THE TRANSCRIPTIONAL ANALYSIS OF *BURKHOLDERIA PSEUDOMALLEI* UPON PROLONGED INCUBATION IN HUMAN SERUM

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ABSTRACT

Burkholderia pseudomallei causes melioidosis, an often-fatal tropical disease endemic in Southeast Asia and northern Australia. The bacteria's ability to survive against the host immune response makes it difficult to be completely eradicated even upon appropriate treatment. In this study, we aim to evaluate *B. pseudomallei's* survival and transcriptional changes upon 28-day incubation compared to 5-day in healthy human serum (HHS) by RNA sequencing. Two clinical *B. pseudomallei* isolates were cultured in HHS and tryptic soy broth for 28 days. RNA was extracted on the 5th and 28th day from HHS followed by RNA sequencing to identify the transcriptional adaptations of *B. pseudomallei* in HHS at day 28 compared to day 5. The gene expression of selected genes was validated by reverse-transcription real-time PCR. In this study, *B. pseudomallei* isolates were able to survive and proliferate in HHS after day 5 in contrast to declining growth in tryptic soy broth (TSB). Among the 1,657 differentially regulated genes, more than half (62%) of the up-regulated genes were of the accessory genome. The highly expressed genes were related to surface determinants especially capsular polysaccharides (CPS II and III). In addition, genes involving ATP synthase operon, acyl-homoserine lactone-mediated quorum sensing system (AHL-QS) mediated secondary metabolites and secretion systems were also up-regulated. These suggest that B. pseudomallei requires activation of various mechanisms for prolonged survival in HHS. This study provides an insight into the possible mechanisms utilised by *B. pseudomallei* for persistent survival in HHS.

KEYWORDS: *B. pseudomalle*i, Serum, Gene expression, RNA-seq, Survival

BACKGROUND

B. pseudomallei, a Gram-negative soil bacterium, is the etiological agent of melioidosis which is a tropical disease endemic in Southeast Asia and northern Australia. However, this disease may have spread to other regions including Southern America, Africa, Middle East, Southern and Eastern Asia as proven by the culture of *B. pseudomallei* from soils (1). This life-threatening disease mainly presents pneumonia and septicaemia (2). There is currently no commercialized vaccine available for melioidosis. The mortality rate of septicemic melioidosis may reach up to 90% upon a delayed diagnosis and inappropriate antimicrobial treatment (3-5). Relapse and recurrent melioidosis still occur suggesting incomplete bacterial clearance despite the administration of appropriate therapy (5).

B. pseudomallei is able to adapt and survive in diverse environments including wide ranges of temperature, pH levels, oxidative stress, osmotic pressures, oxygen, iron and nutrient limitations (6-12). *B. pseudomallei* may reside in humans for many years. The development of latent melioidosis infection is low at <5% but may be underestimated as the patients may remain asymptomatic. It was reported that the latency period may extend up to 29 years (2). Serum activates various components to act against bacterial invasion particularly complement system and antibody-dependent cellular phagocytosis (ADCP). Despite that, *B. pseudomallei* is not only resistant to serum killing and capable of proliferation in healthy human serum (HHS). Capsular polysaccharides (CPS) and lipopolysaccharide (LPS) have been described as contributors to this event (13,14). *B. pseudomallei* can also survive and replicate intracellularly while evading macrophage killing mechanisms (15-17). The interplay between various bacterial virulence factors including type 3 (T3SS), type 6 secretion systems (T6SS), surface polysaccharides such as CPS and LPS, biofilm and toxin-antitoxin systems could be associated with the bacterial survival advantage in different niches (2).

Transcriptional analysis ranging from DNA microarray to RNA sequencing has been utilized to study mechanisms and cellular responses of *B. pseudomallei* under different environmental stress and during the infection process (12). Taking into consideration the ability of B. pseudomallei to survive in various conditions, we aim to identify the transcriptional adaptations of *B. pseudomallei* after prolonged incubation in HHS. Differing from the previous approach to observe the bacteria's viability in a few hours (13,14), we started by investigating the survival of *B. pseudomallei* in HHS for a longer duration. In this study, we aim to evaluate *B. pseudomallei's* survival and transcriptional changes upon 28-day incubation compared to 5-day in HHS by RNA sequencing followed by a subsequent quantitative reverse-transcription PCR (qRT-PCR) to assess and compare the changes in gene expression that are possibly related to the bacterial persistence.

