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Volatile-Sulfur-Compound Profile Distinguishes *Burkholderia pseudomallei* from *Burkholderia thailandensis*

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Solid-phase microextraction gas chromatography-mass spectrometry (SPME-GCMS) was used to show that dimethyl sulfide produced by *Burkholderia pseudomallei* is responsible for its unusual truffle-like smell and distinguishes the species from *Burkholderia thailandensis*. SPME-GCMS can be safely used to detect dimethyl sulfide produced by agar-grown *B. pseudomallei*.

The earthy, truffle-like smell of mature *Burkholderia pseudomallei* colonies on solid agar media has been used to aid identification in the clinical laboratory. Previous attempts to analyze the volatile metabolic products of *B. pseudomallei* have been hampered by the low sensitivity of separation technologies and concerns about laboratory-acquired infection risks. Our earlier gas-liquid chromatography (GLC) analysis of *B. pseudomallei* and *Burkholderia thailandensis* was unsuitable for volatile organic compound (VOC) analysis (1). Recent gas chromatography-mass spectrometry (GCMS) analysis of bacterial fatty acid content corroborated our GLC results (2) but did not analyze VOC production. Combination of solid-phase microextraction capture (SPME) with GCMS presented an opportunity to analyze the volatile products of *Burkholderia* metabolism, identify the distinctive VOCs generated by *B. pseudomallei*, and determine whether the principal components of its odor could be used to aid in its identification.

Bacterial strains. *Burkholderia* species that had been identified by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) MS and species-specific PCR assay (3, 4) were used, including five fully sequenced strains (5) from the Western Australian *Burkholderia* Culture Collection (*B. pseudomallei* strains NCTC 13177, DM98, and BCC 215; *Burkholderia thailandensis* Bt4; and *Burkholderia ubonensis* Bu). Fourteen other *B. pseudomallei* strains and *Burkholderia cepacia*, *Burkholderia vietnamiensis*, and another *B. thailandensis* strain were included. Additional non-*Burkholderia* Gram-negative bacteria, including *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Comamonas testosteroni*, *Ralstonia pickettii*, and *Cupriavidus necator*, were obtained from the Western Australian Culture Collection. Bacterial strains were cultured in air at 37°C for 24 h on 5% horse blood agar, and their identities were verified by MALDI-TOF MS analysis (3). A bacteriological loop was used to touch 10 distinct colonies of each strain and inoculate a fresh 5% horse blood agar plate to guarantee single-colony growth after 24 h of incubation in air at 37°C. These plates were used for SPME-GCMS analysis at 48 h after inoculation. A more detailed 1-week time series compared *B. pseudomallei* NCTC 13171 and *B. thailandensis* Bt4 inoculated at 24-h intervals onto blood agar plates from a single manufacturer's batch (Excel Laboratory Products, Nedlands, WA, Australia). Each plate was incubated in its own labeled zip lock polyethylene bag on the same incubator shelf as the others in the series. SPME-GCMS glass vials were coded to conceal the durations of incubation and identities of the bacteria. We analyzed three discrete bacterial colonies per glass vial by excising 0.80-mm-diameter plugs from 5% horse blood agar plates and transferring them to a sterile glass vial sealed with a diaphragm-protected screw top. The microextraction sheath was inserted into the vial's headspace, and automatic sampling was conducted for 10 min after sheath insertion. To determine the sensitivity and specificity of volatile organic sulfur compound detection as a means of distinguishing *B. pseudomallei* from near-neighbor *Burkholderia* species and more-common laboratory Gram-negative isolates, we repeated the analyses after 24 and 48 h of incubation with 16 *B. pseudomallei* and 11 non-*B. pseudomallei* strains.

