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Biomarker discovery in the biofilm-forming process of *Burkholderia pseudomallei* by mass-spectrometry

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ABSTRACT

A serious human infectious disease called Melioidosis is a result of *Burkholderia pseudomallei* infection. Treatment for infected individuals is difficult due to a wide range of ineffective antibiotics including a high level of antibiotic tolerance which has been known to be caused by biofilm production. However, biofilm forming processes of this bacterium are not well documented despite multiple-methodologies being applied. In this study, we utilized a proteomics strategy called whole cell matrix-assisted laser desorption ionization-time of flight mass spectrometry (whole cell MALDI-TOF MS) to discover a potential biomarker relating biofilm forming in *B. pseudomallei*. The results presented a novel specific type of enzyme amylo-alpha-1, 6-glucosidase, which was demonstrated by a higher level of gene expression during the biofilm development. Our results also suggested a list of candidate markers that might be involved in this scenario. Eventually, this knowledge may expand valuable data to the biofilm study that may increase effective treatments for people infected with *B. pseudomallei* and possibly other antibiotic tolerant bacteria.

1. Introduction

Burkholderia pseudomallei is a gram-negative, aerobic, motile, bacillus, which is the causative agent of the severe human infectious disease called Melioidosis. The clinical symptoms are often associated with pneumonia and bacterial dissemination to distant sites such as skin, the genital system, bone, liver and spleen (Wiersinga et al., 2006). Although there is a possible treatment to cure infected people, the wide range of ineffective antimicrobials such as macrolides, cephalosporins, penicillin, colistin, rifamycins, ceftazidime and the unavailability of vaccination for this bacterial species make treatment and prevention of this disease unreliable (Wiersinga et al., 2006; Chantratita et al., 2011). In addition, the biofilm-forming capacity of *B. pseudomallei* production was previously reported (Vorachit et al., 1995), and is another factor that leads to less bacteria eradicated. This situation occurs because phagocytic cells are interfered with by the extracellular polymeric substances of biofilm cells (Taweechaisupapong et al., 2005). Besides this scenario, biofilm cells are also the cause of antibiotic treatment failure (Chantratita et al., 2011; Sawasdidoln et al., 2010). They can be more tolerant of antimicrobial agents by approximately 100 to 1000 times depending on the ability of biofilm production when compared with their free-living (planktonic) state (Gilbert et al., 1997; Mongkolrob et al., 2015). Thus, the study of biofilm formation has been of interest throughout the past decade. A recent study by Chantratita and coworkers has found a possible relationship of multiple colony appearances and their biofilm productivity levels (Chantratita et al., 2007). They have shown that the type II morphology, out of seven types, exhibited the highest biofilm production. However, the observation in some cases of their biofilm-forming capacities, in the different levels of productivity, did not relate to colony characteristics.

Consideration of a new technology of data acquisition of whole cell matrix-assisted laser desorption ionization-time of flight mass spectrometry (whole cell MALDI-TOF MS) is currently useful to identify microorganisms by measurement of their unique molecular fingerprints (Sauer et al., 2008; Seibold et al., 2010; Cherkaoui et al., 2011; Ferreira et al., 2011; Boggs et al., 2012). Moreover, the technique is also widely used to discover protein markers that are expressed in different situations such as finding resistant peptide markers among bacterial strains (Lu et al., 2012). MALDI-TOF MS also is a method of choice using molecular separation of biomolecules by differential mass. In some cases this method has found that the useful mass range is in the range of 500–15,000 Da (Tanaka et al., 1988).

Here, we aimed to utilize the application of whole cell MALDI-TOF MS to discover a metabolic polypeptide that involves biofilm

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development. Our modified strategies are based on 4 combination criteria (i) the clear mass spectral peaks ranging 2–20 kDa (ii) the Quick Classifier Algorithm (QC) software (iii) the utilizing a biofilm defective strain for comparison and (iv) the setting up criteria of protein candidates involved in biofilm function. In addition, this study provides further information of candidate proteins in biofilm formation and extends the usage of MALDI-TOF MS in the area of microorganism research.