METHODS

Bacteria isolates and growth analysis

Two clinical *B. pseudomallei* isolates (IMRS1 and IMRS2) were inoculated in 1 mL pooled HHS (undiluted) and cultured at 37 oC under static conditions for 28 days without changing the media. The pooled HHS used in this study were not diluted prior to the experiment and tested as negative by in-house IgM and IgG ELISA against heat-inactivated *B. pseudomallei* whole cell antigen (18). In addition to incubation in HHS, both IMRS1 and IMRS2 were also inoculated in tryptic soy broth (TSB) under the same condition to serve as a positive control for growth curve analysis. Briefly, several colonies of *B. pseudomallei* from an overnight culture were suspended in normal saline and adjusted to obtain a starting concentration of approximately 1 x 107 colony-forming units (CFU)/mL in a total of 1 mL HHS or TSB. A higher inoculating bacterial concentration was chosen to allow *B. pseudomallei's* survival throughout 28 days experiment (19). Bacterial colony counts were performed in triplicates on day 1, 3, 5, 10, 14 and 28 upon inoculation by plating diluted bacterial suspension on tryptic soy agar (TSA) and the result was read after 24 hours incubation at 37 °C. All experimental procedures involving live *B. pseudomallei* isolates were conducted in a Biosafety Level 3 (BSL-3) laboratory.

RNA extraction and sequencing

Total RNA was extracted from IMRS1 and IMRS2 after incubation in HHS for 5 days (D5) and 28 days (D28). Briefly, 200 µL of the bacterial culture were pelleted and washed one time by phosphate buffer saline (PBS) prior to extraction using MasterPureTM RNA purification kit according to the manufacturer's protocol. The concentration and quality of the total RNA was assessed by QubitTM RNA HS assay kit (Invitrogen) and Agilent RNA 6000 Pico kit (Agilent Technologies, USA), respectively. This was followed by ScriptSeqTM Complete kit (Epicentre, USA) which contains Ribo-

Zero rRNA Removal Reagents and magnetic core kit to remove ribosomal RNA and ScriptSeqTM v2 RNA-Seq library preparation kit to prepare cDNA library. The final cDNA library was reassessed with QubitTM RNA HS assay kit, Agilent HS DNA kit (Agilent Technologies, USA) and library quantitative PCR (KAPA Biosystems, USA) prior to paired-end sequencing at 2 x 75 bp read length on Miseq system (Illumina, USA). The RNA-seq reads obtained from this study has been deposited in the database of the European Nucleotide Archive with accession number PRJEB49969. The sample accession numbers are ERR7961951, ERR7961952, ERR7961953 and ERR7961954 for IMRS1D5, IMRS1D28, IMRS2D5 and IMRS2D28, respectively.

RNA-seq bioinformatics analysis

The raw read sequences were trimmed to discard sequencing adapters, low quality bases (Q<20) and minimum acceptable read length of at least 35 bp using BBDuk v.36 (20). The clean reads were aligned and mapped to B. pseudomallei reference genome, K96243 (Genbank accession: chromosome 1, NC006350.1 and chromosome 2, NC006351.1) by TopHat2 v. 2.0.11 (21). Expression quantification and profiling was performed on the mapped reads using FeatureCounts v.2.0.1 (22). The biological replicates were assessed for their consistency within groups by sample distances and clustering matrix prior to differential gene expression analysis by DESeq 2 v.3.2 (2). Pair-wise differential expression was performed between isolates harvested at day 5 and day 28. Gene expression with log2 fold change of ≥1 (up-regulated) or ≤ -1(down-regulated) with q value of ≤0.05 were considered as significant. The expressed genes were subjected to identification of biological processes and functional annotation by Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) orthology (KO) using database for annotation, visualization and integrated discovery (DAVID) Bioinformatics Resources 6.8 (24).

Reverse-transcription real-time PCR (qRT-PCR)

Compared to group D5, six up-regulated genes (BPS_RS31320, BPS_RS25250, BPS_RS19075, BPS_RS27075, BPS_RS32200 and BPS_RS26895) of group D28 with various functions and expression level (log2 fold change between +1 to +7) were randomly selected for qRT-PCR. The experiment was performed in duplicates. Briefly, RNA was extracted as described above and converted to cDNA by qPCRBIO cDNA synthesis kit (PCR Biosystems, UK). The qRT-PCR was performed on Biorad CFX96 using SensiFAST SYBR kit (Bioline, UK) with initial denaturation at 95 °C for 3 min followed by 45 cycles of denaturation at 95 °C for 5 sec, annealing and extension at 58 °C to 60 °C for 15 sec. Melting curve analysis was conducted by increasing the annealing temperature by 0.1 °C per step from 65 °C to 95 °C. The primer pair, BPS_RS26895 (hcp1) (25) and the other five pairs were designed using Primer-BLAST. 16S rRNA gene was used as the housekeeping control for data normalization. The primers are shown in Table S1.

RESULTS

***B. pseudomallei* growth in HHS**

Both *B. pseudomallei* isolates, IMRS1 and IMRS2 were able to survive in HHS and TSB until day 28 with approximately 1 log difference of CFU/Lm in HHS compared to TSB at the end of the experiment. The bacterial count showed a slight increase from 6 x 109 CFU/mL (day 5) to 0.3-1 x 1010 CFU/mL (day 28) in HHS. In contrast, the bacterial count continued to decrease from 0.6-3 x 1012 CFU/mL (day 3) to 0.9-1 x 109 CFU/mL (day 28) in TSB (Figure 1). *B. pseudomallei's* persistence and multiplication after day 5 in HHS leads to a further transcriptomic analysis using RNA-seq.