Extraction procedure. Analytes were extracted from the headspace by solid-phase microextraction at room temperature and then analyzed by SPME-GCMS. Sampling was performed with a headspace autosampler (CTC CombiPAL). Samples were placed at the bottom of a 20-ml headspace vial with a screw top lid and extracted with a coated microextraction fiber (DVB/CAR/PDMS; Supelco) for 10 min at room temperature. The needle depth inside the vial was set to 45 mm so that there was no direct contact with bacterial cultures. The microextraction fiber was desorbed in the spectrometer inlet for 1 min at 250°C with a needle depth of 54 mm. The samples were analyzed on a gas chromatograph (Agilent 7890A GC) coupled to a mass spectrometer (Agilent 5975C MSD) set up with a 0.25-μm capillary column (Agilent). We superimposed mass spectrometer recordings on a negative-control trace produced by a sterile incubated agar plug and compared volatile compounds from *B. pseudomallei* with those from *B. thailandensis* and other species. We identified major components from a mass spectrum library (National Institute of Standards and Technology) and verified key volatile products against analytical standards (>98% purity; Sigma-Aldrich). The statistical methods used for quantitative analysis were column statistics, the Wilcoxon

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TABLE 1 Comparison of volatile organic sulfur compounds from *B. pseudomallei* and *B. thailandensis* cultures

VOC	Mean concn (ng/ml) ± SD		Bp/Bt[a]	*P* value WMSRT[b]	*P* value χ^2 trend
	B. pseudomallei	*B. thailandensis*			
DMS	7.73 ± 11.84	0.81 ± 0.62	9.54	0.0117	0.0232
Diethyl sulfide[c]	2.00 ± 1.16	0.75 ± 0.47	2.67	0.0078	NS[d]
Methanethiol[c]	5.35 ± 3.58	2.18 ± 1.78	2.45	0.0156	NS
Dimethyl trisulfide[c]	1.07 ± 0.80	0.48 ± 0.38	2.23	NS	0.0479
Dimethyl disulfide	262.9 ± 415.4	1,609 ± 2,052	0.163	NS	<0.0001
Thiocyanic acid, methyl ester[c]	1.02 ± 1.13	8.53 ± 10.47	0.120	0.0313	NS

[a] Bp/Bt, mean of *B. pseudomallei* product divided by mean of *B. thailandensis* product.
[b] WMSRT, Wilcoxon matched-pair signed-rank test.
[c] Target ion area normalized to internal standard.
[d] NS, not significant.

matched-pair signed-rank test, the Mann-Whitney U test, and χ^2 trend (Prism version 6.0; GraphPad, San Diego, CA).

The major volatile sulfur compound produced by both *B. pseudomallei* and *B. thailandensis* was dimethyl disulfide (Table 1), but its concentration did not distinguish these species. Analytical-grade dimethyl disulfide was odorless, unlike mature *B. pseudomallei* cultures. The volatile sulfur compounds generated in significantly higher concentrations by *B. pseudomallei* cultures were dimethyl sulfide (DMS), diethyl sulfide, methanethiol, and thiocyanic acid, methyl ester. The differentiating volatile compound in the highest absolute and relative concentrations was DMS, which was produced early in the week-long time series (Fig. 1). Analytical-grade DMS produced a subjective odor identical to that of (covered) mature *B. pseudomallei* cultures. No DMS was produced by uninoculated 5% horse blood agar or *B. pseudomallei* colonies inoculated directly onto the inside of sterile glass vials. *B. thailandensis* and other species produced little or no DMS. The other VOCs that distinguished *B. pseudomallei* from *B. thailandensis* were present at lower concentrations than DMS in *B. pseudomallei*. These included anisole; the 2-ethyl-methyl ester of hexanoic acid, 3-hexanone, 5-methyl-3-heptanone, or 3-heptanone; the methyl ester of 2-heptenoic acid methyl ester; and the 2-methyl ester of propanoic acid. Production of DMS, dimethyl disulfide, and trimethyl sulfide varied among strains of *B. pseudomallei* and the other *Burkholderia* species tested. Using a cutoff of 5 area units of DMS at 24 h, the specificity for *B. pseudomallei* detection was 1.00 and the sensitivity was 0.76; at 48 h, they were 0.91 and 0.89, respectively. DMS generation at 48 h was significantly greater than at 24 h of incubation (Wilcoxon test, $P = 0.0008$, 16 pairs, median difference = 7.5), though there was a small overlap in DMS production with the non-*B. pseudomallei* bacteria tested.

