BACTERIAL PATHOGENICITY

Ribotype differences between clinical and environmental isolates of *Burkholderia pseudomallei*

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Burkholderia pseudomallei is isolated frequently from the soil in regions where the disease melioidosis occurs. However, recent surveys in Thailand have shown that the frequency of isolation of the organism from soil samples is not directly related to the incidence of melioidosis in an area. To determine whether strain populations of *B. pseudomallei* prevalent in soil are genotypically related to strains causing clinical disease, rRNA *Bam*HI restriction fragment length polymorphisms (RFLP) of 139 soil environmental isolates and 228 human isolates were compared. Two groups of ribotype patterns were found. Group I comprised 37 different ribotype patterns which were characterised by five to eight hybridisation bands of 2.8–> 23 kb. All of these ribotypes were identified among the clinical isolates, and 18 of them were also found in 59 environmental isolates. Group II was represented by 12 ribotypes found only in environmental strains. These ribotype patterns comprised one to five bands in the size range 9–> 23 kb. All but one of the 73 isolates in this group grew on a minimal medium supplemented with L-arabinose. In contrast, only 3% of the 66 isolates from the environment with group I ribotype patterns could utilise this sugar as their sole energy source. These findings suggest that *B. pseudomallei* strains that utilise arabinose constitute a population that is genetically distinct from other environmental and clinical strains.

Introduction

Melioidosis is an infection caused by *Burkholderia pseudomallei*. Despite the apparent ubiquity of *B. pseudomallei* in the soils of endemic areas of Southeast Asia, there is great variability in the incidence of melioidosis in different regions [1]. This may be due to host susceptibility related to conditions such as diabetes mellitus, a well recognised risk factor [2], and differences in occupational exposure to the organism. Differences in bacterial virulence might also contribute to the geographic distribution of melioidosis.

A recent study by Wuthiekanun *et al.* [3] suggests that

although clinical and environmental isolates of *B. pseudomallei* from Thailand are morphologically similar and antigenically indistinguishable, two populations of the species are revealed by extended biochemical testing. They identified two different biotypes of *B. pseudomallei* that were characterised by two phenotypes based on their ability to utilise the sugar L-arabinose as a sole energy source for growth. This study suggested that the biotype differences are associated with the disease-producing potential of the bacteria for man as arabinose positive (*ara*⁺) isolates were found exclusively in the environment and not from patients with melioidosis, while *ara*[–] isolates were isolated from both man and the environment. The question arises as to whether these varieties of *B. pseudomallei* are genomically related or show sufficient differences to warrant further investigation of species identity. Therefore, the variation in rRNA gene loci of isolates of both phenotypes was examined to determine their degree of genetic relatedness.

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Materials and methods

Bacterial isolates

A total of 367 isolates of *B. pseudomallei* was examined; 139 isolates were from the environment (133 soil, five water, one manure) and their details including sites and geographical origin are given in Table 1. All but 20 of the environmental isolates were from Thailand; the non-Thai isolates originated from nine different countries. The remaining 228 isolates were from a collection of strains from cases of human melioidosis worldwide, the great majority of which were from Southeast Asia.

All isolates were presumptively identified as *B. pseudomallei* by their reaction with Gram's stain and colony morphology on Ashdown's selective medium, production of oxidase and resistance to gentamicin (10 µg disk) and colistin (10 µg) [4]. Species identity was confirmed by API 20NE profiles (bioMérieux, Marcy, France). Arabinose utilisation was determined by growth on minimal salts agar [5] containing L-arabinose 0.2%. Ethanol assimilation was tested by the growth of isolates and production of acid on ethanol 1% in an ammonium salts based medium [6].

Ribotyping

Ribotyping of isolates was performed as described by Kaufmann *et al.* [7]. Chromosomal DNA was extracted with guanidium thiocyanate from the 48-h growth on nutrient agar and digested with *Bam*HI restriction endonuclease. The DNA fragments were separated by electrophoresis in agarose gel and transferred to a nylon membrane. The membrane was treated with a biotin-labelled cDNA of rRNA from *Escherichia coli* (Boehringer Mannheim, Germany) and DNA hybridisation bands were visualised colorimetrically with the BlueGene kit (Gibco-BRL, Uxbridge). The migration distance of each DNA band on the membrane was measured manually and molecular size was calculated with reference to bacteriophage lambda DNA digested with *Hind*III and labelled with biotin (Gibco-BRL). Each profile was differentiated according to the number and position of DNA bands and was assigned an arbitrary number. Pair-wise comparisons of similarity were made by the Dice coefficient [8] and cluster analysis was performed by the unweighted pair group matching band average (UPGMA) and displayed as a dendrogram with the aid of a computer program (MVSP; Kovach Computing Services, Pentraeth, Anglesey).

