2% gel.

DNA sequencing

The PCR products were purified with QIAquick PCR purification kit (Qiagen, Chatsworth, USA) and sequenced from both directions with the primers SR1 and SR5. The sequencing reactions were set up with an ABI Prism BigDye terminator cycle sequencing ready reaction kit (PE Applied Biosystems) and run on the ABI 377 automated sequencer (PE Applied Biosystems). Nucleotide sequences were analysed with the programs in the LASERGENE software package (DNASTAR, Madison, WI, USA) and GenBank (National Center for Biotechnology Information).

Results

Identification and characterisation of a repetitive DNA element

Three clones of the recombinant plasmids, YL1, YL3 and YL5, containing the inserts with sizes of 4.1, 7.2 and 2.4 kb, respectively, were isolated from the genomic library of *B. pseudomallei* strain ATCC23343. Each insert was sequenced in both directions and was found to contain identical sequences, indicating that they were partially digested products from the same location in the genome. A novel repetitive region, containing 34 identical copies of a 10-bp sequence – 5′-CGACGCAGGC-3′ – was identified at a position 33 bp downstream of a predicted open reading frame (Liu *et al*., unpublished data).

Conservation of the repetitive element

The presence of the repetitive element was determined by Southern blot analysis of chromosomal DNA from 12 independent ara$^-$ isolates of *B. pseudomallei* (seven human, four animal and one from soil), the type strain of *B. thailandensis* and representatives of other species. A strong band of 2.0–2.4 kb was given by all ara$^-$ *B. pseudomallei* isolates as well as by a *B. mallei* strain (Fig. 1). One or two faint bands of 2.3–7.0 kb were also detected in some lanes. This may have been due to incomplete digestion of the genomic DNA. No bands were detected in the *B. thailandensis* strain or in strains of other species (Fig. 1). The absence of the repetitive element in DNA from six other ara$^+$ isolates (S95019, TRF681, TRF682, TRF683, TRF686 and TRF666) was further confirmed by Southern blot analysis.

