In silico analysis of essential and non-homologous proteins in Salmonella typhimurium biofilm

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Abstract. Salmonella typhimurium is a Gram negative pathogen that commonly causes severe gastroenteritis. It is resistant to a wide range of antibiotics and is able to form biofilm on both biotic and abiotic surfaces. To date, essential and non-homologous proteins in S. typhimurium biofilm remain not well investigated. Therefore, the present work was performed to analyze essential and non-homologous proteins in S. typhimurium biofilm using a combination of one-dimensional SDS-PAGE, HPLC - ESI - QTOF and bioinformatics. Results demonstrated that seven major protein bands (78.1 kDa, 51.2 kDa, 41.5 kDa, 37.3 kDa, 35.1 kDa, 27.6 kDa, and 25.4 kDa) were present in whole-cell protein extract of S. typhimurium biofilm. A total of 75 proteins were successfully identified from both 25.4 kDa and 51.2 kDa protein bands. Approximately 54.67% of QTOF-identified whole-cell proteins were found to be essential to the survival of S. typhimurium biofilm and were non-homologous to human proteome. Majority of essential and non-homologous S. typhimurium biofilm proteins were associated with transport and protein synthesis. The findings from the present work may be useful for development of novel antibiofilm agent.

1. Introduction
A biofilm refers to a microbial community that attaches to hydrated surface by extracellular polymeric substances (EPS) matrix. This microbial community differs from its floating counterpart in several aspects including protein expression pattern that typically results in distinct metabolic performance [1]. The mixture of monolayer biofilm and three dimensional biofilm often produces highly heterogeneous mature biofilm [2, 3]. It has been established that the spatial heterogeneity in the biofilm structure contributes to failure of antimicrobial treatment, leading to a wide range of global issues. Thus, great efforts in the search for natural products and synthetic chemicals as novel antibiofilm agents still continue [4-7].

Salmonella typhimurium is a Gram-negative pathogen that commonly causes severe gastroenteritis and is an important biofilm producer. Its resistance towards a wide range of antibiotics has previously been reported [8] while EPS matrix has been shown to be crucial for viablity of S. typhimurium biofilm [9]. Analysis by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has revealed protein expression pattern in S. typhimurium biofilm. 25.4 kDa and 51.2 kDa protein bands have been found to be consistently expressed in all stages of S. typhimurium biofilm and respond towards antimicrobial treatment [10], suggesting their crucial roles in biofilm formation. Considering the facts that the essential and non-homologous proteins in pathogenic microorganisms are
potential drug targets [11], there is a need to identify the essential and non-homologous proteins in *S. typhimurium* biofilm.

Therefore, the present work was carried out to identify the essential and non-homologous proteins in 25.4 kDa and 51.2 kDa protein bands from *S. typhimurium* biofilm. A combination of SDS-PAGE and high performance liquid chromatography-electrospray ionization-quadrupole time of flight (HPLC-ESI-QTOF) was used to resolve and identify biofilm proteins whilst protein basic local alignment search tool (BLASTp) search against related databases was performed to identify essential and non-homologous biofilm proteins.

2. Methodology

2.1 Test microorganism

*S. typhimurium* ATCC 14028 was used in the present work. Evaluation of culture purity was performed by Gram-staining and assessment of colony morphology. Bacterial inoculum was adjusted to $12 \times 10^8$ CFU mL$^{-1}$ for biofilm formation assay.

2.2 Biofilm formation assay

Biofilm was developed in 6-well microplate at 37 °C for 24 h as previously described [10].

2.3 Protein electrophoresis and identification

Whole-cell protein extraction, protein determination by Bradford assay, one-dimensional polyacrylamide gel electrophoresis (SDS-PAGE), Coomassie staining, tryptic digestion and high performance liquid chromatography-electrospray ionization-quadrupole time of flight (HPLC-ESI-QTOF) were performed as previously described [10].