Statistical test

Associations between the source of isolates, their ribotype group and utilisation of substrates for growth were tested for statistical significance by the χ^2 test.

Results

The migration distance of each rDNA hybridisation band was measured for all isolates of *B. pseudomallei* from clinical melioidosis and the environment, and their molecular size was calculated with reference to lambda markers. Among the 228 human isolates, 15 distinct band positions were identified, ranging in size from 2.8 to > 23 kb; most isolates were characterised by three to eight bands, and a total of 37 different patterns were distinguishable by visual comparison (Fig. 1). These patterns were termed group I, and 18 of them were also found among 59 of the 139 environmental isolates examined. Seven isolates from the environment had unique ribotypes not found among human isolates and correspondingly 18 human isolates had unique ribotypes that were not found amongst the environmental isolates. Two individual ribotype patterns (types 1 and 3) predominated in both clinical and environmental isolates in group I (Table 2).

A second group of ribotype patterns was recognised exclusively in environmental isolates. This group was characterised by one to five bands in the size range 9–>23 kb (Fig. 1) and only one band position coincided with the 15 bands on which the 37 patterns in group I were based. At least 12 different patterns formed this group II and were distinguished from group I ribotypes by the prefix A (Table 2). Three patterns predominated among the 73 isolates represented by 8, 27 and 10 isolates, respectively, and the DNA of 11 isolates was not digested by *Bam*HI.

Fig. 1 shows that cluster analysis of pair-wise comparisons of ribotypes within group I revealed an overall similarity of 62% and most of these patterns differed by two or more band positions. However, groups I and II ribotype clusters were only 25% related to each other. On the basis of simple matching for the presence or absence of a particular band in the gel track, group II isolates were markedly unrelated to each other and a percentage similarity as low as 30% was required to group the five isolates together. This was due in the main to the fact that only eight band positions were identified within group II ribotypes and despite their limited size distribution, many isolates differed from each other by three to four bands.

Table 3 shows the percentage of environmental and clinical isolates that utilised arabinose or ethanol, or both, and their distribution in groups I and II ribotype patterns. Overall, 74 (53.2%) of 139 environmental isolates utilised arabinose as a sole energy source, but only two of 66 isolates of group I ribotype patterns were ara⁺ compared with 72 of 73 groups II isolates ($\chi^2 = 126.33$, $p < 0.0001$). Similarly, significantly more isolates in group II (63 of 73) assimilated ethanol compared with group I (4/26; $\chi^2 = 42.63$, $p < 0.0001$). Thus, the great majority of ribotype group I isolates from the environment were similar to

Table 1. Sites, geographical origin and dates of isolation of environmental isolates of *B. pseudomallei*