Transcriptional profile of *B. pseudomallei* in prolonged exposure to HHS

RNA-seq experiment was performed in this study to analyse the transcriptomic profile of *B. pseudomallei* isolates in HHS and possible mechanisms involved in the bacterial persistence. The experiment was performed to compare the differentially expressed genes (DEGs) of *B. pseudomallei* exposed to HHS for 5 days and 28 days. The assessment of the two biological replicates showed that they were clustered within groups and exhibited a clear separation between both conditions (Figure 2). Hence, the DEGs profiles could be generated and proceeded for further analysis. The isolates were labelled and grouped according to their incubation period. For example, isolates IMRS1D5, IMRS2D5 and IMRS1D28, IMRS2D28 were collected on day 5 (D5) and day 28 (D28), respectively. The trimmed reads were aligned against *B. pseudomallei* K96243 genome demonstrating overall mapping percentage of IMRS1D5 (49.3%), IMRS2D5 (57.6%), IMRS1D28 (53.5%) and IMRS2D28 (56.4%). A total of 1,657 genes, q≤0.05, were significantly differentiated in group D28 compared to D5 (Table S2). Among them, 873 genes exhibited an

increase in expression (up-regulated) while 784 genes showed a decrease in expression (down-regulated) with log2 fold change ranging from -5 to +7 (Table S2). More than half of the up-regulated genes were of chromosome 2 (62%) whereas 77% of the down-regulated genes were of chromosome 1.

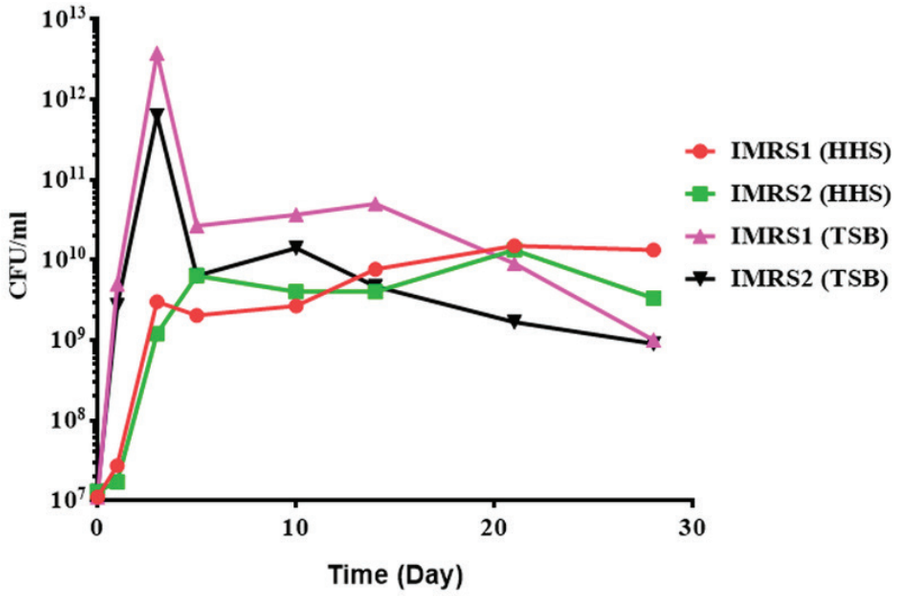

Figure 1. Survival of *B. pseudomallei* in healthy human serum (HHS). Bacterial colony counts were performed in triplicates at seven-time points: Day 1, 3, 5, 10, 14, 21 and 28.

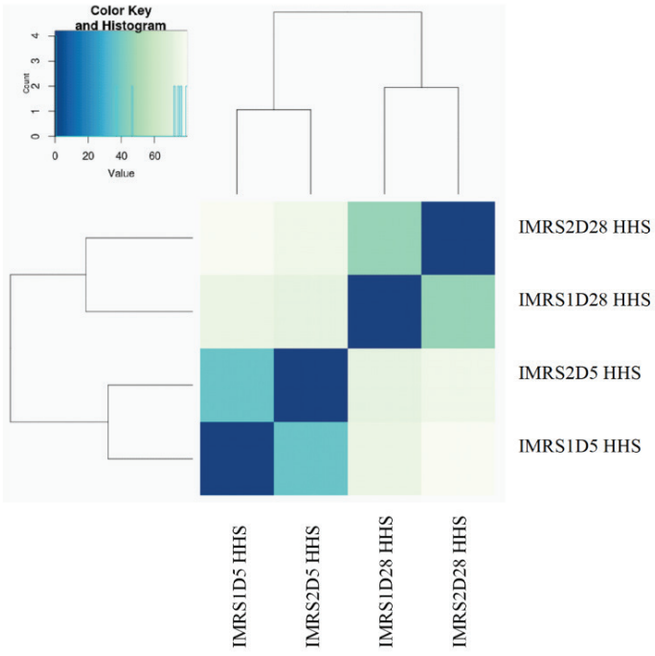

Figure 2. Sample distances and clustering matrix of *B. pseudomallei* biological replicates. A dendrogram based on clustering of sample distances was performed with DESEQ2 package where the discretely distributed expression values were transformed into a regularised log transformation model (rlog). The biological replicates with similar profile were clustered together. The dendrogram showed 2 distinct groups: Day 5 (IMRS1D5 HHS, IMRS2D5) and Day 28 (IMRS1D28, IMRS2D28).