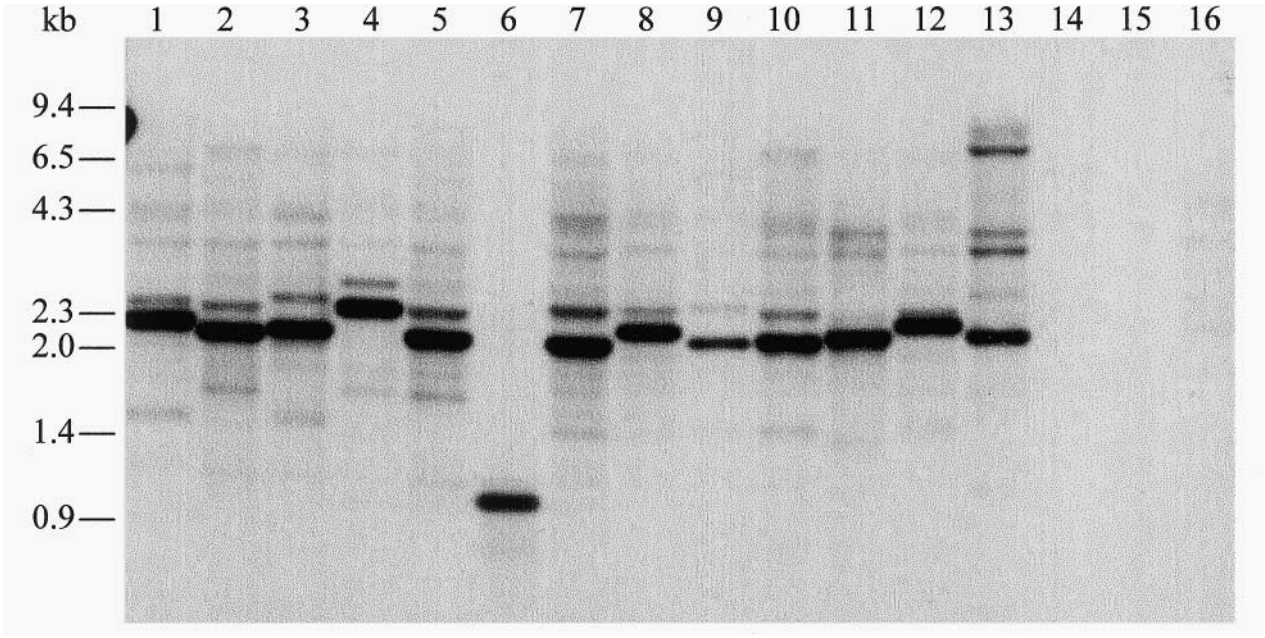

Fig. 1. Southern blot analysis of bacterial DNA digested with *Eco*RI and *Pst*I with a probe (SSR30). Lanes **1–12**, ara$^-$ *B. pseudomallei* isolates ATCC15682, 1, 4, 8, 22, 37, Jumari, 77/96, 115, 612, 21/96 and ATCC23343; **13**, *B. mallei* ATCC10399; **14–16**, *B. thailandensis* ATCC700388, *B. cepacia* ATCC17616 and *P. aeruginosa* ATCC27853.

Length polymorphism of the repeats in ara⁻ isolates

PCR analysis was performed on DNA from 76 human, animal and soil isolates of ara⁻ *B. pseudomallei*, 7 ara⁺ isolates and reference control strains of other species to determine length polymorphisms of the repetitive regions with the primers SR1 and SR5. A specific product was amplified from each of the ara⁻ isolates and the two *B. mallei* strains tested. Fig. 2 shows that the size of the PCR products from the ara⁻ isolates ranged from 400 to 700 bp. Interestingly, a specific product of *c*. 400 bp was also amplified from all ara⁺ isolates. This finding was unexpected, as the repetitive element was not detected in ara⁺ isolates by Southern blotting. No specific DNA fragments were amplified from the isolates of other species.

Heterogeneity of the variable-number tandem repeats

DNA sequence analysis was performed on both strands of the fragments amplified from isolates of both ara⁻ and ara⁺ phenotypes with primers SR1 and SR5. The number of copies of the repetitive unit in ara⁻ isolates was highly variable and 26 distinct alleles were identified ranging from 5′-CGACGCAGGC-3′$_{13}$ to 5′-CGACGCAGGC-3′$_{45}$ (Table 2). Of the 26 alleles, the most common were 18×, 19×, 16× and 26× repeats at frequencies of 10.5%, 10.5%, 9.2% and 8%, respectively. The nucleotide sequence of the PCR fragments amplified from the two *B. mallei* strains, contained 24× and 27× repeats, respectively. Seven distinct alleles were found in 10 human and animal isolates from Malaysia and four different alleles were

