Proteomic analysis of *Staphylococcus aureus* biofilm cells grown under physiologically relevant fluid shear stress conditions

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### Abstract

**Background:** The biofilm forming bacterium *Staphylococcus aureus* is responsible for maladies ranging from severe skin infection to major diseases such as bacteremia, endocarditis and osteomyelitis. A flow displacement system was used to grow *S. aureus* biofilms in four physiologically relevant fluid shear rates (50, 100, 500 and 1000 s⁻¹) to identify proteins that are associated with biofilm.

**Results:** Global protein expressions from the membrane and cytosolic fractions of *S. aureus* biofilm cells grown under the above shear rate conditions are reported. Sixteen proteins in the membrane-enriched fraction and eight proteins in the cytosolic fraction showed significantly altered expression (p < 0.05) under increasing fluid shear. These 24 proteins were identified using nano-LC-ESI-MS/MS. They were found to be associated with various metabolic functions such as glycolysis / TCA pathways, protein synthesis and stress tolerance. Increased fluid shear stress did not influence the expression of two important surface binding proteins: fibronectin-binding and collagen-binding proteins.

**Conclusions:** The reported data suggest that while the general metabolic function of the sessile bacteria is minimal under high fluid shear stress conditions, they seem to retain the binding capacity to initiate new infections.

**Keywords:** Biofilm, *Staphylococcus aureus*, Flow chamber, Shear stress, Proteomics

### Background

A biofilm is an aggregate of bacterial microcolonies adherent to each other and to biotic or abiotic surfaces, embedded in an extracellular matrix produced by the sessile bacteria composing the microcolonies. Biofilm has been found in 65-80% of the bacterial infections, and is considered refractory to host defenses in antibiotic therapy [1]. Among the biofilm forming bacteria, *Staphylococcus aureus* is responsible for severe skin infections to such major diseases as bacteremia, endocarditis and osteomyelitis. Under suitable conditions, *S. aureus* causes serious complications in devices like implants and catheters by producing biofilms on them [2,3]. Treatment of such infections becomes even more challenging given that several *S. aureus* strains show resistance to multiple antibiotics (e.g., methicillin and vancomycin). Donlan and Costerton [4] postulated three mechanisms responsible for antibiotic resistance: (i) delayed penetration of the antimicrobial agent through the biofilm matrix, (ii) altered growth rate of biofilm organisms, and (iii) other physiological changes due to the biofilm mode of growth. Growth rate and physiological changes are considered the major causes of resistance to antibiotics. Characterizing bacterial biofilm architecture and stability in their favorable growth conditions would help elucidate how these bacteria survive during their processes of biofilm formation.

In general, increased antimicrobial tolerance is related to the biofilm physiology and architecture [5]. Architecture depends on (i) the development of an extracellular matrix composed of proteins, DNAs, minerals and/or polysaccharides secreted by the sessile cells and (ii) the shear environment in which matrix development occurs [4]. The stability of the structure lies in its viscoelastic properties and its related ability to withstand mechanical cleansing forces (e.g., fluid shear stress) [6]. A number of previous studies investigated the mechanism of
adhesion and erosion of *S. aureus* biofilm cells under physiologically relevant fluid shear conditions [7-13]. The latter study demonstrated that platelets (thrombocytes) adhere to the collagen (fibrous structured proteins in blood vessels) of the vascular sub-endothelium in distinct patterns. Shear rates of ≤ 800 s⁻¹ were demonstrated to allow better adhesion than the higher shear rates.

In addition to the above, several studies focused on the protein profiles of *S. aureus* biofilm cells under different growth conditions [14-16]. Higher levels of surface proteins, especially the fibrinogen-binding proteins, were observed in biofilm cells when compared to planktonic cells [17]. Similarly, several other studies investigated protein profile of *S. aureus* biofilm cells under different growth conditions most of which employed either shake flasks or nutrient-rich agar plates to produce biofilm [14-16]. Nonetheless, use of shake flasks or agar plates does not seem to provide appropriate environment for the physiologically relevant biofilm studies for several reasons [17,18]. First, it exposes the bacteria to nutritionally rich media. Second, biofilm grown in shake flasks encounters high osmolarity, oxygen limitation and a relatively high cell density that together influence gene expression in *S. aureus* [19-21]. Third, shake flasks do not mimic the in-vivo environment where *Staphylococci* grow as biofilm under fluid shear.