2.4 Analysis of essential and non-homologous proteins

The QTOF-identified whole-cell proteins from *S. typhimurium* biofilm were analyzed using *in silico* subtractive approach as previously described [11].

3. Result and Discussion

In the present work, *in silico* subtractive approach was used to identify essential and non-homologous proteins from *S. typhimurium* biofilm. The QTOF-identified whole-cell proteins were analysed against database of essential genes (DEG) and were compared with the proteome of human host. Application of this method focused on 25.4 kDa and 51.2 kDa protein bands because they have been shown to be important in *S. typhimurium* biofilm [10].

One-dimensional SDS-PAGE analysis showed the presence of seven major protein bands (78.1 kDa, 51.2 kDa, 41.5 kDa, 37.3 kDa, 35.1 kDa, 27.6 kDa, and 25.4 kDa) in whole-cell protein extract of *S. typhimurium* biofilm (Figure 1). A total of 75 proteins were successfully identified from both 25.4 kDa and 51.2 kDa protein bands (Table 1). Approximately 54.67% of QTOF-identified whole-cell proteins were found to be essential to the survival of *S. typhimurium* and were non-homologous to human proteome, making them ideal therapeutic targets for biofilm control (Table 1). Majority of essential and non-homologous *S. typhimurium* proteins were associated with transport and transcription/translation (Table 2).
Figure 1. SDS-PAGE image of whole-cell protein expression in *S. typhimurium* biofilm. 25.4 kDa and 51.2 kDa protein bands were then analysed using HPLC-ESI-QTOF. Estimated protein amount loaded into gel was 4.5 µg.

Table 1. Summary of *in silico* subtractive analysis of QTOF-identified whole-cell proteins.

| Proteins                                                   | Number | %  |
|-------------------------------------------------------------|--------|----|
| QTOF-identified whole-cell proteins                         | 75     | 100|
| QTOF-identified whole-cell proteins without matches in human host | 47     | 62.67|
| QTOF-identified whole-cell proteins with matches in DEG database | 41     | 54.67|
| QTOF-identified whole-cell proteins considered as ideal therapeutic targets | 41     | 54.67|

Numerous protein expression studies have been carried out to study life cycle, antimicrobial resistance and control strategy of *S. typhimurium* due to rapid global spread of multidrug-resistant *S. typhimurium* [8]. In 2010, Aksakal [12] studied 34 Salmonella serovars by one-dimensional SDS-PAGE. They successfully identified consistent expression of protein bands of 78.1 kDa, 51.2 kDa, 41.5 kDa, 37.3 kDa, 35.1 kDa, 27.6 kDa, and 25.4 kDa in all Salmonella serovars. However, they have not identified the expression of these protein bands in biofilm growth mode of Salmonella serovars. A later work by Yahya et al. [10] has demonstrated the expression of these protein bands in *S. typhimurium* biofilm, especially 25.4 kDa and 51.2 kDa protein bands whereby inhibition of 25.4 kDa and 51.2 kDa protein bands has completely kill *S. typhimurium* biofilm. Whole-cell protein expression in *S. typhimurium* biofilm observed herein is consistent with the previous works [10, 12].

One-dimensional SDS-PAGE is an efficient method to separate all types of proteins based on size. It is widely used to profile changes in proteome expression, check sample purity, estimate the size of unknown proteins and monitor protein purification workflow. For protein identification, one-dimensional SDS-PAGE is often combined with HPLC-ESI-QTOF. In the present work, 25.4 kDa and 51.2 kDa protein bands detected by Coomassie staining were excised from the gel and digested with trypsin enzyme for protein identification by HPLC-ESI-QTOF. According to Xiong et al. [13], this combination becomes the most efficient strategy to identify the proteins contained within the membrane fraction. They successfully identified 349 membrane proteins of *Mycobacterium tuberculosis* H37Rv, validated by ≥2 tryptic peptide matches and MOWSE score >75.
Identification has become a method of choice nowadays because it offers greater effectiveness in time based on BLASTp search against DEG database with e-value cutoff score of 1e-06. Homologous proteins when they meet the criteria as follows: i) crucial for the survival of an organism and operational cost. In the present work, biofilm proteins are considered as essential and non-homologous to human.