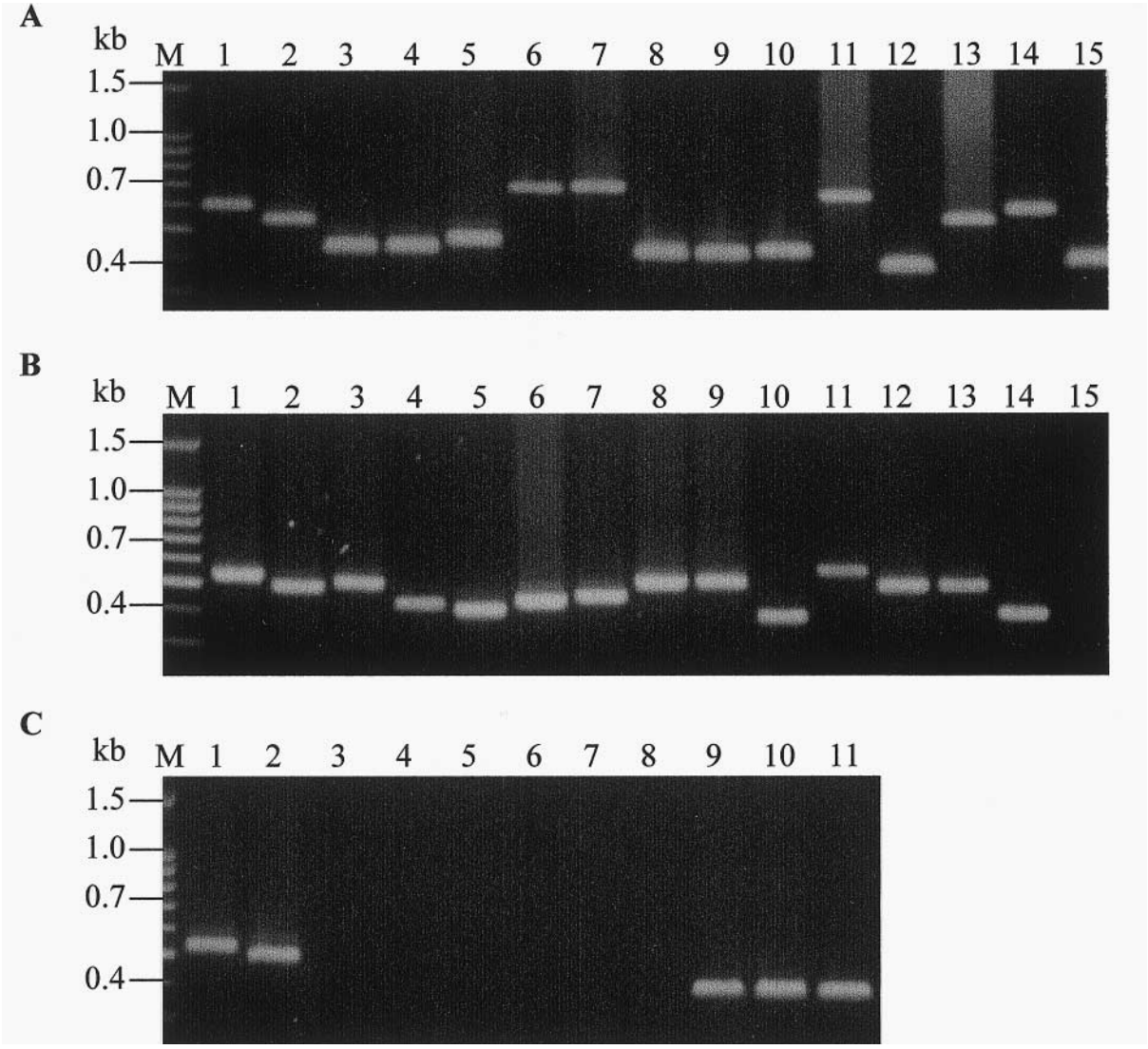

Fig. 2. Gel electrophoresis of the PCR on bacterial DNA with the SR1 and SR5 primers. (**A**) Lanes **1** and **2**, *B. pseudomallei* strains ATCC23343 and ATCC15682; **3–15**, ara⁻ *B. pseudomallei* human isolates 1, 2, 4, 8, 12, 22, 23, 36, 37, 40, 59, 0-D10468 and Jumari. (**B**) Lanes **1–4**, ara⁻ *B. pseudomallei* soil isolates 77/96, 78/96, 79/96 and 15/10; **5–14**, ara⁻ *B. pseudomallei* animal isolates 115, 612, 363679, 153, 488, 3/96, 6/96, 21/96, 22/96 and 28/96; **15**, a negative blank control. (**C**) Lanes **1–8**, other bacterial species (*B. mallei* ATCC23344 and ATCC10399, *B. cepacia* ATCC17616 and ATCC25416, *P. aeruginosa* ATCC27853, *R. pickettii* NC011149, *B. gladioli* NC010073 and *R. pickettii* ATCC27511); **9–11**, ara⁺ *B. pseudomallei* isolates (*B. thailandensis* ATCC700388, S95019 and TRF681); **M**, DNA marker.

Table 2. Categories of the variable-number tandem repeats in 76 ara$^-$ *B. pseudomallei* isolates

Repeat type*	Number (%) of isolates	Reference no. of isolates
13×	1 (1.3)	40
14×	4 (5.3)	38, 9-D38465, 3/96 and DB
15×	3 (4.0)	Jumari, 48 and 28/96
16×	7 (9.2)	5, 43, 26, 54, 28, 115 and 17
17×	4 (5.3)	22, 23, 33 and 34
18×	8 (10.5)	1, 2, 21, 56, 15, 490, 53 and 15/10
19×	8 (10.5)	30, 36, 52, 57, 62, 39, 42 and 612
20×	2 (2.6)	7 and 4-D84602
21×	5 (6.6)	4, 3-D85239, 363679, 216408, and DC
22×	2 (2.6)	4-D82316 and 147
23×	3 (4.0)	19, 78/96 and 109/96
24×	2 (2.6)	9 and 11
25×	5 (6.6)	35, 4-589580, 79/96, 22/96 and 21/96
26×	6 (8.0)	9-A57203, 10, 18, 24, 153 and 488
27×	3 (4.0)	14, 77/96 and GD
28×	2 (2.6)	ATCC15682 and 413
30×	1 (1.3)	59
31×	1 (1.3)	6/96
32×	1 (1.3)	31
33×	1 (1.3)	6
34×	1 (1.3)	ATCC23343
35×	2 (2.6)	0-D10468 and 358
41×	1 (1.3)	37
42×	1 (1.3)	12
44×	1 (1.3)	8
45×	1 (1.3)	20

