JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 2003, p. 4812–4814 Vol. 41, No. 10
0095-1137/03/$08.00+0 DOI: 10.1128/JCM.41.10.4812–4814.2003

Cellular Fatty Acid Profile Distinguishes *Burkholderia pseudomallei* from Avirulent *Burkholderia thailandensis*

Timothy J. J. Inglis,[1]* Max Aravena-Roman,[1] Simon Ching,[2] Kevin Croft,[3] V. Wuthiekanun,[4] and Brian J. Mee[5]

Division of Microbiology and Infectious Diseases[1] and Division of Clinical Pathology,[2] Western Australian Centre for Pathology and Medical Research, and Microbiology, School of Biomedical and Chemical Sciences, University of Western Australia, Nedlands,[5] and Department of Medicine, University of Western Australia, Perth,[3] Western Australia, Australia, and Wellcome Tropical Diseases Research Unit, Mahidol University, Bangkok, Thailand[4]

Received 4 June 2003/Returned for modification 7 July 2003/Accepted 14 July 2003

***Burkholderia pseudomallei*, the cause of melioidosis, can be distinguished from the closely related but nonpathogenic *Burkholderia thailandensis* by gas chromatography (GC) analysis of fatty acid derivatives. A 2-hydroxymyristic acid derivative (14:0 2OH) was present in 95% of *B. pseudomallei* isolates and no *B. thailandensis* isolates. GC mass spectrophotometry confirmed that 2-hydroxymyristic acid was present in *B. pseudomallei*. GC-fatty acid methyl ester analysis may be useful in distinguishing these two closely related species.**

Burkholderia pseudomallei is a gram-negative bacterium that causes melioidosis after soil or water exposure in tropical northern Australia and Southeast Asia (3). Key features that aid recognition of *B. pseudomallei* in primary cultures include a characteristic wrinkling of colonies on solid media and a bipolar gram-negative appearance on microscopy, which is due to accumulation of polyhydroxybutyrate (15). Commonly used methods, such as substrate utilization, have been found to be unreliable for definitive identification of *B. pseudomallei* (6; T. H. Koh, L. S. Y. Ng, J. L. F. Ho, L.-H. Sng, G. C. Y. Wang, and R. V. T. P. Lin, Letter, J. Clin. Microbiol. **41:**1809, 2003). Some reference centers now rely on additional confirmatory tests, such as nucleic acid amplification (10). The closely related, nonpathogenic L-arabinose-utilizing strains have recently been proposed as a distinct species, *Burkholderia thailandensis* (1). *B. thailandensis* has yet to be isolated in Australia. A recent attempt to distinguish pathogenic from nonpathogenic strains of *B. pseudomallei* analyzed cellular fatty acid content (14). Gas-liquid chromatography failed to show any significant difference between Vietnamese clinical and environmental *B. pseudomallei* isolates but was not used to analyze *B. thailandensis* isolates. A recent study of the chemical composition of a virulence-associated hemolysin indicated that 3-hydroxytetradecanoic (3-hydroxymyristic) acid was present in exolipids from *B. pseudomallei* and absent in the closely related but nonpathogenic *B. thailandensis* (5). In preliminary studies we noted possible distinguishing features in the gas chromatography (GC) analysis of *B. pseudomallei* and *B. thailandensis* isolates. In the present study we investigated a collection of *B. pseudomallei* and *B. thailandensis* strains for distinguishing cellular fatty acid patterns, with particular reference to hydroxymyristic acid.

* Corresponding author. Mailing address: Division of Microbiology and Infectious Diseases, Western Australian Centre for Pathology and Medical Research, Nedlands, WA 6909, Australia. Phone: 618 9346 3461. Fax: 618 9381 7139. E-mail: tim.inglis@health.wa.gov.au.