2. Materials and methods

2.1. Bacteria strains and growth conditions

Two clinical isolated bacterial strains, *B. pseudomallei* 354 and *B. pseudomallei* U2704, were used in this study which were obtained from Melioidosis Research Center (Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand). The 354 strain was classified as a high biofilm-producing isolate whereas the U2704 strain was categorized as a low biofilm-producing isolate. They were examined by both conventional crystal violet staining modified from Stepanovic and Merritt (Stepanovic et al., 2000; Merritt et al., 2005) and bacterial colony morphology described by Chantratita (Chantratita et al., 2007). In general, the bacteria were grown and stored on Ashdown's agar plate. In case of biofilm inducing conditions, both strains of *B. pseudomallei* were activated prior to 1% volume per volume inoculation of these cells into 2.5 ml of minimal induced biofilm medium modified by Vogel and Bonner's medium (MVBM) or Luria Bertani (LB) medium as indicated in each experiment. Both culture media were supplemented with 4 μg per ml final concentration of gentamycin. All cultivated cells were aerobically incubated with no shaking at 37 °C and were harvested as indicated in each experiment.

2.2. Whole cell preparation for MALDI-TOF MS

2.2.1. Sample preparation for MALDI-TOF MS analysis

Cultivated bacterial cells in both LB medium and MVBM medium were harvested every 8 h up to 48 h. At each time point, the cell concentration used was approximately 0.2 of absorbance value per 1000 μl. The cultivated cells were harvested by centrifugation at 12000 rpm for 2 min. Then, cells were added with 1 ml of 70% ethanol for sterile bacteria. After cell inactivation, the samples were concentrated by centrifugation at 12000 rpm for 2 min. Supernatant (10 μl) was aspirated and re-suspend in a clear solution. The 5 μl of remaining cell suspensions were then transferred into 200 μl of 0.1% *w/v* sinapinic acid matrix solution, which was composed of 10 mg of sinapinic acid solubilized in 1 ml of solubilized solution containing 50% *v*/v acetonitrile and 2.5% v/v trifluoroacetic acid. The samples were vigorously mixed by pipetting for 2 min. The 2 μl of mixed solution were then spotted onto a MTP 384 ground steel plate (Bruker Daltonik, Germany). The samples were dried in air prior to analyzing by MALDI-TOF MS.

2.2.2. MALDI-TOF MS parameters

In order to evaluate the mass spectral profiles of *B. pseudomallei* cultivated cells, MS profiling spectra were collected from an Ultraflex III MALDI-TOF mass spectrometer (Bruker Daltonik, Germany). Each of the samples were assayed in 29 replications. Otherwise, another one spot of *Escherichia coli* DH5α was included in each set of samples, which were used as external control for MS machine operation. The standards contained in the ProteoMass™ peptide & protein MALDI-TOF MS calibration kits (Sigma-Aldrich, St. Louis, MO) were used as external calibrators including: ACTH fragment, 2465.2 Da; Bovine insulin oxidized β-chain, 3494.7 Da; Bovine insulin, 5735.0 Da; Cytochrome C, 12362 Da; and Apomyoglobin, 16952 Da. The FlexControl software of the MS instrument was set as default parameters recommended by manufacturer. MALDI spectra were acquired in a linear mode. Five hundred 50 Hz laser shots were collected and peaks representing a mass of 2 to 20 kDa were measured.

2.2.3. Data visualization and statistical analysis

Mass spectra profiling was visualized and statistical analysis performed as standard protocol by the FlexAnalysis and ClinProTools™ data processing software (Bruker Daltonik, Germany). Briefly, all spectra were normalized to their own total ion count (TIC). The data was subtracted from the base line and the resolution adjusted to 800 ppm. All spectra profiles were presented in gel-view and mass intensity by color gradient represented an individual mass. Furthermore, a modified statistical procedure was applied to analyze the spectral profiles by the command function 'Peak Statistics'. It included spectra averaging and peak calculation of the loaded data. All spectral profiles were also analyzed with the 3D scatter plot of the Principal Component Analysis (PCA) to determine the similarity and difference of bacterial profiles. Quick Classifier algorithm was also applied to indicate the candidate biomarker. The statistical selection under this command was analyzed by Wilcoxon/Kruskal-Wallis for peak differentiation.

2.3. Proteins database searching

A list of proteins was searched from Protein Knowledgebase (UniProtKB database) at http://www.uniprot.org/uniprot/. The single parameter in the query search was the organism '*Burkholderia pseudomallei*'. The experimentally determined molecular masses (observed masses) of the candidate proteins were matched with theoretical protein masses. If none of the theoretical protein masses from the database could be matched, the experimental protein mass was conducted to search with a theoretical mass of observed mass ± 3 Da instead, which corresponded to a modification of the protonated experimental molecule (Demirev et al., 1999; Ryzhov and Fenselau, 2001).