Table 1: Functional analysis of significantly up-regulated genes (log^2 fold change > +4, $p<0.05$) in group D28

Category	GO term ID	GO term description	Genes	
			New locus tag	Old locus tag
Metabolic and Molecular function[a]	GO:0016491	Oxidoreductase activity	BPS_RS31320, BPS_RS31340, BPS_RS29245, BPS_RS31400, BPS_RS23300, BPS_RS25235, BPS_RS02520	BPSS2284, BPSS2287, BPSS1918, BPSS2299, BPSS0840, BPSS1196, BPSL0480
	GO:0006807	Nitrogen compound metabolic process	BPS_RS25250	BPSS1199
	GO:0008152	Metabolic process	BPS_RS20220, BPS_RS02540, BPS_RS21020, BPS_RS21025, BPS_RS25540, BPS_RS25240, BPS_RS25625	BPSS0291, BPSL0484, BPSS0425, BPSS0426, BPSS1251, BPSS1197, BPSS1266
	GO:0006351	Transcription	BPS_RS29240	BPSS1917
	GO:0006633	Fatty acid biosynthetic process	BPS_RS29440	BPSS1955
	GO:0004022	Alcohol dehydrogenase	BPS_RS29380	BPSS1944
	GO:0005975	(NAD activity)	BPS_RS29450, BPS_RS29300	BPSS1957, BPSS1928
	GO:0006082	Carbohydrate metabolic	BPS_RS29445	BPSS1956
	GO:0019439	process	BPS_RS29070	BPSS1888
	GO:0006725		BPS_RS29065	BPSS1887
	GO:0006529	Organic acid metabolic process	BPS_RS22380	BPSS0677
	GO:0016740	Aromatic compound catabolic	BPS_RS28720	BPSS1828
	GO:0019673	process	BPS_RS27950	BPSS1688
	GO:0042619		BPS_RS29235, BPS_RS29435	BPSS1916, BPSS1954
	GO:0009103	Cellular aromatic compound metabolic process	BPS_RS20985, BPS_RS28730	BPSS0418, BPSS1830
	GO:0009058	Asparagine biosynthetic process	BPS_RS20990	BPSS0419
	GO:0016614	Transferase activity	BPS_RS03725	BPSL0707
	GO:0003995	GDP-mannose metabolic process	BPS_RS02535	BPSL0483
	GO:0019441		BPS_RS30615	BPSS2156
	GO:0003824	Poly-hydroxybutyrate biosynthetic process	BPS_RS21000, BPS_RS19750	BPSS0421, BPSS0214

Category	GO term ID	GO term description	Genes	
			New locus tag	Old locus tag
	GO:0030163	Lipopolysaccharide biosynthetic process	BPS_RS24805	
	GO:0015986	Biosynthetic process	BPS_RS29425	
	GO:0016042	Oxidoreductase activity acting on CH-OH group of donors	BPS_RS18930	BPSS0067
	GO:0046872		BPS_RS29060	BPSS1886
	GO:0000271	Acyl-CoA dehydrogenase activity	BPS_RS28745	BPSS1833
	GO:0004725		BPS_RS28740	BPSS1832
	GO:0009190	Tryptophan catabolic process to kynurenine	BPS_RS03150	BPSL0597
	GO:0019538		BPS_RS21525	BPSS0522
	GO:0008080	Catalytic activity	BPS_RS21005	BPSS0422
		Protein catabolic process		
		ATP synthesis coupled proton transport		
		Lipid catabolic process		
		Metal ion binding		
		Polysaccharide biosynthetic process		
		Protein tyrosine phosphatase activity		
		Cyclic nucleotide biosynthetic process		
		Protein metabolic process		
		N-acetyltransferase activity		
Cellular componenta	GO:0016021	Integral component of membrane	BPS_RS29415, BPS_RS23250 BPS_RS25545, BPS_RS30930, BPS_RS27935, BPS_RS31325, BPS_RS22870, BPS_RS02505 BPS_RS00535	BPSS1951, BPSS0831, BPSS1252, BPSS2213, BPSS1685, BPSS2285, BPSS0766, BPSL0477 BPSL0106
	GO:0042597	Periplasmic space		
Biological processa	GO:0006950	Response to stress	BPS_RS23295, BPS_RS23285	BPSS0839, BPSS0837
	GO:0006810	Transport	BPS_RS02510, BPS_RS09870	BPSL0478, BPSL1855
	GO:0009372	Quorum sensing	BPS_RS23535	BPSS0885
	GO:0008643	Carbohydrate transport	BPS_RS26095	BPSS1350
	GO:0007155	Cell adhesion	BPS_RS22875	BPSS0767