Investigations discussed thus far provided significant information to the molecular mechanisms of biofilm formation, erosion and dissemination. However, little is known how the overall protein profiles in *S. aureus* biofilm cells are influenced under different fluid shear rates.

It is hypothesized that fluid shear stress influences gene regulatory networks controlling protein expression and thereby contributing to bacterial adhesion/survival during infection. In this manuscript, a proteomic approach to study the expression of membrane and cytosolic protein fractions in biofilm cells grown under physiologically relevant fluid shears is reported. The protein expression profiles of biofilm cells under different shear conditions provide novel insights into the cellular processes related to bacterial adhesions/survival during infection in vivo.

**Results and discussion**

**2-DE profile, protein identification and classification**

For the comparison of protein profiles under different shear rates, a two dimensional electrophoresis (2-DE) profile was generated using membrane protein fractions from cells grown in shake flasks for two hours. It is known that most of the proteins associated with bacterial adhesion to the substratum are located in the membrane fraction. Of the 200 ± 5 protein spots resolved in four biological replicate gels, 64 were confidently identified using nano-LC-MS/MS (Figure 1 and Table 1). The remaining 136 protein spots could not be identified due to their low abundance and were below the limit of identification. As shown in Table 1, most of the proteins were identified with high XC score and sequence coverage. Using PSORT [22] and TMpred [23], the subcellular location of 16 proteins were identified as membrane and 20 proteins as transmembrane. Twenty-six proteins were also predicted to be of cytoplasmic origin, which may have originated from lysed cells. The exact reason for the contamination of proteins