Computational analysis of essential and non-homologous proteins for therapeutic target identification has become a method of choice nowadays because it offers greater effectiveness in time and operational cost. In the present work, biofilm proteins are considered as essential and non-homologous proteins when they meet the criteria as follows: i) crucial for the survival of an organism based on BLASTp search against DEG database with e-value cutoff score of 1e-06 and ii) present in the microorganisms but do not exist in Homo sapiens based on BLASTp search against non-redundant database with e-value threshold of 1e-06.

Succinate dehydrogenase iron-sulfur subunit is an enzyme involved in conversion of succinate to fumarate. It constitutes respiratory enzyme complex embedded in inner mitochondrial membrane and is the only enzyme that functions in both the TCA cycle and oxidative phosphorylation. The present work identified succinate dehydrogenase iron-sulfur subunit [SDHB_SALTY] as an essential and non-homologous protein in *S. typhimurium* biofilm. This finding is in agreement with Yahya et al. [11]. They identified essential and non-homologous TCA cycle enzymes such as citrate lyase subunit alpha/citrate CoA-transferase and succinate dehydrogenase iron-sulfur subunit from *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Helicobacter pylori*, *Staphylococcus aureus*, *Escherichia coli*, *Campylobacter jejuni* and *Porphyromonas gingivalis*.

**Table 2.** QTOf-identified whole-cell proteins which are essential to the survival of *S. typhimurium* and non-homologous to human.