No.	Ref. no	Site	Origin	Date	No.	Ref. no.	Site	Origin	Date
1	978-C	Soil	Australia	...	70	D260 S3/30	Soil	Australia	...
2	Pingy	Water	Thailand S	...	71	D30 S3/40	Soil	Australia	...
3	EE091	Soil	Thailand NE	1990	72	D34 S3/50	Soil	Australia	...
4	E250	Soil	Thailand NE	1992	73	D75 S3/50	Soil	Australia	...
5	E183	Soil	Thailand NE	1992	74	D85 S2/30	Soil	Australia	...
6	E198	Soil	Thailand NE	1992	75	D109 S3/40	Soil	Australia	...
7	E212	Soil	Thailand NE	1992	76	D112 S3/30	Soil	Australia	...
8	E010	Soil	Thailand NE	1990	77	E13	Soil	Thailand NE	1991
9	E086	Soil	Thailand NE	1990	78	E06	Soil	Thailand NE	1990
10	E090	Soil	Thailand NE	1990	79	E08	Soil	Thailand NE	1990
11	Somredee	Water	Thailand S	...	80	E13	Soil	Thailand NE	1991
12	E021	Soil	Thailand NE	1990	81	E15	Soil	Thailand NE	1991
13	E043	Soil	Thailand NE	1990	82	E16	Soil	Thailand NE	1991
14	E044	Soil	Thailand NE	1990	83	E20	Soil	Thailand NE	1992
15	E195	soil	Thailand NE	1992	84	E255	Soil	Thailand NE	...
16	E208	Soil	Thailand NE	1992	85	E257	Soil	Thailand NE	...
17	E213	Soil	Thailand NE	1992	86	E053	Soil	Thailand NE	1990
18	E218	Soil	Thailand NE	1992	87	E064	Soil	Thailand NE	1990
19	E223	Soil	Thailand NE	1992	88	E072	Soil	Thailand NE	1990
20	E225	Soil	Thailand NE	1992	89	E074	Soil	Thailand NE	1990
21	E231	Soil	Thailand NE	1992	90	E094	Soil	Thailand NE	1991
22	E235	Soil	Thailand NE	1992	91	E099	Soil	Thailand NE	1991
23	E243	Soil	Thailand NE	1992	92	E100	Soil	Thailand NE	1991
24	E249	Soil	Thailand NE	1992	93	E102	Soil	Thailand NE	1991
25	E023	Soil	Thailand NE	1990	94	E107	Soil	Thailand NE	1991
26	E190	Soil	Thailand NE	1992	95	E127	Soil	Thailand NE	1991
27	E215	Soil	Thailand NE	1992	96	E149	Soil	Thailand NE	1991
28	E039	Soil	Thailand NE	1990	97	E178	Soil	Thailand NE	1991
29	Taib	Water	Thailand S	...	98	E260	Soil	Thailand C	1993
30	E182	Soil	Thailand NE	1992	99	E261	Soil	Thailand C	1993
31	E242	Soil	Thailand NE	1992	100	E263	Soil	Thailand C	1993
32	E245	Soil	Thailand NE	1992	101	E267	Soil	Thailand C	1993
33	E237	Soil	Thailand NE	1992	102	E229	Soil	Thailand NE	1992
34	E220	Soil	Thailand NE	1992	103	E201	Soil	Thailand NE	1992
35	E222	Soil	Thailand NE	1992	104	E216	Soil	Thailand NE	1992
36	93-D2	Soil	Australia	...	105	R179	Soil	Thailand NE	1991
37	149	Soil	Australia	...	106	E251	Soil	Thailand NE	...
38	E181	Soil	Thailand NE	1992	107	E048	Soil	Thailand NE	1990
39	E187	Soil	Thailand NE	1992	108	E075	Soil	Thailand NE	1990
40	E015	Soil	Thailand NE	1990	109	E078	Soil	Thailand NE	1990
41	E244	Soil	Thailand NE	1992	110	E082	Soil	Thailand NE	1990
42	74101	Water	Iran	1974	111	E116	Soil	Thailand NE	1991
43	E087	Soil	Thailand NE	1990	112	E171	Soil	Thailand NE	1991
44	E240	Soil	Thailand NE	1992	113	E175	Soil	Thailand NE	1991
45	E038	Soil	Thailand NE	1990	114	E125	Soil	Thailand NE	1991
46	E041	Soil	Thailand NE	1990	115	E049	Soil	Thailand NE	1990
47	E139	Soil	Thailand NE	1991	116	E143	Soil	Thailand NE	1991
48	E227	Soil	Thailand NE	1992	117	E184	Soil	Thailand NE	1992
49	E238	Soil	Thailand NE	1992	118	E262	Soil	Thailand C	1993
50	E224	Soil	Thailand NE	1992	119	E266	Soil	Thailand C	1993
51	E037	Soil	Thailand NE	1990	120	E180	Soil	Thailand NE	1991
52	E186	Soil	Thailand NE	1992	121	E202	Soil	Thailand NE	1992
53	E193	Soil	Thailand NE	1992	122	E221	Soil	Thailand NE	1992
54	E206	Soil	Thailand NE	1992	123	E134	Soil	Thailand NE	1991
55	C2	Soil	Australia	...	124	E160	Soil	Thailand NE	1991
56	NT20	Soil	Burkina Faso	1973	125	E141	Soil	Thailand NE	1991
57	770429	Soil	Madagascar*	1977	126	E264	Soil	Thailand C	1993
58	NT08	Soil	Niger*	1973	127	E246	Soil	Thailand NE	1992
59	7605	Manure	France*	1976	128	E285	Soil	Thailand C	1993
60	E247	Soil	Thailand NE	1992	129	E058	Soil	Thailand NE	1990
61	E226	Soil	Thailand NE	1992	130	E066	Soil	Thailand NE	1990
62	C7532	Soil	USA, Oklahoma	...	131	E286	Soil	Thailand C	1993
63	E233	Soil	Thailand NE	1992	132	E054	Soil	Thailand NE	1990
64	E032	Soil	Thailand NE	1990	133	E057	Soil	Thailand NE	1990
65	E203	Soil	Thailand NE	1992	134	E061	Soil	Thailand NE	1990
66	Somredee	Soil	Thailand S	...	135	E062	Soil	Thailand NE	1990
67	M7665/91	Soil	Singapore	1991	136	E071	Soil	Thailand NE	1990
68	Kenya	Water	Kenya	1992	137	E080	Soil	Thailand NE	1990
69	D228	Soil	Australia	...	138	E122	Soil	Thailand NE	1991
					139	E124	Soil	Thailand NE	1991