2.4. Quantitative RT-PCR for validation of gene expression

The *B. pseudomallei* 354 and the U2704 strains were grown for 24 h in both MVBM medium and LB medium representing biofilm lifestyle and planktonic lifestyle, respectively. Cultivated cells were harvested and RNA extracted using Trizol reagent (Invitrogen, California, USA). Following the purification procedure, extracted RNA was treated with RNase-free DNase (Promega, Medison, USA) according to recommended procedure prior to quantification of gene expression with two steps qRT-PCR.

The first step of the RT-PCR was conducted as a singleplex reverse transcription in a total volume 20 μl. Amylo F/R and 23 s F/R (Table 1)

Table 1
Primers used for PCR, RT-PCR and qRT-PCR reactions.

Target	Primer	Sequence (5′ – 3′)	Amplicon size (bp)
Amylo-alpha-1,6-glucosidase DNA/RNA (this study)	Amylo-F	ATT CCG GTG TCA CGC TTT ATC	163
	Amylo-R[a]	GAT GTC CAC AAG GTT GCC AAC	
23 s rDNA/23 s rRNA (described elsewhere)	23 s-F	CGA ATG GGG AAA CCC GGC CC	198
	23 s-R	GGC CGC ACT TTC CAG AGC GT	

[a] Remark Some nucleotide base of Amylo-R primer was transversionally changed from the template. The primer was designed to substitute C to G at position 13 (5′ → 3′) in order to prevent the secondary structure formation.

were used as RT-PCR primers for quantitative expression of the candidate gene and the internal control. The reaction contained 500 ng RNA, 1× ImProm-II™ Reaction Buffer (Promega, Medison, USA), 3.0 mM $MgCl_2$, 0.5 mM dNTP, 1 µl of ImProm-II™ Reverse Transcriptase enzyme (Promega, Medison, USA), and 1 µM of gene-specific primers (Table 1). In addition, two reactions of negative control (no RNA template and no RT enzyme) were included for quality control. The reaction cycling was programmed according to the manufacturer's recommendations.

The qRT-PCR reaction mixture was performed in a total volume of 10 µl. The real-time mixture was composed of 2 µl of 100× diluted cDNA, 0.1 µM of gene-specific primers (Table 1), 1× ROX reference dye low (KAPA Biosystems, Massachusetts, USA) and 1× of KAPA SYBR FAST qPCR Master Mix (KAPA Biosystems, Massachusetts, USA).

Real-time PCR amplification and detection were performed with the Stratagene Mx3000P cycler (Agilent Technologies, California, USA) with the following parameters: (i) one cycle of 95 °C for 2 min, (ii) 40 cycles of 95 °C for 30 s, (iii) 60 °C for 30 s, (iv) 72 °C for 30 s. The quality of amplification was determined using the default settings of the qPCR machine for the detection of fluorescent products and the melting curve analysis. The Real-time amplification results were evaluated and presented in the $\Delta\Delta C_t$ calculation, which represented the comparative quantitation of amylo-alpha-1,6-glucosidase gene per 23 s rRNA and compared the expression level among the bacterial strains. Calculation also used some samples within the indicated experiment as a calibrator. Standard calculation formula was used from the comparative Ct method or ΔΔCt method as described by the manufacturer's recommendations (Applied Biosystems).

3. Results

3.1. Comparative biofilm productivity of the two bacterial strains

The ability of biofilm production between the two bacterial strains was confirmed by a conventional method using crystal violet; while the colony morphology method was difficult to confirm compared to the reference type II morphology. These results led us to search for a suitable and more reliable method for comparing biofilm productivity.

3.1.1. Mass spectral profiles from whole cell MALDI-TOF MS

We therefore attempted to use a whole cell MALDI-TOF MS technique starting with monitoring the time-course development of spectral profiles within two culture media, LB and MVBM medium. After bacterial mass spectral profiles were graphed, both of the *B. pseudomallei* strains (U2704 and 354) grown in LB medium were detected in increasing protein expressions through the monitoring times as shown in Fig. 1A and B. These might indicate the changing stages of protein expressions during bacterial growth and development in LB medium. Unlike the above profiles, the bacterial growth in MVBM medium represented different results that exhibited stepwise cyclic behavior, depending on the incubation times (Fig. 1C and D). At the incubation times of 8, 24 and 40 h, spectral patterns detected quantifiable peptide expression, whereas undetectable peptide expression was observed at 16, 32 and 48 h. This characteristic might refer to developmental processes of biofilm formation.