Category	GO term ID	GO term description	Genes	
			New locus tag	Old locus tag
Genetic Information Processing[b]	Bps03110	Chaperones and folding catalysts	BPS_RS31345	BPSS2288
Metabolism[b]	Bps01008	Polyketide biosynthesis proteins	BPS_RS25175	BPSS1183
Unknown	NA	NA	BPS_RS19745, BPS_RS14520, BPS_RS29455, BPS_RS19735, BPS_RS30950, BPS_RS24810, BPS_RS22795, BPS_RS02580, BPS_RS27940, BPS_RS02525, BPS_RS29250, BPS_RS29725, BPS_RS31025, BPS_RS29270, BPS_RS27725, BPS_RS21585, BPS_RS22385, BPS_RS23265, BPS_RS25245, BPS_RS31405, BPS_RS30260, BPS_RS30290, BPS_RS19740, BPS_RS05940, BPS_RS25205, BPS_RS26930, BPS_RS02565, BPS_RS24415, BPS_RS24180, BPS_RS25220, BPS_RS03115[c], BPS_RS25200[c], BPS_RS12040[c], BPS_RS24715[c], BPS_RS08580[c], BPS_RS24735[c], BPS_RS04620[c], BPS_RS26125[c], BPS_RS27650[c]	BPSS0213, BPSL2703, BPSS1958, BPSS0211, BPSS2217, BPSS1115, BPSS0753, BPSL0492, BPSS1686, BPSL0481, BPSS1919, BPSS2001, BPSS2230, BPSS1923, BPSS1649, BPSS0534, BPSS0678, BPSS0834, BPSS1198, BPSS2300, BPSS2098, BPSS2103, BPSS0212, BPSL1133, BPSS1188, BPSS1505, BPSL0489, BPSS1044, BPSS1003, BPSS1192, NA

[a] Gene orthology (GO) analysis

[b] Kyoto encyclopedia of genes and genomes (KEGG) orthology (KO)

[c] Old locus tag not available

NA, not available

Structural and energy metabolism

Both IMRS1D28 and IMRS2D28 exhibited a significantly high level of gene expressions involving energy and carbon metabolism, for instance, F-type ATP synthase operon in chromosome 2 (*BPS_RS29380-BPS_RS29450*), surface polysaccharides biosynthetic clusters (*BPS_RS28715-BPS_28750, BPS_RS20980-BPS_RS21040*), glycolysis and gluconeogenesis including aromatic hydrocarbons catabolism (*BPS_RS29055-BPS_RS29070*) and several of fatty acid or acyl-CoA metabolism associated genes (*BPS_RS18930, BPS_RS29235*) (Table 1) (Table S2). Of the four CPS operons Reckseidler-Zentenon et al. detected in *B. pseudomallei*, there was overexpression in CPS II (*BPS_RS20980-BPS_RS21040*) and CPS III (*BPS_RS28715-BPS_RS28755*) in group D28 (13). Genes encoding for alternative energy sources were expressed at a comparably slightly lower level, for instance, arginine deiminase pathway, fumarate and nitrate reduction, lipid metabolism and NAD-dependent formate dehydrogenase.

Quorum sensing, secondary metabolites and stress response genes

Bacterial quorum sensing (QS) machinery has been associated with bacterial survival during over crowding and adaptation in hostile environments especially acyl-homoserine lactone-mediated quorum sensing system (AHL-QS) (26). In this study, we observed significant up-regulation of bpsI genes (*BPS_RS23535, BPS_RS25160, BPS_RS27290*) however no changes indicated the difference in both groups with respect to the bpsR genes except bpsR5 (*BPS_RS12550*). *B. pseudomallei* encodes for diverse clusters of secondary metabolites in its genome. Significant over expression of a number of QS-regulated secondary metabolites genes was observed including bactobolin cluster and adjacent unknown cluster (*BPS_RS25085-BPS_RS25250* at log2 fold change +2 to +7) but malleilactone (*BPS_RS20270-BPS_RS20340*) and alkyl quinolone (*BPS_RS21340-BPS_RS21370*) clusters were not differentially expressed in this study. Also noted was the up-regulation at log2 fold change +2 to +5, in several genes of other secondary metabolites including malleipeptin (*BPS_RS27625-BPS_RS27645*), syrbactin (*BPS_RS25625-BPS_RS25660*), rhamnolipids (B*PS_RS21595-BPS_RS21630, BPS_RS26075-BPS_RS26105*) and several putative secondary metabolites. Increased transcripts were also observed in several stress-related proteins (*BPS_RS01585, BPS_RS07460, BPS_RS23280-BPS_RS23295, BPS_RS24945, BPS_RS28775, BPS_RS29240, BPS_RS29330, BPS_RS31130, BPS_RS31345*). Iron is critical for bacterial growth yet we do not observe the activation of genes related to iron acquisition mechanisms. Instead, in group D28, we observed down-regulation of siderophore biosynthesis pathways and transporters (*BPS_RS09420-BPS_RS09475, BPS_RS25280, BPS_RS28840*), FtrABCD system (*BPS_RS20630-BPS_RSBPS_RS20645*), hemin transport (*BPS_RS19935-BPS_RS19940*) and FeS cluster (*BPS_RS12205-BPS_RS12240*).