NE, Northeast; S, South; N, North; C, Central.

..., Date not known.

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Fig. 1. Hybridisation patterns of group I and II ribotypes. Lanes 1–5, group I; 6–10, group II. The dendrogram shows percentage similarity of patterns within and between ribotype groups.

Table 2. *B. pseudomallei* isolates in group I and II ribotypes isolated from environmental and human sources

Ribotype	Number (%) of isolates from	
	Environment	Human sources
Group I		
1	10 (7.2)	64 (28.1)
3	17 (12.8)	45 (20.2)
4	7 (5.6)	14 (6.1)
6	6 (4.3)	7 (3.1)
9	1	2 (0.9)
11	0	2 (0.9)
12	0	5 (2.2)
14	1	5 (2.2)
15	6 (4.3)	20 (8.8)
16	3 (2.1)	3 (1.3)
19	0	4 (1.8)
26	4 (2.8)	3 (1.3)
27	2 (1.4)	15 (6.6)
32	0	2 (0.9)
33	0	2 (0.9)
34	0	7 (3.1)
35	0	2 (0.9)
37	2 (1.4)	8 (3.5)
Unique	7 (5.0)	0
Unique	0	18 (7.9)
Group II		
A5	27 (19.4)	0
A6	10 (7.1)	0
A8	4 (2.8)	0
A9	2 (1.4)	0
A10	3 (2.1)	0
A11	3 (2.1)	0
Unique	13 (9.3)	0
Untypable	11 (7.9)	0
Total	139	228

Table 3. Arabinose and ethanol utilisation by *B. pseudomallei* isolates grouped according to ribotype patterns

Source	Number (%) of isolates showing growth on	
	arabinose	ethanol
Environment		
All ribotypes	74/139 (53.2%)	67/99 (67.7%)
Group I ribotypes	2/66 (3.0%)	4/26 (15.4%)*
Group II ribotypes	72/73 (98.6%)*	63/73 (86.3%)
Clinical		
Group I ribotypes	0/228	1/83 (1.2%)

* $p < 0.0001$.

isolates from patients with melioidosis and were of the *ara*⁻ phenotype, whereas group II isolates were invariably *ara*⁺.

Discussion

This study clearly shows the presence of two phylogenetically different populations among isolates that were identified by standard tests as *B. pseudomallei*. Approximately half of the isolates from soil specimens were of ribotypes similar to those from patients with melioidosis but were markedly different from other environmental isolates. These two ribotype groups also differed in their ability to assimilate arabinose and ethanol.

B. pseudomallei is genomically heterogeneous with respect to rRNA genes and ribotype patterns can be allocated to nearly all strains. Lew and Desmarchelier [9] found 22 patterns among 100 isolates of *B. pseudomallei* from Australia and Southeast Asia and described a similar distribution of hybridisation band sizes to some of the ribotypes described here; however, there is no correspondence between the two numbering systems. In their study, only six environmental isolates from Australia (four soil, two water) were studied and these were grouped in three ribotypes, each of which contained strains from human or animal melioidosis.

It is evident from sequence data that restriction fragment length polymorphisms of rRNA gene provide valuable taxonomic information by which bacteria can be classified. At the species level, these sequences are sufficiently conserved that differences within them often reflect intra-species variation or types. The results obtained here suggest that some environmental organisms classified as *B. pseudomallei* by conventional biochemical and cultural tests are distinguishable from the isolates associated with disease by their ribosomal operons. These genomically atypical isolates are not uniform in ribotype although c. 60% fell into three ribotypes only.