3.1.2. The principle component analysis (PCA)

The stepwise spectral profile and its dynamic pattern found in MVBM induced biofilm medium might be related to metabolic development of biofilm formation. We therefore applied another function of ClinProTools™ software called Principle Component Analysis (PCA) to plot data in 3D scatter for comparing the scattering profiles of the two bacterial strains in MVBM induced biofilm medium. As expected, the results gave different grouping profiles. The data of *B. pseudomallei* U2704 were clumped except at 8 h of cultivation (Fig. 2A), while the 354 strain showed a more distributed scattering profile especially at 8, 24, and 40 h (Fig. 2B). This suggested that the profiles of *B. pseudomallei* 354 are more statistically different from *B. pseudomallei* U2704. Thus, the 354 strain was chosen to be used in further analysis.

3.2. Protein and mass database searching

After determination of the 354 strain as the appropriate strain, peptide mass candidates were selected from this strain using PCA analysis. The specific-profiling mass spectra were then obtained as shown in Fig. 3. To indicate the protein marker, however, it was not possible to choose the mass directly from gel-view or profiling mass spectra. Accordingly, an analysis by Quick Classifier algorithm was applied to reveal the candidate markers as summarized in Table 2. Candidate proteins were identified by using the experimental masses as search parameters mentioned in the method. The results of the database protein search, which matched the observed mass at approximately 2000 to 9665 Da of the settled mass criteria, could be responsible for more than a single protein type (Table 2). However, the observed peak at 7177.81 Da corresponding to amylo-alpha-1,6-glucosidase (7174 Da; UniProtKB/TrEMBL accession number **A4LI80**) was picked up as the first acceptable protein candidate.

3.3. Quantitative RT-PCR for validation of gene expression

From the above analysis an amylo-alpha-1,6-glucosidase was determined to be a possible candidate. However in the NCBI protein database only *B. pseudomallei* 305 possessed this protein in the coding genome. Therefore, it was necessary to verify an existence of this gene in the genome of the 2 experimental strains (354 and U2704) by PCR technique prior to validation of the gene expression by quantitative RT-PCR. The amplification results of genomic DNA presented an amplicon size at 163 bp in both strains, indicating the existence of the gene on both bacterial genome (data not shown). Consequently, the DNA sequencing of both amplification products were confirmed with 96.9% sequence identity compared to the *B. pseudomallei* 305 reference strain (data not shown).

Apart from genomic DNA verification, a qRT-PCR was performed to determine the transcriptional levels of the discovered marker. Moreover, a comparison of gene expression was conducted in different media, which represented the planktonic state (LB medium) and biofilm state (MVBM medium) as shown in Fig. 4. When quantitative expressions of an amylo-alpha-1,6-glucosidase gene were calculated, the transcriptional level in MVBM medium were higher by approximately three-fold and one-fold more than LB medium found in the 354 and the U2704, respectively (Fig. 4). This finding indicated that amylo-alpha-1,6-glucosidase expression is required during biofilm development and that is related to biofilm production capacity.

To ensure an amylo-alpha-1,6-glucosidase expression profile relating to the observation of cyclic behavior found in the mass spectral profiles when cultivated cell was grown in MVBM medium and harvested in each period. Transcriptional level of an amylo-alpha-1,6-glucosidase of the 354 strain at 8, 16, 24, 36, 40, and 48 h were determined by qRT-PCR and calculated as shown in Fig. 5. Surprisingly, it did not show a stepwise increase of gene expression as expected. In contrast, the expression profile trends increased at all time-points except at 16 h where a reduction was observed.

4. Discussions

Biofilm formation in some microorganisms has been studied for more than a decade (Flemming et al., 2007; Monds and O'Toole, 2009) in *Pseudomonas aeruginosa* (Friedman and Kolter, 2004) *Escherichia coli* (Zogaj et al., 2001) and *Staphylococcus pneumoniae* (Allegrucci et al., 2006), but the formation of matrices in biofilms is not well understood. To address this question, gel electrophoresis was applied (Orme et al., 2006), but it did not reveal the candidate for biofilm formation.