The bacteria used in this study were from the Western Australian Burkholderia Culture Collection (BCC). Bacterial isolates were maintained in 20% glycerol broth at −70°C and recovered by subculture onto 5% horse blood agar plates incubated in air at 37°C for 24 h. *B. pseudomallei* NCTC 10276 and NCTC 13177 are reference strains (7, 11). The remaining *B. pseudomallei* and *B. thailandensis* isolates are distinct strains from the BCC that were previously characterized by automated *Eco*R1 ribotyping and DNA macrorestriction analysis (8). The *B. thailandensis* strains were geographically unrelated isolates from environmental specimens collected by one of the authors (V.W.) in Thailand. In view of the unreliability of conventional substrate utilization as a means of identifying *B. pseudomallei* and other members of the genus *Burkholderia* (6), the identity of all *B. pseudomallei* strains used in the study was confirmed by a previously described PCR protocol (10). L-Arabinose utilization by *B. thailandensis* was demonstrated by a modification of the previously described method in which L-arabinose (Sigma, St. Louis, Mo.) was used as the sole carbon source in a defined minimal medium (17). Nile blue was used as an indicator for the detection of polyhydroxyalkanaote (16), and *B. pseudomallei* isolates were used as a negative control. All *B. pseudomallei* and *B. thailandensis* isolates were ribotyped by the *Eco*R1 protocol previously reported (8). Cellular fatty acids were analyzed by fine capillary column gas-liquid chromatography of fatty acid methyl esters (FAME) using a dedicated GC analyzer (MIDI Systems Inc., Newark, Del.) and the corresponding proprietary interpretive software (Sherlock, version 2.01; MIDI Systems Inc.). Bacterial strains were inoculated onto Trypticase soy broth agar (Oxoid) and incubated at 28°C in air for 24 h. Bacterial cells were harvested with sterile, disposable bacteriology loops and smeared around the lower 2 cm of a borosilicate Wheaton tube (MIDI Systems Inc.). Saponification, methylation, extraction, and washing steps were performed according to the manufacturer's instructions. Extracted FAME preparations were run in batches with a calibration control before each set of 10 analysis vials. FAME

TABLE 1. Principal GC FAME products of *B. pseudomallei* and *B. thailandensis*

FAME peak[a]	% of total FAME content[b] for:	
	B. pseudomallei (n = 87)	*B. thailandensis* (n = 13)
18:1 w7c	32	32
16:0	23	25
17:0 cyclo	5.7	5.5
16:0 3OH	4.1	4.6
19:0 cyclo w8c	3.7	3.8
14:0	3.5	2.6
18:0	0.9	1.1
14:0 2OH[c]	0.58	Not detected

[a] FAME (GO retention time) peak corresponding to the respective FAME derivative.

[b] Values are percentages of the total bacterial FAME content made up by the derivative of the named fatty acid. Additional fatty acid derivatives quantified but present in only trace amounts (<1%) are not shown but contribute to the total cellular fatty acids on which percentage calculations were based.

[c] 14:0 2OH (2-hydroxymyristic acid) was present in 95 and 0% of *B. pseudomallei* and *B. thailandensis* isolates tested, respectively.

analysis was expressed both as a graph of peak activity against retention time and as a percentage of total FAME for each isolate. The FAME products of bacterial cellular fatty acids were confirmed by GC-mass spectrometry analysis of the two *B. pseudomallei* reference strains, one clinical *B. pseudomallei* strain, and one *B. thailandensis* strain. Bacterial cells were saponified with 1 ml of 3.75 M NaOH for 30 min at 100°C. The mixture was neutralized with concentrated HCl to pH ~3 before extraction with 5 ml of high-performance liquid chromatography grade hexane. After thorough extraction by shaking, the mixture was centrifuged and the organic layer was separated. The hexane in the organic layer was evaporated at 40°C under nitrogen. The bacterial cell extract was derivatized with 50 μl of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide and 50 μl of pyridine at 40°C for 60 min. Mass spectrometric analysis of the fatty acids in the bacterial cellular extract as trimethylsilyl derivatives was carried out with a Hewlett-Packard (HP) 5890 series II gas chromatograph coupled to an HP 5989B mass spectrometer. The instrument was run in the electron impact scan mode, monitoring a mass range of 50 to 550 mass units. Chromatographic separations were performed with a 30-m by 0.25-mm HP5-MS capillary column with 0.25-μm film thickness. The helium carrier gas flow rate was 1 ml/min, and the temperature was programmed from an initial value of 160 to 300°C at 10°C/min. Extracted ion chromatograms were obtained for the major unique fragments of the 2- and 3-hydroxymyristic acids of *m*/*z* 271 and 233, respectively.