B. pseudomallei is an environmental saprophyte that has been isolated widely from soil and water in Southeast Asia [10, 11]. The environment has been suggested as an important source for acquisition of the organism by patients, and this is supported by the finding of similar strain genotypes in isolates from both environmental and clinical sources. However, the relationship between environmental contamination and clinical melioidosis has not been established and it is not known whether an association exists between the distribution of types of *B. pseudomallei* in the environment and clinical presentations of melioidosis.

Species of the genus *Burkholderia* are distinguished from those of *Pseudomonas* by their cellular lipid and fatty acid composition, 16S rRNA sequences, DNA-DNA homology values and phenotypic characteristics [12]. At least 10 different species have been classified within the genus *Burkholderia*, and these range from strict plant pathogens (e.g., *B. solanacearum*) to species of medical interest such as *B. cepacia* [13]. *B. pseudomallei* may be confused with *B. cepacia*, as both exhibit relatively similar biochemical test results and have intrinsic resistance to polymyxin and gentamicin. The study by Wuthiekanun *et al.* [3] clearly showed that *ara*⁺ isolates of *B. pseudomallei* are not *B. cepacia* and they do not differ sufficiently in biochemical activity from *B. pseudomallei* to warrant their classification in another species by this criterion alone. However, the evidence presented here appears to be inconsistent with a single species concept. Therefore, it will be necessary to examine

environmental and human isolates of both ribotype groups further for their 16S rRNA sequence similarity and other properties to elucidate their taxonomic status and its relationship to pathogenicity. Furthermore, the significance of arabinose and ethanol utilisation by clinical strains of *B. pseudomallei* is not known, but they may prove to be potential markers of pathogenicity.

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References

1. Dance DAB. Melioidosis: the tip of the iceberg? *Clin Microbiol Rev* 1991; **4**: 52–60.
2. Chaowagul W, White NJ, Dance DAB *et al.* Melioidosis: a major cause of community-acquired septicemia in Northeastern Thailand. *J Infect Dis* 1989; **159**: 890–899.
3. Wuthiekanun V, Smith MD, Dance DAB, Walsh AL, Pitt TL, White NJ. Biochemical characteristics of clinical and environmental isolates of *Burkholderia pseudomallei*. *J Med Microbiol* 1996 **45**: 408–412.
4. Dance DAB, Wuthiekanun V, Naigowit P, White NJ. Identification of *Pseudomonas pseudomallei* in clinical practice: use of simple screening tests and API 20NE. *J Clin Pathol* 1989; **42**: 645–649.
5. Clowes RC, Hayes W (eds). Experiments in microbiol genetics. Oxford, Blackwell Scientific Publications. 1968: 184–192.
6. Barrow GI, Feltham RKA (eds). Cowan and Steel's Manual for the identification of medical bacteria, 3rd edn. Cambridge, Cambridge University Press. 1993: 115.
7. Kaufmann ME, Pitcher DG, Pitt TL. Ribotyping of bacterial genomes. In: Chart H (ed) Methods in practical laboratory bacteriology. Boca Raton, CRC Press. 1994: 123–138.
8. Dice LR. Measures of the amount of ecologic association between species. *Ecology* 1945; **26**: 297–302.
9. Lew AE, Desmarchelier PM. Molecular typing of *Pseudomonas pseudomallei*: restriction fragment length polymorphisms of rRNA genes. *J Clin Microbiol* 1993; **31**: 533–539.
10. Wuthiekanun V, Smith MD, Dance DAB, White NJ. Isolation of *Pseudomonas pseudomallei* from soil in north-eastern Thailand. *Trans R Soc Trop Med Hyg* 1995; **89**: 41–43.
11. Van Phung L, Quynh HY, Yabuuchi E, Dance DAB. Pilot study of exposure to *Pseudomonas pseudomallei* in northern Vietnam. *Trans R Soc Trop Med Hyg* 1993; **87**: 416.
12. Yabuuchi E, Kosako Y, Oyaizu H *et al.* Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. *Microbiol Immunol* 1992; **36**: 1251–1275.
13. Gillis M, Van TV, Bardin R *et al.* Polyphasic taxonomy in the genus *Burkholderia* leading to an emended description of the genus and proposition of *Burkholderia vietnamiensis* sp. nov. for N₂-fixing isolates from rice in Vietnam. *Int J Syst Bacteriol* 1995; **45**: 274–289.