Here, we proposed a variation of MALDI-TOF MS for the discovery

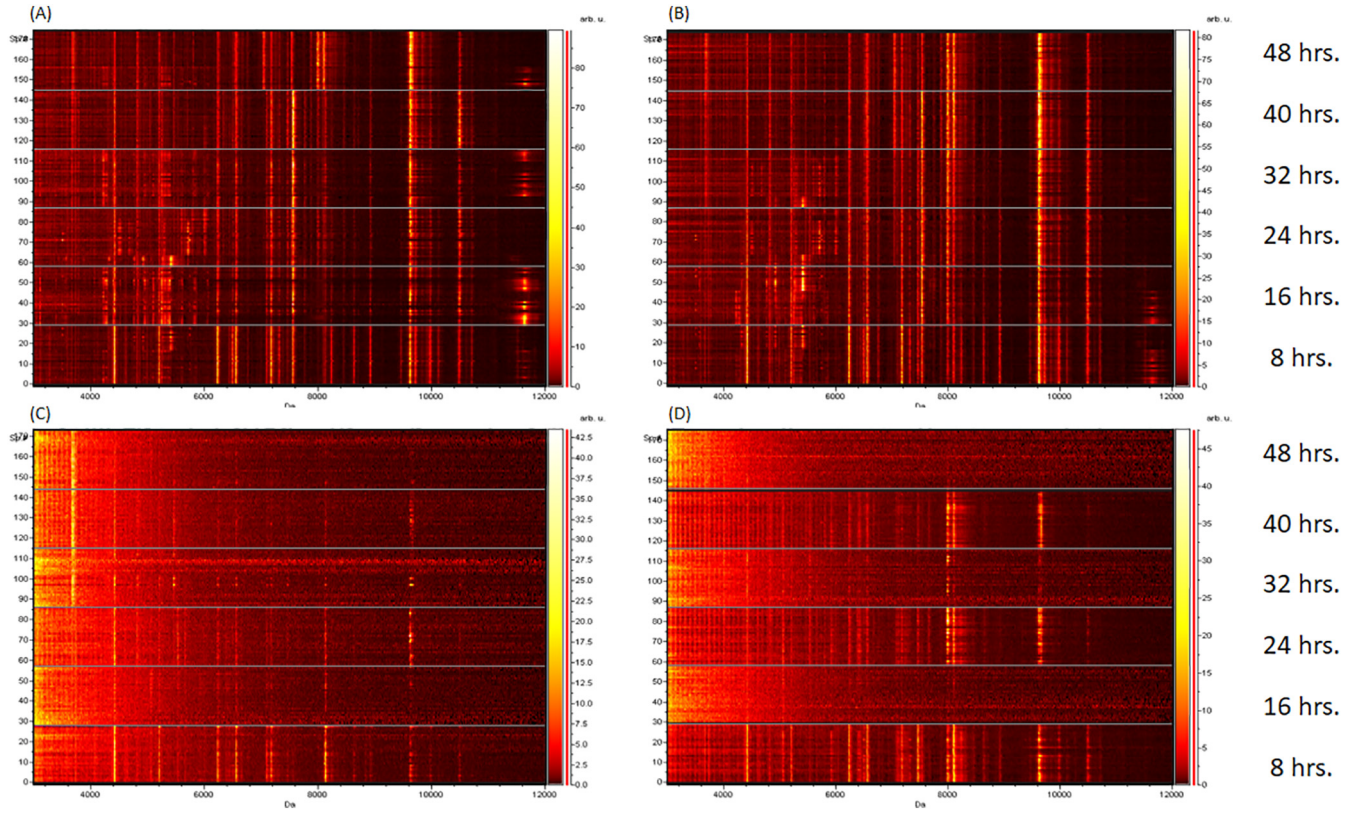

Fig. 1. Mass spectral profiles of the two *B. pseudomallei* strains cultivated in different media at various times; [A] the U2704 strain cultivated in LB and [C] in MVBM, [B] the 354 strain cultivated in LB and [D] in MVBM. Bacterial mass spectral profiles obtained from MALDI-TOF MS were monitored from 8 h up to 48 h. Each sample was analyzed with 29 replicative assays.

of biomarkers involved in biofilm-matrix formation called whole cell MALDI-TOF MS, which expand on its use as a tool for identifying *B. pseudomallei* (Sogawa et al., 2011; Lau et al., 2012; Inglis et al., 2012; Niyompanich et al., 2014; Niyompanich et al., 2015). It may also be useful as a tool for identifying other microorganisms. This technique the advantage of being able to analyze very small molecules to very large molecules (Inglis et al., 2012). Although, the biofilm-matrices consist of many types of biomolecules such as polysaccharides, proteins, glycolipids and nucleic acids, the technique also has a high separation capacity and analysis power based on the mass variation of analyzed molecules. With the utilization of this methodology for analysis of molecular masses between 2 and 20 kDa, it is possible to identify novel protein candidates involved in biofilm-matrix formation.