Virulence factors associated with intracellular survival

B. pseudomallei infection in host involves initial attachment to enter into the host cell via endocytic vesicles followed by a replication process and eventually spread to other cells (2). We observed up-regulation of genes ranging from log2 fold change +1 to +4 involving fimbriae biogenesis, adhesion, flagellar associated genes (*BPS_RS19075BPS_RS19085, BPS_RS22875, BPS_RS23025, BPS_RS23650, BPS_RS19215, BPS_RS09545, BPS_RS09555, BPS_RS00130, BPS_RS01185, BPS_RS01205, BPS_RS01415*) and secretion systems in group D28 (Table S2). *B. pseudomallei* type 3 secretion systems (T3SS) are important for bacterial invasion and escape from endocytic vesicles (17). A number of genes of clusters: T3SS-1 (*BPS_RS26340-BPS_RS26435*) and T3SS-3 (*BPS_RS27000-BPS_RS27190*) were significantly up-regulated in group D28. *B. pseudomallei* encodes for six types of 6 secretion system (T6SS) that facilitates replication in the cytosol and spreading to neighbouring cells (27). Majority of the genes from T6SS clusters were significantly up-regulated in group D28 including several genes of tss-2 (*BPS_RS19090-BPS_RS19195*), tss-3 (*BPS_RS19495-BPS_RS19590), tss-4 (BPS_RS21490- BPS_RS21580*), tss-5 (*BPS_RS26870-26960), tss-6 (BPS_RS30235- BPS_RS30320*). The genes and log2 fold change were listed in Table S2.

Quantitative real time PCR (qRT-PCR) analysis

Independent analysis was performed by qRT-PCR on group D28 with reference to group D5 to assess and compare the relative expression of six randomly selected up-regulated genes with varying differential gene expression ranging from +1 to +7 on the log2 scale. The comparison between the log2 fold change of RNA-seq and qRT-PCR is shown in Figure 3. Four genes, *BPS_RS19075, BPS_RS27075, BPS_RS32200*

and *BPS_RS26895* encoding for a fimbriae-related chaperone, BsaZ, BimA and Hcp1 respectively, showed good relative gene expression compared to RNA-seq. Overall, the qRT-PCR results showed up-regulation in gene expression albeit of a different magnitude from RNA-seq.