![Figure 1](http://www.proteomesci.com/content/12/1/21)
| Spot ID | Protein name                          | Functional category              | XC-score | pl/Mr | Cov (%) | Accession number | Location       |
|---------|--------------------------------------|----------------------------------|----------|-------|---------|-----------------|----------------|
| 1       | Fibronectin-binding protein           | Virulence mechanism              | 150      | 4.4/114 | 18      | gi:15925493     | Membrane       |
| 2       | Aconitate hydratase                   | Carbohydrate metabolism          | 396      | 4.7/98 | 51      | gi:15924340     | transmembrane  |
| 3       | Elongation factor G                   | Transcription and replication     | 548      | 4.6/77 | 49      | gi:15923537     | transmembrane  |
| 4       | Collagen Binding Protein (Cna)        | Virulence mechanism              | 90       | 5.8/133 | 11      | gi:21284341     | Membrane       |
| 5       | Trigger factor                        | Virulence mechanism              | 140      | 4.2/49 | 36      | gi:15924665     | Cytosolic       |
| 6       | DnaK protein                          | Stress protein                   | 348      | 4.5/66 | 67      | gi:15924570     | Membrane       |
| 7       | DnaK protein                          | Stress protein                   | 218      | 4.5/66 | 51      | gi:15924570     | Membrane       |
| 8       | PEP-protein phosphatase               | Carbohydrate metabolism          | 60       | 4.5/63 | 20      | gi:15926670     | Membrane       |
| 9       | PEP-protein phosphatase               | Carbohydrate metabolism          | 30       | 4.5/63 | 18      | gi:15926670     | Membrane       |
| 10      | Pyruvate kinase                       | Carbohydrate metabolism          | 150      | 4.1/63 | 25      | gi:15924687     | transmembrane  |
| 11      | Prolyl-tRNA synthetase                | Cell division                    | 228      | 4.9/64 | 54      | gi:15924253     | transmembrane  |
| 12      | Prolyl-tRNA synthetase                | Cell division                    | 58       | 4.9/64 | 13      | gi:15924253     | transmembrane  |
| 13      | 30S ribosomal protein                 | Protein synthesis                | 130      | 4.4/43 | 37      | gi:15924666     | transmembrane  |
| 14      | Glutamyl- amidotransferase            | Amino acid metabolism            | 84       | 4.9/53 | 25      | gi:21283570     | transmembrane  |
| 15      | Glycyl-tRNA synthetase                | Cell division                    | 90       | 4.9/54 | 20      | gi:15924555     | Cytosolic       |
| 16,17   | Elongation factor Tu                  | Virulence mechanism              | 214      | 4.6/43 | 60      | gi:15923538     | Membrane       |
| 18      | Pyruvate dehydrogenase                | Virulence mechanism              | 180      | 4.8/41 | 48      | gi:15924083     | Membrane       |
| 19      | Phosphoglycerate kinase               | Carbohydrate metabolism          | 120      | 5.1/42 | 42      | gi:15923763     | transmembrane  |
| 20      | Coenzyme A ligase                     | Protein synthesis                | 140      | 5.0/43 | 42      | gi:21282234     | transmembrane  |
| 21      | DNA-directed RNA polymerase           | Transcription and replication     | 110      | 4.5/35 | 38      | gi:15925214     | Cytosolic       |
| 22      | Glyceraldehyde-3-PDH                 | Carbohydrate metabolism          | 108      | 4.8/36 | 44      | gi:15923762     | Cytosolic       |
| 23      | Coenzyme A synthase                   | Carbohydrate metabolism          | 30       | 4.8/36 | 20      | gi:49484747     | transmembrane  |
| 24      | Pyrophosphatase                       | Carbohydrate metabolism          | 30       | 4.5/34 | 15      | gi:15924090     | Membrane       |
| 25      | Phosphotransacetylase                 | Others                           | 102      | 4.6/35 | 55      | gi:15923578     | Cytosolic       |
| 26      | Elongation factor Ts                  | Transcription and replication     | 190      | 5.0/32 | 58      | gi:15924247     | Cytosolic       |
| 27      | General stress protein Ctc            | Stress protein                   | 50       | 4.2/24 | 29      | gi:15923491     | Cytosolic       |
| 28      | 30S ribosomal protein                 | Protein synthesis                | 106      | 5.3/30 | 30      | gi:15924035     | transmembrane  |
| 29      | Triosephosphate isomerase             | Carbohydrate metabolism          | 58       | 4.7/27 | 32      | gi:15923764     | Cytosolic       |
| 31      | Superoxide dimutase                   | Stress protein                   | 80       | 5.0/23 | 35      | gi:15924543     | Cytosolic       |
| 32      | Cysteine synthase                     | Carbohydrate metabolism          | 60       | 5.2/33 | 24      | gi:15923503     | transmembrane  |
| 33      | Malatequinone oxidoreductase          | Carbohydrate metabolism          | 220      | 6.1/56 | 38      | gi:15925597     | Membrane       |
| 34      | SOS ribosomal protein                 | Protein synthesis                | 174      | 4.5/13 | 96      | gi:15923530     | transmembrane  |
| 35      | SOS ribosomal protein                 | Protein synthesis                | 210      | 9.8/20 | 77      | gi:15925228     | Cytosolic       |
| 36,37,43| SOS ribosomal protein                 | Protein synthesis                | 78       | 9.9/20 | 52      | gi:15925225     | Cytosolic       |
| 38      | SOS ribosomal protein                 | Protein synthesis                | 50       | 10.8/16 | 43     | gi:15925221    | Cytosolic       |
| 39      | SOS ribosomal protein                 | Protein synthesis                | 70       | 9.5/15 | 42      | gi:15923527     | Cytosolic       |
| 40      | SOS ribosomal protein                 | Protein synthesis                | 40       | 10.3/11 | 50     | gi:15924637     | Cytosolic       |
| 41      | SOS ribosomal protein                 | Protein synthesis                | 10       | 9.5/24 | 87      | gi:15923528     | Cytosolic       |
| 42      | SOS ribosomal protein                 | Protein synthesis                | 438      | 5.4/91 | 57      | gi:15923515     | Cytosolic       |
| 43      | SOS ribosomal protein                 | Protein synthesis                | 100      | 10.1/24 | 44     | gi:15925234     | Cytosolic       |
| 44      | Inositol-monophosphate DH              | others                           | 178      | 5.5/53 | 45      | gi:15923800     | transmembrane  |
| 45      | 30S ribosomal protein                 | Protein synthesis                | 80       | 10.2/24 | 36     | gi:15925240     | Cytosolic       |
| 46      | Catalase                              | Stress protein                   | 164      | 5.2/58 | 40      | gi:21282950     | Cytosolic       |
| 47      | Pyruvate dehydrogenase E1            | Virulence mechanism              | 90       | 4.8/41 | 24      | gi:15924083     | Membrane       |
from cytoplasmic origin is not clear. One possible reason is the inherent chemistry of lysostaphin, the enzyme used in digesting the membrane fraction. A recent study reported that the treatment of bacterial cells with lysostaphin decreases cell wall stiffness by digesting peptidoglycan and could eventually lead to the formation of osmotically fragile cells [24]. A detailed explanation of how lysostaphin causes cell autolysis during digestion is osmotically fragile cells [24].