We found that the dominant odor-producing compound that distinguished *B. pseudomallei* from its near neighbor *B. thailandensis* was DMS, an important biological signal compound. Methyl disulfide is highly volatile and produces an offensive odor at high concentrations. SPME-GCMS has been used previously to determine the range of VOCs that contribute to the organoleptic properties of truffles (6), the major constituents of which are DMS and methanethiol (7). DMS is responsible for the earthy odor produced by a range of soil bacteria and explains historical descriptions of the smell of *B. pseudomallei* cultures. Quantitative test performance analysis of *B. pseudomallei*'s earthy odor as a preliminary detection method shows that its sensitivity improves over 48 h of incubation. Though we do not recommend sniffing or smelling bacterial cultures in order to detect the species, we realize that the long-held belief that a pungent odor given off early by mature colonies of bacteria may be used to recognize potential *B. pseudomallei* cultures. From our data, SPME-GCMS appears to be a safer alternative to sniffing culture plates and is clearly a more objective way of ruling in possible *B. pseudomallei* where an SPME-GCMS service is readily available. This technique complements other rapid phenotyping methods such as MALDI-TOF MS for rapid culture characterization. It has been applied previously to more commonly encountered bacterial species (8–10) and used for their direct detection in exhaled breath samples (11). Production of DMS by marine algae is thought to act as a defense against oxidative stress (12) and induces apoptosis in malignant human

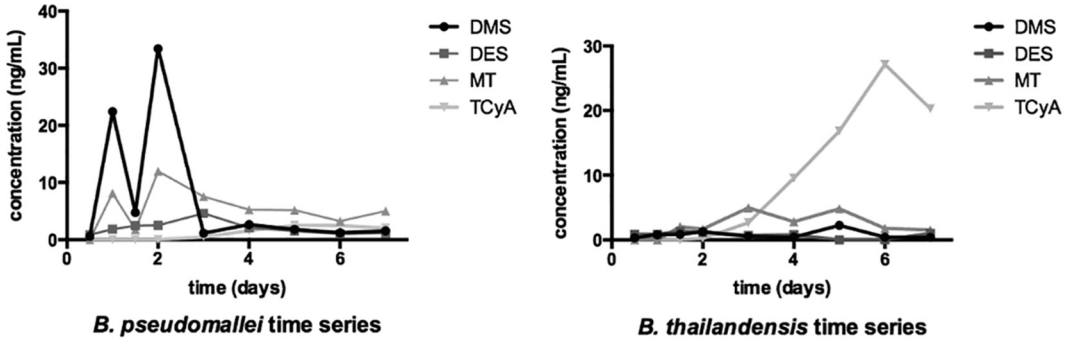

FIG 1 Comparison of concentrations of the volatile organic sulfur compounds produced by cultures of *B. pseudomallei* NCTC 13177 and *B. thailandensis* Bt4, showing the time-dependent trends that distinguish the two species. DMS, dimethyl sulfide; DES, diethyl sulfide; MT, methanethiol; TCyA, thiocyanic acid, methyl ester.

cell lines (13), suggesting a possible bacterial survival function that may contribute to the pathophysiology of melioidosis. Our results are a step toward clinical evaluation of VOC detection as a means of point-of-care diagnosis of acute melioidosis by SPME-GCMS.