Preliminary phenotypic and genotypic characterization of the bacterial isolates used in this study confirmed that the isolates were distinct strains of *B. pseudomallei* or *B. thailandensis*. No *B. pseudomallei* isolates utilized L-arabinose, while all *B. thailandensis* isolates did, as determined by the above method. The FAME products from the two species differed only in the presence of small quantities of 2-hydroxymyristic acid in almost all the *B. pseudomallei* strains (Table 1). No 2-hydroxymyristic acid was detected in any of the *B. thailandensis* strains. The 2- and 3-hydroxymyristic acids as trimethylsilyl derivatives in the bacterial cellular extract were confirmed by GC-mass spectrometry analysis of the two *B. pseudomallei* reference strains, one clinical *B. pseudomallei* strain, and one *B. thailandensis* strain. The abundance ratio of *m*/*z* 271 fragment of 2-hydroxymyristic acids to *m*/*z* 233 fragment of 3-hydroxymyristic acids from extracted ion chromatograms produced by GC-mass spectrometry of trimethylsilylated bacterial cellular 2- and 3-hydroxymyristic acids are shown in Table 2.

Our investigation is the first known direct comparison of cellular fatty acid profiles from *B. pseudomallei* and *B. thailandensis* and has uncovered a previously unrecognized distinguishing feature. The practical difficulties encountered in the identification of *Burkholderia* species from environmental specimens have been described previously (2, 9, 14). Those studies noted the presence of 2-hydroxymyristic acid in *B. pseudomallei* strains but did not reveal any difference between *B. pseudomallei* and *B. thailandensis* because the latter species was not included in the study. The presence of 2-hydroxymyristic acid in *B. pseudomallei* was also noted by the Centers for Disease Control and Prevention (18), but the work was performed prior to the transfer of *Pseudomonas pseudomallei* to the new genus *Burkholderia*. At that time the case for a new species of L-arabinose-utilizing nonpathogenic *B. pseudomallei* had not yet been made. It is of interest that Haussler and colleagues did not detect 2-hydroxymyristic acid while working on *B. pseudomallei* exoproducts, despite the discovery of 3-hydroxymyristic acid residues (5). This apparent discrepancy in the findings may reflect differences in the extraction procedures used or in the cellular location of the two compounds. 2-Hydroxymyristic acid is a cellular fatty acid component of bacteria and has been found recently in nonpathogenic species *Sphingomonas paucimobilis* and *Bacillus subtilis* (12, 13). In *Salmonella enterica* serovar Typhimurium 2-hydroxymyristic acid appears to modify the lipid A component of lipopolysaccharide and may therefore contribute to disease pathogenesis (4). A second fatty acid identification method was used to confirm the identity of peaks produced during GC analysis of FAME. Neither method can be regarded as fully quantitative. The absence of 2-hydroxymyristic acid in all *B. thailandensis*

TABLE 2. Hydroxymyristic acids from *B. pseudomallei* and *B. thailandensis* detected in duplicate (replicate) tests by mass spectrometer

Bacterium	Strain	Replicate	Abundance ratio of fragment	
			2-Hydroxymyristic acid (*m*/*z* 271)	3-Hydroxymyristic acid (*m*/*z* 233)
B. pseudomallei	NCTC 10276	a	0.14–0.16	1
B. pseudomallei	NCTC 13177	a	0.12–0.15	1
B. pseudomallei	BCC 122	a	0.09–0.13	1
B. thailandensis	BCC 48	a	0	1

strains analyzed must therefore be regarded as subject to the lower limits of detection of both the methods we used. Nevertheless, these results suggest that GC analysis of fatty acid methyl esters can be used to distinguish between these species and possibly supplement other bacterial identification strategies used to identify members of the genus *Burkholderia*. This preliminary investigation indicates a critical difference between *B. pseudomallei* and the nonpathogenic *B. thailandensis* in the presence or absence of 2-hydroxymyristic acid, a minor component of the cellular fatty acid content. 2-Hydroxymyristic acid and its metabolites are worth further investigation as potential contributors to the pathogenesis of *B. pseudomallei* infection.