Regardless of the origins, the presence of these cytoplasmic proteins does not diminish the significance of the membrane proteins that were identified in this study. In fact, there are reports that suggest that some of the *S. aureus* cytoplasmic proteins may have a moonlighting function and play roles in virulence [30,31].

Proteins visualized in the 2-DE profile were also classified based on their functions using PSORT and TMpred. About 30% of the identified proteins were involved in carbohydrate metabolism, 20% in protein synthesis and 12% in stress tolerance. KEGG prediction confirmed the functional categories of three virulence mechanism associated proteins: fibronectin-binding protein (spot 1), collagen-binding surface protein (Cna; spot 4) and trigger factor (spot 5). Fibronectin-binding protein in *S. aureus* is central to the invasion of endothelium. During infection, *S. aureus* forms a bridge by using fibronectin-binding proteins with the host cell receptors [29]. The collagen binding protein (Cna; spot 4) identified in this study is well studied as well for its binding with collagen [32-34]. Unlike fibrinogen- and collagen-binding proteins, the associations of elongation factor Tu (spots 16 and17) and pyruvate dehydrogenase (spot 18) with virulence mechanism could not be predicted by KEGG. On the contrary, association of these proteins with virulence mechanism were reported from other findings [35].

**Effect of fluid shear rate on protein expression in biofilms**

Fluid shear, a hydrodynamic force also generated by blood flow against the vessel wall, is an important physiological phenomenon in the vasculature system [36-38]. Using physiologically relevant fluid shear rate conditions, adequate number of biofilm cells could be harvested to carry...
out 2-DE for protein separation from both cytoplasmic and membrane cell fractions (Figure 2).

Fluid shear rates range from 40 s$^{-1}$ to 2,000 s$^{-1}$ in physiological conditions [39]. To investigate the effect of fluid shear on protein profile in biofilm, the protein expression patterns of biofilm cells grown at 50 s$^{-1}$, 100 s$^{-1}$, 500 s$^{-1}$ and 1000 s$^{-1}$ were compared. Of the 200 protein spots resolved in 2-DE gels from the biofilm cells grown in shake flasks, 16 protein spots showed significant differences in their abundance as shear rate was increased ($p < 0.05$, Student’s t-test). These proteins were identified using nano-LC-MS/MS. Their localization and possible functions were predicted by PSORT and TMpred (Figure 3). All the proteins with a differential spot volume in membrane fraction were confirmed as either membrane or transmembrane in their sub-cellular localization. Similarly, eight cytosolic proteins that displayed lower abundance with an increase in the fluid shear rates were identified. Sub-cellular location and function of these proteins in cytoplasm were also confirmed by PSORT and TMpred.

As shown in Figure 4, three membrane proteins showed higher abundance in biofilm cells at 1000 s$^{-1}$ when compared to those at 50 s$^{-1}$, 100 s$^{-1}$, and 500 s$^{-1}$. These proteins were identified by LC-MS/MS as malate dehydrogenase (spot 20), branched-chain alpha-keto acid dehydrogenase (spot 33) and 50S ribosomal protein (spot 42; Figure 4 and Table 2). Unlike the membrane associated fraction, cytoplasmic proteins that showed higher abundance with an increase in the fluid shears could not be detected.

Proteins that showed decreased expression with increasing fluid shear rates were also observed. Thirteen membrane associated proteins showed lower expression in biofilm cells at 1000 s$^{-1}$ when compared to those at 50 s$^{-1}$, 100 s$^{-1}$ and 500 s$^{-1}$ (Figure 4 and Table 2). A closer look at the functional categories of proteins revealed that this decrease in protein expression represented a regulated state of lower metabolic activity. Nine of the 13 identified proteins are associated mainly in glycolysis/TCA cycle, indicating that utility of the glycolysis/TCA pathways were minimized under high shear rates. Glyceraldehyde 3-phosphate dehydrogenase decreased (spot 2 and 3) by 4.2 to 5.8 folds in 1000 s$^{-1}$ when compared to that in 50 s$^{-1}$.

Similar to phosphate dehydrogenase, alanine dehydrogenase (spot 4), another important protein that catalyzes the NAD-dependent reversible reductive amination of pyruvate into alanine, was decreased by 4.2 fold in 1000 s$^{-1}$ when compared to 50 s$^{-1}$ (Table 2 and Figure 4). In addition, a significant decrease in malate:quinone oxidoreductase and succinyl-CoA synthase were observed under high fluid shear rates.