REFERENCES

1. **Inglis TJ, Merritt A, Chidlow G, Aravena-Roman M, Harnett G.** 2005. Comparison of diagnostic laboratory methods for identification of *Burkholderia pseudomallei*. J Clin Microbiol **43:**2201–2206. http://dx.doi.org/10.1128/JCM.43.5.2201-2206.2005.
2. **Li D, March JK, Bills TM, Holt BC, Wilson CE, Lowe W, Tolley HD, Lee ML, Robison RA.** 2013. Gas chromatography-mass spectrometry method for rapid identification and differentiation of *Burkholderia pseudomallei* and *Burkholderia mallei* from each other, *Burkholderia thailandensis* and several members of the *Burkholderia cepacia* complex. J Appl Microbiol **115:**1159–1171. http://dx.doi.org/10.1111/jam.12310.
3. **Inglis TJ, Healy PE, Fremlin LJ, Golledge CL.** 2012. Use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis for rapid confirmation of *Burkholderia pseudomallei* in septicemic melioidosis. Am J Trop Med Hyg **86:**1039–1042. http://dx.doi.org/10.4269/ajtmh.2012.11-0454.
4. **Merritt A, Inglis TJ, Chidlow G, Harnett G.** 2006. PCR-based identification of *Burkholderia pseudomallei*. Rev Inst Med Trop Sao Paulo **48:**239–244. http://dx.doi.org/10.1590/S0036-46652006000500001.
5. **Mukhopadhyay S, Thomason MK, Lentz S, Nolan N, Willner K, Gee JE, Glass MB, Inglis TJ, Merritt A, Levy A, Sozhamannan S, Mateczun A, Read TD.** 2010. High-redundancy draft sequencing of 15 clinical and environmental *Burkholderia* strains. J Bacteriol **192:**6313–6314. http://dx.doi.org/10.1128/JB.00991-10.
6. **Díaz P, Ibáñez E, Señoráns FJ, Reglero G.** 2003. Truffle aroma characterization by headspace solid-phase microextraction. J Chromatogr **1017:**207–214. http://dx.doi.org/10.1016/j.chroma.2003.08.016.
7. **Zarivi O, Bonfigli A, Cesare P, Amicarelli F, Pacioni G, Miranda M.** 2003. Truffle thio-flavours reversibly inhibit truffle tyrosinase. FEMS Microbiol Lett **220:**81–88. http://dx.doi.org/10.1016/S0378-1097(03)00065-X.
8. **Tait E, Perry JD, Stanforth SP, Dean JR.** 2014. Identification of volatile organic compounds produced by bacteria using HS-SPME-GC-MS. J Chromatogr Sci **52:**363–373. http://dx.doi.org/10.1093/chromsci/bmt042.
9. **Trefz P, Koehler H, Klepik K, Moebius P, Reinhold P, Schubert JK, Miekisch W.** 2013. Volatile emissions from *Mycobacterium avium* subsp. *paratuberculosis* mirror bacterial growth and enable distinction of different strains. PLoS One **8:**e76868. http://dx.doi.org/10.1371/journal.pone.0076868.
10. **Tait E, Hill KA, Perry JD, Stanforth SP, Dean JR.** 2014. Development of a novel method for detection of *Clostridium difficile* using HS-SPME-GC-MS. J Appl Microbiol **116:**1010–1019. http://dx.doi.org/10.1111/jam.12418.
11. **Boots AW, Smolinska A, van Berkel JJ, Fijten RR, Stobberingh EE, Boumans ML, Moonen EJ, Wouters EF, Dallinga JW, Van Schooten FJ.** 2014. Identification of microorganisms based on headspace analysis of volatile organic compounds by gas chromatography-mass spectrometry. J Breath Res **8:**027106. http://dx.doi.org/10.1088/1752-7155/8/2/027106.
12. **Sunda W, Kieber DJ, Kiene RP, Huntsman S.** 2002. An antioxidant function for DMSP and DMS in marine algae. Nature **418:**317–320. http://dx.doi.org/10.1038/nature00851.
13. **Zhang G, Wu H, Zhu B, Shimoishi Y, Nakamura Y, Murata Y.** 2008. Effect of dimethyl sulfides on the induction of apoptosis in human leukemia Jurkat cells and HL-60 cells. Biosci Biotechnol Biochem **72:**2966–2972. http://dx.doi.org/10.1271/bbb.80453.