Interestingly, mass spectral profiles found in MVBM medium exhibited stepwise cyclic behavior (Fig. 1C and D). This information suggested that the biofilm development cycle of *B. pseudomallei* had completed their cycle within 24 h. This discovery was also partially supported with a previous study in biofilm metabolic measurement of *Pseudomonas sp.* (Bester et al., 2010). Specifically, the recovery level of CO_2 production is returned to its pre-perturbation levels within 24 h. We therefore suggested that the stepwise protein expressions found in each period are the proteins required for each biofilm development cycle. Although Sauer and coworkers have claimed in their report that the complete cycle of biofilm formation being 12 days (Sauer et al.,

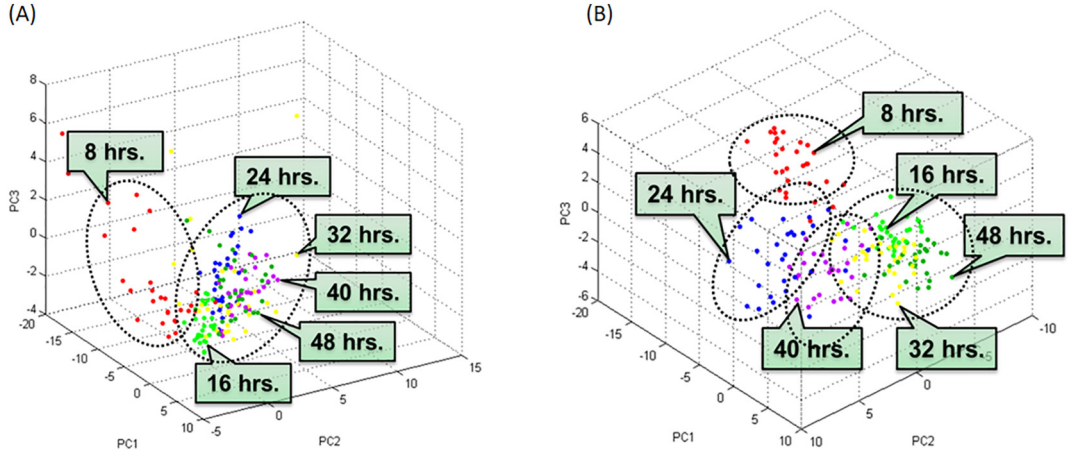

Fig. 2. Principle Component Analysis (PCA) between the two *B. pseudomallei* strains, [A] the U2704 strain and [B] the 354 strain cultivated in MVBM biofilm inducing medium at various times monitored from 8 h up to 48 h. The same color dots present the evaluated profiles from each 29 replicative assays.

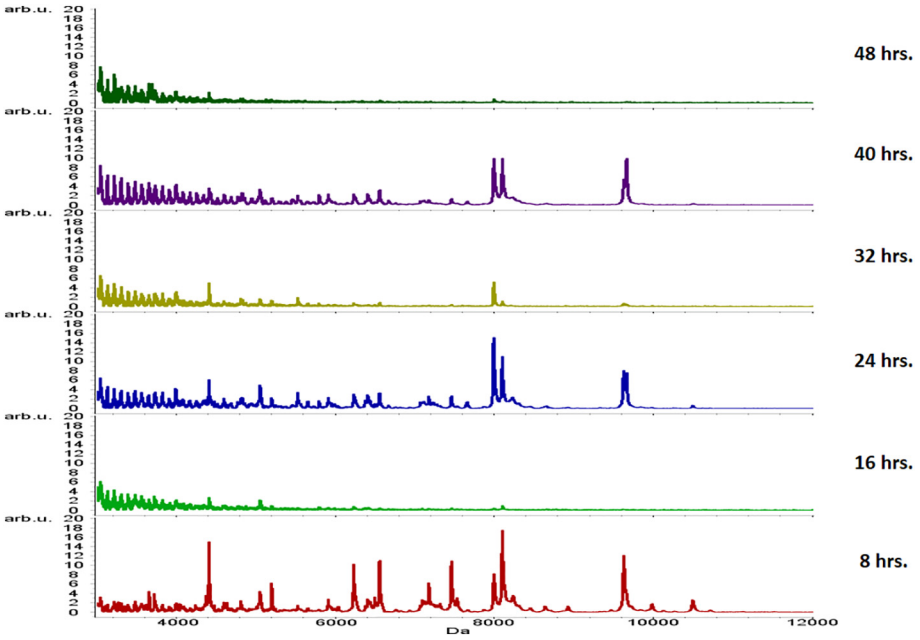

Fig. 3. Average specific-profiling mass spectra of the *B. pseudomallei* 354 cultivated in MVBM biofilm inducing medium at various times from 8 h up to 48 h. Amount of cell was equally adjusted prior to MALDI-TOF MS analysis.