| Accession | Protein name | Mascot score | Peptides matched | Functional categories |
|-----------|--------------|--------------|-----------------|----------------------|
| EKT05435  | Hypothetical protein | 25 | 18 | Unknown |
| OTSA_SALTY| Alpha-alpha- trehalose-phosphate synthase [UDP-forming] | 36 | 4 | Carbohydrate metabolism |
| SYR_SALTY | Arginine- tRNA ligase | 45 | 4 | Proline metabolism |
| FHIA_SALTY| Formate hydrogenlyase transcriptional activator | 17 | 3 | Transcription/Translation |
| PHOP_SALTY| Vindelic transcriptional regulatory protein | 210 | 6 | Cell signaling |
| CAH68205  | citrate kinase like protein | 74 | 7 | Carbohydrate metabolism |
| CC176853 | Putative transposase | 15 | 2 | Cell proliferation |
| OMPA_SALTY| Outer membrane protein A | 306 | 18 | Transport |
| CQE27718  | Outer membrane protein ToC | 33 | 4 | Transport |
| METQ_SALTY| D-methionine-binding lipoprotein MetQ | 105 | 2 | Transport |
| ARGV_SALTY| Lysine/arginine/ornithine-binding periplasmic protein | 82 | 4 | Transport |
| NFSB_SALTY| Oxygen-insensitive NAD(P)H nitroreductase | 51 | 2 | Cofactor metabolism |
| DGA_A_SALTY| 2,5-diketo-D-glucaric acid reductase A | 49 | 2 | Vitamin metabolism |
| AHPQ_SALTY| Alkyl hydroperoxide reductase subunit F | 169 | 10 | Cofactor metabolism |
| RIDA_SALTY| 2-iminobutanioate/2-imino propanoate deaminase | 74 | 2 | Amino acid metabolism |
| SDHB_SALTY| Succinate dehydrogenase iron-sulfur subunit | 41 | 2 | Carbohydrate metabolism |
| FLJB_SALTY| Phase 2 flagellin | 126 | 6 | Cell motility |
| AZZ79774  | Phase 1 flagellin | 42 | 4 | Cell motility |
| CUR77306  | Trigger factor | 1176 | 74 | Transcription/Translation |
| CUG07950  | 30S ribosomal protein | 169 | 5 | Transcription/Translation |
| KM121978 | 50S ribosomal protein L1 | 142 | 3 | Transcription/Translation |
| ESE73385  | ATP synthase subunit alpha | 208 | 13 | Transport |
| AFD30527  | Heat shock protein GroEL | 58 | 2 | Defense response |
| AKI93747 | Heat shock protein 60kDa | 428 | 25 | Defense response |
| KNI28456  | Elongation factor Tu | 358 | 25 | Transcription/Translation |
| AFD60284  | DNA directed RNA polymerase | 58 | 2 | Transcription/Translation |
| CQJ80355  | Heat shock protein GspL | 124 | 3 | Defense response |
| KN13063   | Uridine phosphorylase | 50 | 5 | Nucleotide metabolism |
| AHE4707   | Phosphoglycerate mutase | 73 | 2 | Carbohydrate metabolism |
| AHE89904  | Rho protein | 204 | 7 | Transcription/Translation |
| KPF04252  | Alkyl hydroperoxide reductase | 79 | 7 | Cofactor metabolism |
| CQJ68416  | Negative regulator of multiple antibiotic resistance | 80 | 9 | Transcription/Translation |
| CQK73986  | Flagellar biosynthesis protein FltC | 119 | 4 | Cell motility |
| CCN37166  | Protein-export membrane protein | 106 | 14 | Transport |
| KNI37322  | Recombinase A | 74 | 3 | DNA repair |
| CQJ45143  | Acetylglutamate kinase | 50 | 2 | Amino acid metabolism |
| KNM51232  | Biopolymer transporter ExbB | 39 | 4 | Transport |
| CQJ47592  | Alcohol dehydrogenase | 45 | 3 | Cofactor metabolism |
| KPF04525  | Membrane protein insertase | 236 | 11 | Transport |
| KLT32309  | ABC transporter permease | 68 | 4 | Transport |
| KPF20587  | Cystine transporter subunit | 67 | 3 | Transport |
10 membrane proteins were successfully identified as essential and non-homologous proteins in *S. typhimurium* biofilm, namely outer membrane protein A [OMPA_SALTY], outer membrane protein TolC [CQE27718], membrane protein insertase [KPE94525], protein-export membrane protein [CCW73166], Biopolymer transporter ExxB [KMM51232], ABC transporter permease [KLT32369], Cystine transporter subunit [KPF20588], Lysine/arginine/ornithine-binding periplasmic protein [ARGT_SALTY], D-methionine-binding lipoprotein MetQ [METQ_SALTY] and ATP synthase subunit alpha [ESE73385]. A previous study [14] has identified ATP synthase subunit alpha as an essential and non-homologous protein in *Bacillus anthracis* A0248.

Heat shock protein GroEL is a protein produced by the living cells in response to various stressful conditions such as heat, cold and ultraviolet light. It is also known to be crucial in folding of numerous proteins mediating vital cellular functions. The folding process performed by heat shock protein GroEL involves multiple cycles of substrate and GroES binding and release. In the present work, heat shock protein GroEL [AFD30527] was identified as an essential and non-homologous protein in *S. typhimurium* biofilm. This protein has previously been shown to be an essential and non-homologous protein in *Corynebacterium pseudotuberculosis* [15].

30S ribosomal protein is a protein that assists ribosome assembly during protein synthesis. This protein functions in aligning anticodons of tRNAs with codons of mRNA. It has been established that tetracycline antibiotics inhibit bacterial protein synthesis by targeting 30S ribosomal proteins. The present work successfully identified 30S ribosomal protein [CUC07050] as an essential and non-homologous protein in *S. typhimurium* biofilm. This finding corroborates a previous work showing that 30S ribosomal protein is essential and non-homologous in *Corynebacterium diphtheriae* [16].

### 4. Conclusions

We have demonstrated successful identification of essential and non-homologous proteins from *S. typhimurium* biofilm. The findings from the present work may be useful for development of novel antibiofilm agent.

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