In cytosolic fraction, eight proteins showed lower abundance at 1000 s$^{-1}$ when compared to 50 s$^{-1}$. Like with the membrane protein fraction, glyceraldehyde-3-phosphate dehydrogenase decreased significantly at 1000 s$^{-1}$ when compared to 50 s$^{-1}$. In addition, a lower abundance of alcohol dehydrogenase, succinyl-CoA synthetase, formate acetyltransferase, and malate dehydrogenase was observed under high fluid shear stress.

In the proteome profile reported here, six virulence-associated surface proteins were identified: fibronectin-binding protein (spot 1), collagen-binding surface protein (Cna; spot 4), trigger factor (spot 5), elongation factor Tu (spots 16 and17) and pyruvate dehydrogenase (spot 18). However, changes in expression of these proteins were not detectable under fluid shear stress. On the contrary, most of the glycolysis / TCA cycle associated proteins decreased significantly under higher shear rates. The data thus demonstrate for the first time the relationship between metabolic activity and virulence potential. The glycolysis/TCA metabolic activity of S. aureus was low under fluid high shear, while the abundance of surface proteins, especially the fibronectin-binding and collagen binding surface proteins, remained unchanged. Although the ability of these cells in neo-colonization was not specifically tested, the unchanged abundance of these surface binding proteins implies that these cells retain their ability for further infection. Alternatively, there might be an interaction between virulence mechanism associated proteins and glycolysis/TCA associated proteins under high fluid shear that is yet to be determined. As glucose is the main metabolite of the glycolysis/TCA pathways, it is anticipated that carbohydrate metabolism might have potential contribution to the virulence mechanism associated proteins. Such an interaction of carbohydrate structures and their associated
proteins with cell wall-anchored proteins was predicted from other findings [25].

**Metabolic pathways of identified proteins**

Some of the identified proteins were mapped in their metabolic pathways (Figure 5). As observed in Table 2, most of the changed proteins were associated with carbohydrate metabolism especially glycolysis and TCA cycle. Glyceraldehyde 3-phosphate dehydrogenase (GapC; spots M2 and M3, C1) reduced significantly as fluid shear rates were increased. This protein is responsible for the inter-conversion of 1,3-diphosphoglycerate and glyceraldehyde-3-phosphate (GAPDH), a central step in glycolysis which utilizes NAD for phosphorylation. As fluid shear significantly decreased the abundance of this protein, less energy is anticipated to be available for the transfer of inorganic phosphate to high phosphoryl transfer product, indicating that the bacteria exhibits lower metabolic activity under higher fluid shear stress. Although cytosol is considered to be a primary location for this protein, it was recently reported that GAPDH and several other glycolytic enzymes assemble in complexes on the inside of the cell membrane [26]. This protein is also considered as a metabolic switch for rerouting the carbohydrate flux to counteract stress [40].
In addition to a reduction in GAPDH under high fluid shears, significant decreases in alanine dehydrogenase and formate acetyltransferase were also observed. Both of these enzymes provide pyruvate for glycolytic pathways. Reduction in these enzymes also implies lower acetyl-CoA that drives TCA cycles.

There were significant changes in three enzymes of TCA cycle with the increased rate of fluid shear (Figure 5).

Malate dehydrogenase that catalyzes the conversion of malate into oxaloacetate (using NAD+) increased significantly. This increase in malate dehydrogenase seems to contradict with the decreased level of expression of other glycolytic and TCA cycle enzymes in this study. However, the reaction mediated by this enzyme is reversible, indicating that the increased level in malate dehydrogenase might have resulted due to the reverse reaction. This is also consistent with the reduction in malatequinone oxidoreductase (spot 7). In addition, a significant decrease in the expression of succnly-CoA synthetase was also observed. Taken together, it can be concluded that fluid shear stress have a significant effect on the abundance of some key enzymes in the TCA cycles, implying lower TCA cycle activity.

Implications of reduced TCA cycle activity in regulating or affecting staphylococcal virulence and/or virulence determinant biosynthesis are well studied [41-45]. Higher amount of capsular polysaccharides in relation to TCA cycle inactivation was reported [41]. It was anticipated that TCA cycle inactivation results lower amount of oxaloacetate for conversion to phosphoenolpyruvate to be used in gluconeogensis pathway [41].