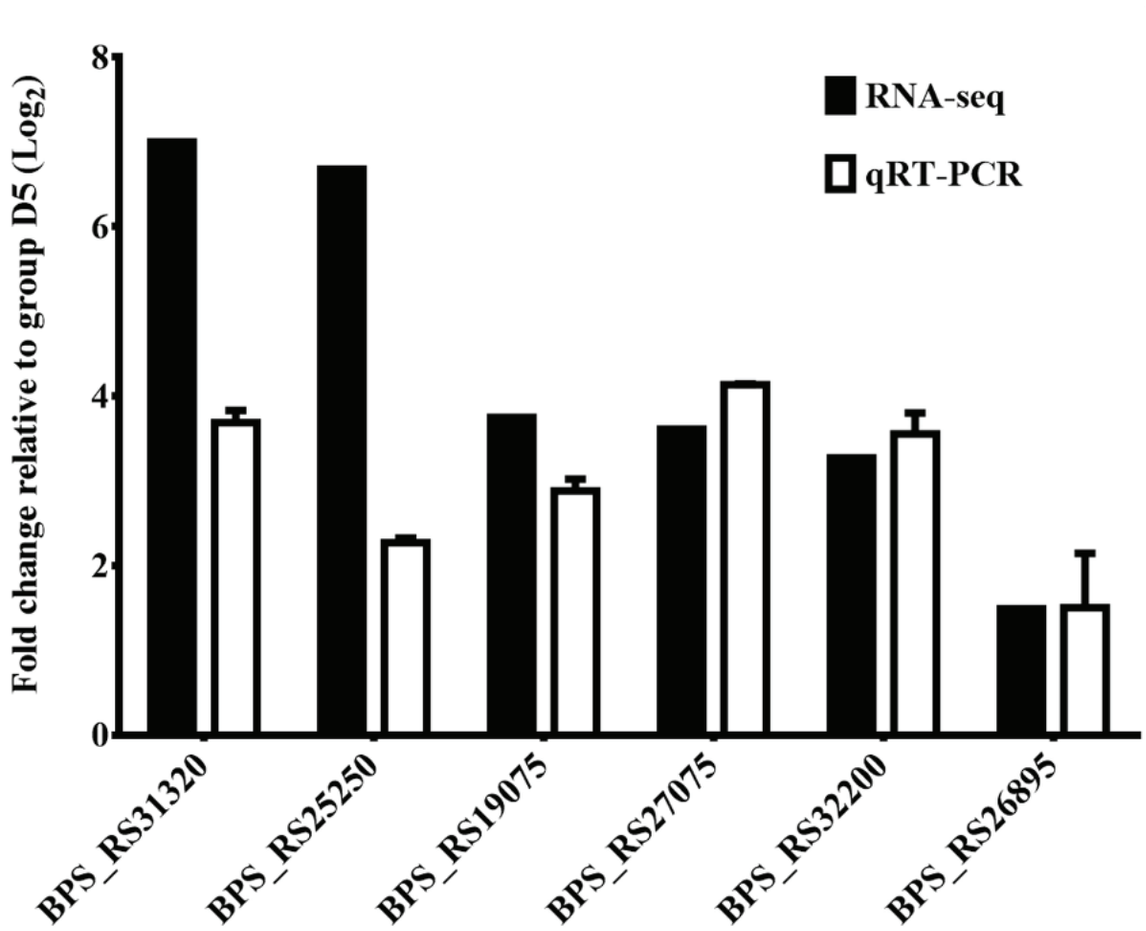

Figure 3. Verification of several up-regulated genes in RNA-seq data by qRT-PCR. Six genes encoding various proteins with different functions were assessed. Bars represent the gene expression level on a log2 scale for RNA-seq (black bar) and qRT-PCR (open bar). The data shown were after normalization with 16S rRNA and error bars indicate the standard deviations between two independent experiments.

DISCUSSION

The ability of *B. pseudomallei* to survive in unfavourable environments could have led to its persistence and dormancy in accidental hosts, humans. *B. pseudomallei* was observed to be viable in distilled water for more than 16 years (7) and in humans, relapse melioidosis still exists despite the completion of appropriate antibiotic therapy (5). Continuous investigation of this bacterium with a large genome, approximately 7.2 mb is crucial to explore its virulence factors and adaptations in various conditions. In this study, we investigated the transcriptional adaptation of *B. pseudomallei* at a later stage in HHS by RNA-seq. Both IMRS1 and IMRS2 survived in HHS over 28 days and had higher bacterial count at day 28 (0.3-1 x 1010 CFU/mL) than starting bacterial concentration (approximately 1x107 CFU/mL) (Figure 1). Survival of *B. pseudomallei* upon overnight incubation in HHS was also observed by DeShazer et al. (28), yet bacterial multiplication was only observed at 10-60% HHS (28). Therefore, a longer incubation period in HHS might give a different presentation as well as gene expression. We also observed that compared to chromosome 1 which encodes for the core functions, most of the differential expressions was observed in chromosome 2 which was reported to be responsible for accessory functions

including adaptation to environmental conditions (2). Further validation by qRT-PCR on six randomly selected genes with different expression level analyses reflected comparable gene expression patterns to RNA-seq despite smaller magnitude.

Isolation of mixed colony morphotypes from clinical specimens were not uncommon and colony switching could occur during infection or under nutritional starvation (29). We also observed the emergence of several colony morphotypes during incubation in HHS after D5 (data not shown). Colony switching was associated with variation in metabolic activity, intracellular and extracellular survival, replication fitness, virulence and persistence (29-31). Correspondingly, the transcriptome analysis showed that prolonged incubation in HHS greatly affected the expression of surface determinant genes. Surface polysaccharides which consist of CPS and LPS are the first barrier to act and protect bacteria from hostile surroundings. However, alteration in CPS or LPS may affect the deposition of complement factor c3b on the bacterium surface therefore it leads to ineffective phagocytic killing and persistence in serum (13,32). We observed that CPS II and III were overexpressed in this study. These gene clusters were described to enhance survival rate in immunized mice (27) but Reckseidler-Zenteno et al. (33) showed that CPS III was being suppressed in 30% human serum (33). This discrepancy could be due to the variation in experimental procedures whereby different bacterial strains, higher serum concentrations and longer duration of exposure were applied in this study. CPS I and LPS operons which had been associated with serum resistance (13,33) were instead down-regulated in this study. In addition, we observed an increased expression of a putative outer membrane porin (*BPS_RS09155*) and glycosyltransferase (*BPS_RS31115*) which were found to resist serum killing (34).

Surprisingly, we observed a lack of activation and repression of iron acquisition which is crucial for bacterial growth and survival (32,35,36,37). We found contrasting results indicating prolonged incubation in HHS possibly does not induce an iron-limiting environment or iron may not be required to maintain viability in HHS for a longer period. The F-type ATP synthase operons are involved in ATP-dependent bioenergetic processes and are available in both chromosomes 1 and 2. In this study, the F-type ATP synthase operon of chromosome 2 is utilized, possibly to counteract oxygen depletion in HHS incubation, a phenomenon also observed during the anaerobic condition and short ultraviolet light irradiation. Likewise, the other F-type ATP-synthase operon on chromosome 1 was either repressed or not significantly differentiated (9,37,38). However, Ooi et al. (9) observed the repression in both operons upon prolonged anaerobic challenge until two weeks and nutrient starvation (9). The operon at chromosome 2 acts as a highly efficient proton pump and was described to assist bacterial survival in hostile conditions, for instance, acidic environments in phagosomes (39). Opposed to our result, in the oxidative stress challenge, the bacteria up-regulated F-type ATP synthase of chromosome 1 instead of chromosome 2 (9,10). These contradicting results affirm that *B. pseudomallei* is able to alternate different mechanisms under varying challenges and the duration of exposure is also believed to contribute towards the outcome pattern.