2002), however, their results were gained from visualization under microscopic observation. It may be interesting to investigate further if both evidences might be true depending on what levels are being focused on.

Obviously the 3D plot of Principle Component Analysis (PCA) for grouping the mass profiles (Fig. 2) indicated significantly different levels of overall protein expression at each time point between the 354 and the U2704 strains. This confirms the U2704 strain produced less biofilm, because correspondingly there was less protein expression when compared with the higher biofilm production of the 354 strain. Thus, the *B. pseudomallei* 354 strain was chosen to identify protein markers for further analysis.

Following the PCA analysis, the Quick Classifier Algorithm (QC) software was used to identify the mass candidates. In detail, it was recommended by the manufacturer, who claimed that this algorithm reported the mass candidates by statistical calculation at certain peak positions. Consequently, a protein name search was conducted in UniProtKB/TrEMBL protein database. However, this methodology was not able to identify the markers by automatic search because it does not have the function of protein database for protein indication by mass search. Moreover, the theoretical mass might not directly correspond to the observed mass since it might have a covalent attachment via a thiol ester linkage of 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid or SA), which is recommended for protein analysis by MALDI-TOF MS, to cysteine-containing protein (Fagerquist et al., 2010). Therefore, protein marker identification should be searched in wide-range prior to choosing the candidate by interested function. Nevertheless, we conducted a search with the theoretical mass ± 3 Da instead, which was recommended by previous studies (Demirev et al., 1999; Ryzhov and Fenselau, 2001).

Table 2 showed that there was more than a single protein type corresponding to the same experimental mass. In the list, there were some interesting proteins such as acetate permease and outer membrane porin *Opc*P, but these two proteins have less supporting evidence. Based on the functional criteria, the observed peak at 7177.81 Da corresponding to amylo-alpha-1,6-glucosidase (7174 Da; UniProtKB/TrEMBL accession number **A4LI80**) was therefore chosen to be the first acceptable protein candidate. According to our hypothesis, the candidates have to be involved in biofilm formation. Apart from the amylo-alpha-1,6-glucosidase candidate, the others within ± 3 Da having corresponding functions, such as hexapeptide transferase family protein (7175 Da; from *B. pseudomallei* Pakistan 9 BUH. Contig 270) and integrase catalytic region (7176 Da; *B. pseudomallei* MSHR 346). Nonetheless, there were some interesting points that need to be verified. We therefore verified the gene existence and the level of gene expression of all three candidates. The results demonstrated that an integrase catalytic region did not appear on *B. pseudomallei* 354's genome as well as *B. pseudomallei* U2704's genome (data not shown), whereas a hexapeptide transferase family protein was not consistently expressed in both strains (data not shown). To confirm this our results showed a consistent level of amylo-alpha-1,6-glucosidase mRNA expression (data not shown) and presence in the genome of both strains. We further analyzed the gene encoding of amylo-alpha-1,6-glucosidase from PCR amplification products by DNA sequencing analysis. The results illustrated more than 96.9% DNA sequence identity between our experimental strains and the target model, *B. pseudomallei* 305 from the genome database. Currently, only a single strain *B. pseudomallei* 305 from genome database reports this gene coding sixty-five amino acid residues. Our findings strongly suggests the existence of the amylo-alpha-1,6-glucosidase gene in both the *B. pseudomallei* 354 and the *B. pseudomallei* U2704 genomes.

In order to compare the expression of the candidate protein in two states, planktonic and biofilm, the results demonstrated that an amylo-alpha-1,6-glucosidase was highly expressed and required during biofilm formation and its gene expression in the biofilm was higher than the planktonic lifestyle (Fig. 4). Moreover, both candidates the level of gene expression in biofilm and the overall level of biofilm capacity quantified by conventional crystal violet staining were had the same trend (data not shown). Obviously, the 354 had higher gene expression and biofilm capacity than the U2704 strain. Evidence was also presented that there were relationships between the level of gene expression and the level of biofilm production, which was indicated by the MALDI-TOF MS spectral intensity (Fig. 3). These results presented approximately three-fold difference once we compared the results from the two experimental strains, *B. pseudomallei* 354 and *B. pseudomallei* U2704, (data not shown). Consequently, we also monitored the level of amylo-alpha-1,6-glucosidase gene expression during the growing stages from 8 h until 48 h of incubation. Unexpectedly, the expression pattern did not present the cyclic behavior (Fig. 5). This result suggested that an amylo-

Table 2
Candidate proteins masses using parameter in query search of *B. pseudomallei* database.