Inhibiting TCA cycle activity dramatically increases polysaccharides intercellular adhesins (PIA) biosynthesis through genetic regulation of icaADBC, a group of genes responsible for production of PIA [43,45]. It is anticipated that regulatory proteins responding to TCA cycle-mediated changes in the metabolic status regulate icaADBC transcription, indicating that the TCA cycle acts as a signal transduction pathways for regulating virulence factor synthesis and biofilm formation [41].

Lower glycolytic and TCA cycle activities of bacteria under fluid shear stress is also consistent with lower amount of cell that was harvested at 500 and 1000 s⁻¹ (Figure 2).

Here, it is demonstrated that under fluid shear conditions, bacteria maintain a dormant mode of growth by reducing glycolytic and TCA cycles activities for their survival while maintaining their capacity for further infection. From clinical perspective, the dormant mode of growth is considered a major role that bacteria plays to escape the effect of antibiotics [46].

Conclusions
The expression levels of two important surface binding proteins, fibronectin-binding and collagen-binding proteins, were not influenced by varying levels of physiological fluid shear rates. On the other hand, some of the S. aureus proteins that are associated with metabolic functions such as carbohydrates, protein synthesis, and stress tolerance significantly changed their level of expression. These results imply that bacteria maintain slow metabolic activity under high fluid shear stress. To
our knowledge, this is the first report that catalogs the differential protein expression under various physiologically relevant fluid shear stress conditions. As revealed from this study, most of the altered proteins were directly related to the energy balance of different metabolic pathways. A future study that elucidates post-translational protein modifications (especially phosphorylation) may provide a further understanding of the highly variable biofilm proteomes under fluid shear forces.

### Methods

#### Bacterial culture

*S. aureus* Phillips, a biofilm forming bacteria, was used in this study. The strain was isolated from a patient diagnosed with osteomyelitis and has been extensively studied [9,10,33]. *S. aureus* cultures were started by inoculation (10 μL) from glycerol stocks into tryptic soy broth (TSB; 50 μL) supplemented with 0.25% (w/v) glucose and grown at 37°C with constant rotation at 141 rpm in shake flasks. The growth of the bacterial strains was monitored by measuring the absorbance of the broth at 600 nm on a spectrophotometer. The cells were harvested at mid-exponential phase (OD600 = 0.3 to 0.35), centrifuged (4000 rpm) for 15 min at 4°C and resuspended in phosphate-buffered saline (DPBS; 138 mM NaCl, 2.7 mM KCl, pH 7.4). Cell concentrations were determined using a Coulter Multisizer (Beckman Coulter).

#### Flow chamber system

A 40 cm silicon tube (SILASTIC) was used as the reactor. Briefly, after sterilization, the reactor tube was filled with collagen type I for coating and washed with sterile PBS after an hour. The reactor was then inoculated with 20-fold diluted mid-exponential phase cells.

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### Table 2 Differentially expressed membrane and cytosolic protein fractions were identified with linear ion trap nano-LC-ESI-MS/MS