Although bacterial virulence may be compensated for longer survival in the host, we observed up-regulation of several T3SS and T6SS-related genes which are important for bacterial intracellular survival and pathogenicity. Increased expression of bsaZ could lead to delayed phagosome escape and intracellular survival (17). T6SS clusters were enriched except for tss-1. Of them, T6SS-5 cluster act as a virulence determinant to mediate cell-cell fusion and form multi-nucleated giant cells, a hallmark of B. pseudomallei infection. The induction of tss-5 had been reported during macrophage invasion and intercellular spread (15,40). Up-regulation of T6SS clusters (tss-3, tss-4) and T3SS-3 were also observed under conditions of nutrient starvation (9). In addition, tss-4 coupled with TseM were found to play an antioxidant role and this mechanism was observed to be negatively-regulated by oxidative stress regulators (14,41). This is coherent with our observation that these genes including sigma factors RpoE (*BPS_RS13045*) and RpoS (*BPS_RS27150*), KatG-DpsA operon (*BPS_RS15365-BPS_RS15360*), KatB (*BPS_RS24130*), AhpC (*BPS_RS21370*), OxyR (*BPS_RS15370*) and SodC (*BPS_RS05275*) were either down-regulated or not significantly differentiated (2,10). Another interesting finding was that genes repressed under oxidative stress were highly up-regulated in our study (10). These divergences suggest that *B. pseudomallei* may activate an alternative mechanism to combat possible oxidative damage, for example, T6SS, however, further validation is required.

The survival of *B. pseudomallei* in HHS upon the end of the experiment could be affected by the up-regulation of QS mechanism particularly in AHL signalling that plays a crucial part in secondary metabolism

(26,42,43). Over expression of oxalate (*BPS_RS26130*) by AHL-QS observed in this study may help to neutralize the increasing surrounding pH due to the excretion of metabolism by-product during bacterial growth (44). QS-regulated bactobolin may also be toxic to host protein translation and its disruption resulted in reduced growth competition of *B. thailandensis* to *C. substugae* (45). The activation of these genes was also observed in *in vivo* study from relapse isolates of melioidosis patients (CF6, CF9, CF11) (46) but down-regulated during oxygen and iron deficiency, oxidative and heat stress (9,10). Syrbactin and malleipeptin which act as proteasome inhibitors and biosurfactants, may aid in bacterial attachment and invasion (42). Collectively, these suggest that the production of secondary metabolites is commonly observed as bacteria adapt in a diverse environment, but is not limited to specific set of gene. There are still uncharacterized or putative secondary metabolites in *B. pseudomallei.* It is important to note that the serum and human immune system are not static and therefore the data comparing day 5 and day 28 may not be entirely extrapolated. However, the outcome of this study could provide some insights into the possible mechanisms involved in *B. pseudomallei* survival and persistence in human sera mimicking a condition of chronic infection. Nevertheless, the usage of a low number of isolates as well as the extensive possible variations present under both *in vitro* and *in vivo* conditions remains the main limitation of this study that should be further investigated.

CONCLUSION

In summary, *B. pseudomallei* is able to persist and survive in HHS for 28 days by alteration of its gene expression mainly of the accessory genome. Interestingly, the bacterial count remained higher than the starting inoculation number until the end of the experiment. The genes that are affected involve alteration of surface determinants especially CPS II and CPS III, activation of ATP synthase operon, AHL-QS systems-mediated secondary metabolites and secretion systems. Overall, *B. pseudomallei* exhibits a multifaceted transcriptomic profile under various conditions and there may be more possible mechanisms for this versatile bacterium to be further explored.

ETHICAL DECLARATIONS

The GenBank accession numbers for strain K96243 are NC_006350.1 and NC_006351.1. The reads generated from this study have been deposited in European Nucleotide Archive with accession number PRJEB49969. The sample accession numbers are ERR7961951, ERR7961952, ERR7961953 and ERR7961954 for IMRS1D5, IMRS1D28, IMRS2D5 and IMRS2D28, respectively. Table S1 and S2 have been deposited in NIH-Data Repository System (NIH-DaRS).

CONFLICT OF INTERESTS

The authors declare that they have no competing interests. This work was supported by the Ministry of Health Malaysia Research grant [NMRR-15-1618-27725 and NMRR-17-505-35327]. NA and SHYF designed and coordinated the project. FA, RH and SHYF provided the samples and reagents. SHYF performed the experimental procedures. SHYF and MKN analysed and interpreted the transcriptome data. SHYF wrote the manuscript. NA, RH and MKN reviewed and revised the drafts of the manuscript. All authors have read and approved the manuscript.

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