REFERENCES

1. **Brett, P. J., D. DeShazer, and D. E. Woods.** 1998. *Burkholderia thailandensis* sp. nov., a *Burkholderia pseudomallei*-like species. Int. J. Syst. Bacteriol. **48:**317–320.
2. **Brook, M. D., B. Currie, and P. M. Desmarchelier.** 1997. Isolation and identification of *Burkholderia pseudomallei* from soil using selective culture techniques and the polymerase chain reaction. J. Appl. Microbiol. **82:**589–596.
3. **Dance, D. A.** 2000. Melioidosis as an emerging global problem. Acta Trop. **74:**115–119.
4. **Gibbons, H. S., S. Lin, R. J. Cotter, and C. R. Raetz.** 2000. Oxygen requirement for the biosynthesis of the S-2-hydroxymyristate moiety in *Salmonella typhimurium* lipid A. Function of LpxO, a new Fe^{2+}/alpha-ketoglutarate-dependent dioxygenase homologue. J. Biol. Chem. **275:**32940–32949.
5. **Haussler, S., M. Nimtz, T. Domke, V. Wray, and I. Steinmetz.** 1998. Purification and characterization of a cytotoxic exolipid of *Burkholderia pseudomallei.* Infect Immun. **66:**1588–1593.
6. **Inglis, T. J., D. Chiang, G. S. Lee, and C.-K. Lim.** 1998. Potential misidentification of *Burkholderia pseudomallei* by API 20NE. Pathology **30**:62–64.
7. **Inglis, T. J., S. C. Garrow, C. Adams, M. Henderson, and M. Mayo.** 1998. Dry-season outbreak of melioidosis in Western Australia. Lancet **352:**1600.
8. **Inglis, T. J. J., L. O'Reilly, N. Foster, A. Clair, and J. Sampson.** 2002. Comparison of rapid, automated ribotyping and DNA macrorestriction analysis of *Burkholderia pseudomallei.* J. Clin. Microbiol. **40:**3198–3203.
9. **Kondo, E., P. Naigowit, P. Phanichruttiwong, W. Petkanchanapong, P. Chetanachan, N. Thirawattanasuk, and K. Kanai.** 1991. Fatty acid profile and acid phosphatase activity of fresh isolates of *Pseudomonas pseudomallei.* Jpn. J. Med. Sci. Biol. **44:**195–211.
10. **Kunakorn, M., and R. B. Markham.** 1995. Clinically practical seminested PCR for *Burkholderia pseudomallei* quantitated by enzyme immunoassay with and without solution hybridization. J. Clin. Microbiol. **33:**2131–2135.
11. **Maegraith, B. G., and C. S. Leithead.** 1964. Melioidosis: a case report. Lancet **i:**862–863.
12. **Matsunaga, I., N. Yokotani, O. Gotoh, E. Kusunose, M. Yamada, and K. Ichihara.** 1997. Molecular cloning and expression of fatty acid alpha hydroxylase from *Sphingomonas paucimobilis.* J. Biol. Chem. **272:**23592–23596.
13. **Matsunaga, I., A. Ueda, N. Fujiwara, T. Sumimoto, and K. Ichihara.** 1999. Characterization of the ybdT gene product of *Bacillus subtilis*: novel fatty acid beta-hydroxylating cytochrome p450. Lipids **34:**841–846.
14. **Phung, L. V., T. B. Tran, H. Hotta, E. Yabuuchi, and I. Yano.** 1995. Cellular lipid and fatty acid compositions of *Burkholderia pseudomallei* strains isolated from human and environment in Viet Nam. Microbiol. Immunol. **39:**105–116.
15. **Pitt, T. L.** 1998. *Pseudomonas*, *Burkholderia* and related genera, p. 1109–1138. *In* L. Collier, A. Balows, and M. Sussman (ed.), Microbiology and microbial infections, vol. 2. Systematic bacteriology. Arnold, London, United Kingdom.
16. **Spiekermann, P., B. H. Rehm, R. Kalscheuer, D. Baumeister, and A. Steinbuchel.** 1999. A sensitive, viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. Arch. Microbiol. **171:**73–80.
17. **Trakulsomboon, S., D. A. Dance, M. D. Smith, N. J. White, and T. L. Pitt.** 1997. Ribotype differences between clinical and environmental isolates of *Burkholderia pseudomallei.* J. Med. Microbiol. **46:**565–570.
18. **Weyant, R. S., C. W. Moss, R. E. Weaver, D. G. Hollis, J. G. Jordan, E. C. Cook, and M. I. Daneshvar (ed.).** 1996. Identification of unusual pathogenic gram-negative aerobic and facultative anaerobic bacteria, 2nd ed. Lippincott Williams & Wilkins, Baltimore, Md.