| Spot ID | Fold changed | Protein name                                      | XC score | Cov (%) | pI/Mr | Acc no      | Functional category               |
|---------|--------------|---------------------------------------------------|----------|---------|-------|-------------|-----------------------------------|
| Membrane fraction: higher abundance in 1000S-1 |
| 20      | 3.1          | Malate dehydrogenase 170                          | 54       | 4.9/44  | gi:15924692 | Carbohydrate metabolism         |
| 33      | 2.5          | Branched-chain alpha-keto acid dehydrogenase 260  | 42       | 4.8/46  | gi:15924085 | Carbohydrate metabolism         |
| 42      | 2.2          | 50S ribosomal protein 80                          | 58       | 4.6/17  | gi:15923529 | Amino acid metabolism            |
| Membrane fraction: lower abundance in 1000S-1 |
| 2       | 5.8          | Glyceraldehyde 3-phosphate dehydrogenase 106      | 30       | 5.9/37  | gi:15924677 | Carbohydrate metabolism         |
| 3       | 4.2          | Glyceraldehyde 3-phosphate dehydrogenase 48       | 21       | 5.9/37  | gi:15924677 | Carbohydrate metabolism         |
| 4       | 4.2          | Alanine dehydrogenase 258                         | 66       | 5.5/40  | gi:21283381 | Carbohydrate metabolism         |
| 7       | 3.6          | Malatequinone oxidoreductase 443                   | 62       | 6.1/56  | gi:15925597 | Carbohydrate metabolism         |
| 8       | 3.6          | Alcohol dehydrogenase 510                         | 76       | 5.2/36  | gi:21282297 | Carbohydrate metabolism         |
| 14      | 3.3          | Ornithine-oxo-acid transaminase 340                | 81       | 5.1/43  | gi:15923947 | Amino acid metabolism            |
| 15      | 3.2          | Ribosomal subunit interface protein 200           | 68       | 5.0/22  | gi:15923742 | Amino acid metabolism            |
| 17      | 3.1          | Nucleoside diphosphate kinase 90                   | 61       | 4.9/17  | gi:15924459 | Nucleotide Metabolism            |
| 21      | 2.9          | Malatequinone oxidoreductase 236                   | 46       | 6.1/56  | gi:15925597 | Carbohydrate metabolism         |
| 25      | 2.8          | Succinyl-CoA synthetase 166                        | 49       | 5.4/32  | gi:15924236 | Carbohydrate metabolism         |
| 27      | 2.7          | Alanine dehydrogenase 148                         | 47       | 5.0/40  | gi:15924429 | Carbohydrate metabolism         |
| 34      | 2.5          | Superoxide dismutase 118                          | 62       | 5.2/23  | gi:15923123 | Stress protein                   |
| 37      | 2.4          | FO2F1 ATP synthase 80                              | 43       | 5.7/32  | gi:15925094 | Carbohydrate metabolism         |
| Cytosolic fraction: lower abundance in 1000S-1 |
| 1       | 0.71         | Glyceraldehyde 3-phosphate dehydrogenase 48       | 21       | 5.9/37  | gi:15924677 | Carbohydrate metabolism         |
| 7       | 0.60         | Ornithine-oxo-acid transaminase 340                | 81       | 5.1/43  | gi:15923947 | Amino acid metabolism            |
| 12      | 0.59         | Alcohol dehydrogenase 516                         | 76       | 5.2/36  | gi:21282297 | Carbohydrate metabolism         |
| 14      | 0.78         | Succinyl-CoA synthetase 106                        | 30       | 5.4/32  | gi:15924236 | Carbohydrate metabolism         |
| 19      | 0.77         | Formate acetyltransferase 148                     | 22       | 5.2/84  | gi:15923216 | Carbohydrate metabolism         |
| 20      | 0.63         | Malate dehydrogenase 170                          | 54       | 4.9/44  | gi:15924692 | Carbohydrate metabolism         |
| 26      | 0.55         | Alcohol dehydrogenase 510                         | 76       | 5.2/36  | gi:21282297 | Carbohydrate metabolism         |
| 33      | 0.67         | 50S ribosomal protein 80                          | 36       | 10.2/24 | gi:15925240 | Amino acid metabolism            |

Fold change was calculated using proteins in 50 s⁻¹ compared to those in 1000 s⁻¹. Protein functional categories were annotated from KEGG database.
for 10 minutes. The unattached cells were then rinsed out of the silicon tubing with sterile PBS buffer. One end of the reactor was then connected to a continuous supply of TSB using a peristaltic pump which was calibrated for different flow rates to provide the desired shear stresses. The wall shear rate ($\dot{\gamma}$) in inverse seconds, assuming Newtonian fluid behavior and constant density and viscosity, was calculated by the formula: $\dot{\gamma} = 32Q/\pi D^3$, where $Q$ is the volumetric flow rate and $D$ is the tube internal diameter. The equation and further explanation of flow rates and shear stress calculations can be found in a recent review [47]. During all experiments, the entire flow system was maintained at 37°C. After 24 hours, the reactor tube was gently removed from the system. Biofilm cells were extruded by squeezing the reactor tube. The tube was washed three times with phosphate-buffered saline to ensure recovery of all biofilm cells from the tube. After brief sonication, the cell concentration was measured using the Coulter Multisizer.
Protein extraction
Cells were washed with PBS containing 0.1% sodium azide and then with PBS without azide followed by a brief wash with digestion buffer containing 10 mM Tris HCl, 1 mM EDTA, 5 mM MgCl₂. Approximately $5 \times 10⁸$ bacterial cells were resuspended in 1 mL of digestion mixture containing 35% raffinose (Sigma), protease inhibitor cocktail (Roche; 1 tablet/mL of digestion buffer), and lysozyme (Sigma; 5 units/mL). The cells were incubated in this lysis buffer at 37°C for 30 min. Cell debris was removed by centrifugation at 8,000 g for 20 minutes. The digest was kept at −20°C overnight and then centrifuged at 8,000 g for 20 min; precipitated raffinose was discarded. The protein solution was subjected to ultrafiltration (Millipore) per manufacturer's instructions. Membrane proteins were extracted in 2-DE solubilization buffer urea (8 M; Sigma), thiourea (2 M; Sigma), ASB 14 (1% Sigma) and DTT (1% Sigma), carrier ampholytes (0.08%; GE Healthcare)] with sonication. Protein concentration in the solution was determined using 2D Quant (GE Healthcare), and the resulting solution was stored at −80°C for 2-DE.