Observed mass (Da)	Database mass (Da)	Protein names	Observed mass (Da)	Database mass (Da)	Protein names
4413.15	4410	50S ribosomal protein L36	7099.15	7102	Aminoglycoside acetyltransferase (6) type I (EC 2.3.1.82)
5055.60	5056	Acetate permease	7177.81	7174	Amylo-alpha-1,6-glucosidase
5055.60	5058	Conserved domain protein	7177.81	7175	Hexapeptide transferase family
5913.33	5911	Transcriptional regulatory protein	7177.81	7176	Integrase catalytic region
5913.33	5916	Type III secretion protein HrpB4	7177.81	7180	Conserved domain protein
6232.73	6230	Transposase, mutator type	7177.81	7180	Outer membrane porin OpcP
6409.79	6408	Phage tail completion protein	9631.02	9632	Transcriptional regulator, LysR family
6559.00	6559	Plasmid pRiA4b ORF-3 family protein	9664.84	9662	Alcohol dehydrogenase
7099.15	7101	CheY-like receiver	2184.48; 2357.50; 2922.29; 6379.89; 6439.40; 6539.31; 7072.97; 7115.84; 7277.63; 7463.16; 7995.86; 8102.81; 8203.64; 8234.73; Hypothetical protein		
7099.15	7101	Ribonuclease E			

Fig. 4. A comparison of quantitative amylo-alpha-1,6-glucosidase gene's expression in different cultivation media between LB (enrich media) represented the planktonic stage and in MVBM (biofilm media) represented the biofilm stage. An analysis was amplified by amylo F/R and 23 s F/R primers prior to the ΔC_t calculation. The sample of *B. pseudomallei* enrich media was set as the calibrator for $\Delta\Delta C_t$ calculation.

Fig. 5. A transcriptional level of an amylo-alpha-1,6-glucosidase of the *B. pseudomallei* 354 strain in MVBM biofilm inducing medium at various time from 8 h until 48 h of incubation. An analysis of qRT-PCR was amplified by amylo F/R and 23 s F/R primers prior to the ΔC_t calculation. The sample at 8 h was set as the calibrator for $\Delta\Delta C_t$ calculation.

alpha-1,6-glucosidase gene is important and has to function in all stages of biofilm processes. From this evidence, an amylo-alpha-1,6-glucosidase was therefore a novel metabolic polypeptide candidate in our discovery.

Although, this particular enzyme has been well-studied for its functions, for example glycogen storage disease type III in humans which resulted from the mutation of AGL gene (abbreviation for amylo-alpha-1,6-glucosidase, 4-alpha-glucanotransferase gene) (Goldstein et al., 2010) and hydrolysis activity of alpha-1,6-glucosidic linkage to produce alpha-glucose in *Streptococcus mutans* (Hondoh et al., 2008). In general, the protein family alpha-glucosidase, has been mentioned as one of the biofilm-matrix components in various natural biofilm types such as river biofilms, sewer biofilms, stream sediment biofilms, lake sediment biofilms, waste-water biofilms, marine aggregates biofilms and activated sludge (Rendueles et al., 2013). It is implied that the enzyme amylo-alpha-1,6-glucosidase presents its role outside the body (inside the biofilm matrix), or translates the protein inside the cell and secrets out of the body. Utilizing a whole cell MALDI-TOF MS

technique, the discovered candidates mostly present in the category of trans-membrane proteins or surface-attached proteins, therefore this enzyme potentially has to function inside the biofilm matrix or outside the body. As a function of this particular enzyme involving polysaccharide degradation pathway, a final product of glucose is generated by digesting the polysaccharide at alpha-1,6 linkage then releasing glucose and a linear chain of glycogen (Hondoh et al., 2008; Yamamoto et al., 2007). A recent study by Mongkolrob, has provided the information of carbohydrate in biofilm's compositions of *B. pseudomallei* and has illustrated that glucose is one of the major components (Mongkolrob et al., 2015).

5. Conclusion

This study describes a method for identifying biofilm using a protein that is specific for biofilm with MALDI-TOF MS. The achieved modified strategies are based on 4 combination criteria and the most important criteria is the utilization of a biofilm defective strain for comparison of the mass spectral peaks. Finally, our findings are the first report showing another relative role of this enzyme in respect to the biofilm-forming process that is able to be used as a metabolic biomarker for identification of biofilm forming capacity.

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Disclosure

No authors have any conflicts of interest to declare.

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