*Repeat types are designated according to the copy number of 5′-CGACGCAGGC-3′ in the tandem repeats; ×, represents the copy number.

present in five isolates from domestic pigs. However, while the eight soil isolates from Singapore were spread among six allele types, two isolates (78/96 and 109/96) obtained from the same football field in Singapore contained the same repeat (23×). Moreover, isolates 22 and 23 from the two soldiers who developed melioidosis during training in the same field at the same time contained the 17× repeat.

Sequencing of PCR fragments amplified directly from two to six individual bacterial colonies of 11 random isolates maintained on nutrient agar plates for multiple passages confirmed the stability of the repeat type.

Sequence analysis of PCR fragments from ara$^+$ isolates

Sequence analysis of the amplified PCR fragments demonstrated that a specific non-repetitive region was present in the ara$^+$ isolates. The nucleotide sequences of the 402-bp product, including a 361-bp sequence in the middle and the sequences for the PCR primers at both ends, showed 100% identity and confirmed the absence of the repetitive element in these isolates. The flanking sequences, 79 bp upstream and 134 bp downstream of the repetitive region which excluded the two primer sites in ara$^-$ isolates shared *c.* 78% and 59% identity with the respective regions of the 361-bp sequence in ara$^+$ phenotypes (Fig. 3). A database search, with the WWW BLAST server (National Centre for Biotechnology Information) indicated that the 361-bp non-repetitive sequence from ara$^+$ isolates was novel. The sequence was submitted to GenBank under accession no. AF325538.

Discussion

Repetitive elements have been used as genetic markers for strain typing in a large variety of micro-organisms

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                  Primer SR1
Ara+ B. pseu ACCGCGTATGAAGGGATGTCGGGGCCGTTGACGGTCGAGGTGCGCGACGCGGTCTACGCC 60
Ara- B. pseu ....................C.....................G......

Ara+ B. pseu GTGTGAGTCGCGAGGCGGGGC--GCGCGGCGCGACGAA-AGCTGCGCGCTCGGCATCGTC 120
Ara- B. pseu ......CG.A..GC.TC.CC.CG.....C..T.C..GGG--------------------

Ara+ B. pseu CCGCGCAGATGGCGCCAACGGCGCGCGCGCGTCGCCGCCGCGCGCCGGAGCGCCGTGGCG 180
Ara- B. pseu ------------------------------------------------------------

Ara+ B. pseu GAATGCGTGGGCCCGCCGCCTTGCCCGCCGAGCCGGGCCGTGATTCGGCGGACGCGGTCG 240
Ara- B. pseu ------------------------------------------------------------

Ara+ B. pseu CATGCCCCGCGCGCGAACCGGCCCGACGCCCGGCCCGAGCCTCTACTTCATCGTCGGACC 300
Ara- B. pseu ------.......C.C.G.ATG..G..---...A..G...GTCG.G.-GC..CGC.G.A

Ara+ B. pseu GGCCCTCGGCCCGGCCCGGCGCAGGCGTTTTTCCGCC-GCCGCTTACTGTGTCAGCCCGT 360
Ara- B. pseu C...GCGC...G.C....T.C.GC..C.GCGG....TT.....C...C.C.A...A....

Ara+ B. pseu CCGGCCCGGCATGCTCGGGCGCGCGGTTCAGCAGGTGCGTGCGCGT 402
Ara- B. pseu .......C..G.............................

                                    Primer SR5
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Fig. 3. Comparison of nucleotide sequences of the 402-bp fragment amplified from ara$^+$ isolates with the repetitive region of the ara$^-$ *B. pseudomallei* isolates. Dots indicate identical nucleotides and a dash shows the deletion of a nucleotide. Lines represent the variable-number tandem repeats in the ara$^-$ isolates. Numbers on the right represent the nucleotide positions in the 402-bp sequence from the ara$^+$ *B. pseudomallei* isolates.

[18]. Recombination and polymerase slippage are two mechanisms that have been widely proposed to generate these variable regions. In this study, a novel 10-bp repetitive element – 5′-CGACGCAGGC-3′ – was identified in *B. pseudomallei* strain ATCC23343. This is the first repetitive sequence that has been reported for this species.