Two dimensional Gel electrophoresis
In preparation for 2-DE, 150 μg proteins were resolubilized by adding 2-DE solubilization buffer. The mixture was vortexed intermittently until the protein was completely solubilized. The resulting solution was then diluted to the desired volume with destreak rehydration solution (GE Healthcare). Rehydration of IPG strips (pH 3–11, NL 24 cm; GE Healthcare) with the sample was carried out in the Immobiline Dry Strip Re-swelling Tray (GE Healthcare) according to the manufacturer’s instructions. The rehydrated strips were then subjected to IEF using IPGphor (GE Healthcare) operated at 20°C in gradient mode (97 kVhr). After focusing, the strips were stored at −80°C for later use. Prior to the second dimension SDS-PAGE, IPG strips were equilibrated for 15 minutes in equilibration solution (15 mL) containing 50 mM Tris–HCl, pH 8.8, 6 M urea, 30% w/v glycerol, 2% w/v SDS and traces of bromophenol blue with 10 mg/mL (w/v) of DTT. A second equilibration was carried out for 15 minutes by adding iodoacetamide (25 mg/mL) instead of DTT in equilibration solution. Second dimension SDS-PAGE was performed using large format (26.8 × 20.5 cm) gels (12.5% T/2.6% C) according to the manufacturer’s instructions. Electrophoresis was carried out with an initial constant voltage of 10 mA/gel applied for 30 minutes followed by 20 mA/gel for overnight until the bromophenol band exited the gel. The gels were stained with Colloidal Coomassie brilliant blue (BioRad). Briefly, gels were washed two times using distilled water and then placed on Dodeca high throughput gel staining tray (BioRad) followed by destaining using distilled water. All steps were carried out at room temperature. Gels were scanned as 12-bit TIFF images using GS-800 densitometer (BioRad) and analyzed by Nonlinear Dynamics SameSpots (v.3.2). Spot volumes were normalized by the software to a reference gel. At least three gels (biological replicates) for each treatment were used for analyses. Student’s t-test was performed, and spots with more than 2-fold were considered as significant.

Protein identification
For mass spectrometric identification, gel spots were excised, destained, and digested with sequencing grade trypsin (Promega) as described elsewhere [48]. Peptide samples were then analyzed by nano-LC-ESI-MS/MS using LTQ (Finnigan, Thermo, USA). Nano-LC was performed at reversed phase conditions using Ultimate 3000 (Dionex corporation, USA) C18 column with a flow rate of 1–5 μL/min in 70-90% acetonitrile containing 0.1% formic acid. MS and MS/MS data were collected and interrogated against the NCBI non-redundant protein database for *S. aureus* using SEQUEST with a peptide tolerance of 1.4 amu. Search results were then filtered using three criteria: distinct peptides, XC score vs Charge state (1.50, 2.00, 2.50, 3.00) and peptide probability (0.001). The confirmation of the protein identification was based on the XC score value of more than 50 and Sf score for individual peptide of more than 0.8. It is to be noted that more than one protein were found in each spot, which is not unusual for a gel-based protein separation. Therefore, protein with the highest XC for each spot was selected as positive identification.

Protein localization and function
The prediction of protein localization sites in cells was determined by PSORT, a computer program which analyzes the input sequence by applying the stored rules for various sequence features of known protein sorting signals. The transmembrane protein domain was predicted by TMpred (http://www.ch.embnet.org/software/TMPRED_form.html). To analyze functional categories of the identified proteins, they were submitted to the KEGG database (http://www.genome.ad.jp/kegg/pathway.html) using BRITE hierarchy. KEGG BRITE is a collection of hierarchical classifications of proteins based on their biological function.

Competing interests
The authors declare that there are no competing interests.

Authors’ contributions
NI designed and conducted the experiment and is the corresponding author of this manuscript. JMR and MRM developed the concept. NI and YK analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.
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