The repetitive element was found in all ara$^-$ *B. pseudomallei* and *B. mallei* isolates tested, but was absent from ara$^+$ isolates of *B. pseudomallei* and representatives of other *Burkholderia* or *Pseudomonas* spp. analysed. *B. mallei* is very closely related to *B. pseudomallei* [19]. It is the causative agent of glanders, an infection of equine species. The failure to isolate this organism from soil and water samples collected in Thailand during an intensive environmental survey [15] may suggest that *B. mallei* is rare in this environment.

The difference in the ability to utilise L-arabinose between the two biotypes has been shown to be strongly associated with virulence in the experimental mouse and hamster models of infection [13, 14]. An apparently low LD50 for the ara$^-$ *B. pseudomallei* strains was observed in both experimental animal models. This association was also highlighted by two recent findings on the strong correlation of insertion elements and type III secretion genes with ara$^-$ *B. pseudomallei*. Mack and Titball [20] showed the presence of the homologues of two *B. cepacia* insertion sequences, IS*406* and IS*407* in all ara$^-$ isolates examined, but these were absent from the two ara$^+$ isolates examined. These insertion sequences have been shown to be associated with transmissible strains of *B. cepacia* isolated from cystic fibrosis patients [21, 22]. More recently, Winstanley and Hart [23] identified type III secretion genes, which are present on pathogenicity islands in a number of gram-negative bacteria, in the ara$^-$ phenotype. Taken together with these findings, the strong correlation of the 10-bp repetitive element with ara$^-$ isolates further supports the divergence of the two biotypes of *B. pseudomallei*.

A broad heterogeneity in banding patterns has been observed among *B. pseudomallei* isolates in previous studies with different molecular approaches. Based on the restriction patterns of *Bam*HI ribotyping, Pitt *et al*. [7] identified 44 distinct patterns among 350 isolates gathered over 71 years from 23 countries. These 44 ribotypes could be subdivided into 226 subtypes based on the macrorestriction patterns generated by PFGE of *Xba*I digests. RAPD also has been used to subdivide isolates of the same ribotype, as well as to help to identify the source of an infection or outbreak [9, 24].

In this study, PCR amplification coupled with DNA sequencing with the same pair of primers, SR1 and SR5, to amplify flanking sequences of the repetitive region, provided a simple system for rapid strain differentiation of ara$^-$ *B. pseudomallei* isolates. A broad heterogeneity of the variable-number repeats with 26 distinct alleles was identified. Based on the sampling size there was no obvious association between the repeat types with the source, geographical origin and year of collection. The stability of a specific repeat type in individual isolates even after multiple passage of the culture, indicates that changes of alleles as a result of genetic instability at this locus during culture is rare. Such sequence stability suggests that the 10-bp repetitive element is a reliable molecular marker to determine genetic variations among isolates of ara$^-$ *B. pseudomallei*.

In contrast to the ara$^-$ isolates, a non-repetitive region containing 361 bp of DNA was identified in all ara$^+$ isolates examined. This feature may prove to be a reliable marker for the differentiation of the two biotypes. PCR methods have been used to detect *B. pseudomallei* infection in a number of studies [25–28]. Most of the primers used in these studies targeted sequences in 16S rRNA genes or selected specific DNA fragments. Dharakul *et al*. [15] reported recently the development and use of a multiplex PCR to differentiate the two biotypes from each other and from other bacteria. With a set of primers based on 16S rRNA gene sequences, they showed that two DNA fragments of 405 and 243 bp were amplified in ara$^-$ *B. pseudomallei*, whereas only the 243-bp fragment was present in ara$^+$ strains. This method was also reported to be highly sensitive for the detection of *B. pseudomallei* in clinical specimens.

In conclusion, the PCR method described here is a simple and highly specific procedure for the identification of *B. pseudomallei*. Both biotypes of *B. pseudomallei* could be identified with only one pair of primers and, with the exception of *B. mallei*, other related species were unreactive. A recent study of local environmental sampling by this laboratory suggests that the SR1 and SR5 primers offer higher specificity than 16S rRNA primers for direct identification of *B. pseudomallei* in soil samples (data not shown). To further discriminate between isolates of the two biotypes, a multiplex PCR procedure has been developed by combining the SR1 and SR5 primers with a primer based on the non-repetitive sequence from the ara$^+$ biotype. Its use as a simple and rapid molecular tool in both clinical diagnosis and environmental survey for *B. pseudomallei* is currently being explored.

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