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Blood Serum Affects Polysaccharide Production and Surface Protein Expression in *S. Aureus*

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Abstract

Background—*S. aureus* biofilm serves a major role in pathogenesis. Two of the major components of bacterial biofilm are Polysaccharides intercellular adhesions (PIA) and surface proteins. It is not known how PIA and surface proteins expressions are affected in presence of blood serum. Analyses of surface proteins expressions will provide more effective biomarker discovery that might lead to development of antimicrobial therapeutics to meet the challenges of biofilm-related infections.

Method—Secondary cultures of *S. aureus* Philips, a biofilm-forming bacterium, were generated by inoculating 1 ml of overnight culture into 50 ml of TSB. Bacteria were cultured at several concentrations of blood serum and found that 12.5% supplemented blood serum provide s similar growth curve as normal TSB (100%). One and 2 D SASPAGE were used to separate proteins and the differentially expressed proteins were identified by nano-LC/MS.

Results—Polysaccharide intercellular adhesions production was significantly increased due to the addition of blood serum in the media. We also identified two serum proteins, apolipoprotein and globulin (Fc and Fab), that remained attached with the membrane fraction of bacterial proteins.

Conclusion—These results have strongly demonstrated that blood serum influences the exopolysaccharide expression in *S. aureus.*

Keywords

Biofilm; Serum; *Staphylococcus aureus*; Proteome

Author's contributions

Nazrul Islam designed and conducted the experiment, and corresponding author for this manuscript. Julia M. Ross, Khwaja G. Hossain and Mark R. Marten developed the concept.

Background

A biofilms are micro Colonies of bacteria adhere to each other and to biotic or biotic surfaces, embedded in an extracellular matrix produced by the sessile bacterial cells [1]. Extracellular matrix (ECM) and ECM proteins in bacterial biofilm play crucial roles in contaminating the agricultural produce starting from the field to the packing. In addition, ECM mediates adhesions protect bacteria from external threats and other stressors of adverse environment. Some of the ECM enzymes hydrolyze macro biomolecules into smaller biomolecules which subsequently is taken up by bacteria [2,3].

Both polysaccharide and protein embedded in extracellular matrix of biofilm play critical roles in biofilm stability Martin-Cereceda et al. (2001); Tsuneda et al. (2003). The polysaccharide intercellular adhesns are the major components (90%) of biofilm. Gutberlet et al. (1997); Gross et al. (2001); Weidenmaier & Peschel (2008); Rupp et al. (1995) [4]. Two types of PIA have been reported based on structure,. PIA type I (typically>80%) is a unique linear beta-1, 6 glucosaminoglycan which is predominantly positively charged. PIA type II (typically<20%) is structurally similar to type I, but contains phosphate and ester-linked succinate, and thus carries a mild negative charge Rupp et al. (1995); Mack et al. (1996). The biofilms are stabilized by the linear structure of these PIAs electrostatic interaction between positively and negatively charged residues Mack et al. (1996). In addition, surface proteins appear to play a critical role in contributing to biofilm stability. For example, nearly all S. aureus clinical isolates possess and express the genes necessary for PIA production (ica-operon, described below), yet many do not form biofilms Fitzpatrick et al. (2005, 2006). This implies that surface proteins may act as additional biofilm stabilizers, possibly cooperating with PIA to mediate intercellular adhesion O'Gara (2007).

In antibiotic therapy, biofilm has been found in 65–80% of the bacterial infections, and is considered refractory to host defenses [4]. Staphylococcus s. aureus, a biofilm forming bacteria, is responsible for severe skin infections to such major diseases as bacteremia, endocarditis and osteomyelitis. Under favorable conditions, S. aureus causes serious complications in devices like implants and catheters by producing biofilms on them [5]. Treatment of such infections becomes even more challenging given that several S. aureus strains show resistance to multiple antibiotics (e.g., methicilin and vancomycin). Extracellular matrix (ECM) proteins in bacterial biofilm play crucial roles in biofilm stability. In addition, ECM mediates adhesins to protect bacteria from external threats and also other stressors under adverse environment.

The mechanisms that how bacteria survive in their diverse natural habitats by using ECM and ECM proteins are yet to be fully understood. In a recent study, Floyd et al. [6] studied spatial proteome of surface-associated single-species biofilms formed by uropathogenic Escherichia coli and concluded the presence of at least two regulatory mechanisms controlling type 1 pili expression in response to oxygen availability. Similarly, a recent study on ECM proteome of Bacteroides fragilis, a widely distributed member of the human gut micro biome, identified several lipoproteins, TonB-dependent transporters and auto transporters [7]. Similar to theses investigations, several studies on ECM proteome in E coli were also performed [8–10]. Although these investigations have provided in-depth

information about the certain ECM proteins, it is not known how surface proteins are affected in presence of serum. We, therefore, investigated how blood serum affects ECM and polysaccharide production and surface protein expression in S. aureus using proteomic techniques.

Materials and Methods

Bacterial strain

S. aureus Philips, a biofilm-forming bacterium, was used in this study. In previous studies, we successfully used this strain, which was originally isolated from a patient diagnosed with osteomyelitis Patti et al. (1994); George et al. (2006); George et al. (2007). Secondary cultures was generated by inoculating 1ml of overnight culture into 50ml of TSB and growing at 37 °C with constant rotation in shake flasks for 16 hours. We grew the bacteria at several concentrations of blood serum and found that 12.5% supplemented blood serum similar growth curve as normal TSB. The growth of the bacterial strains was monitored by measuring the absorbance of the broth at 600nm on a spectrophotometer. The cells were then harvested and resuspended in phosphate-buffered saline (D-PBS; 138mm NaCl, 2.7mM KCl, pH 7.4). Cell concentrations was be determined using a Coulter Multisizer.

Measuring PIA

The cell plate was created from one ml of the culture, transferred to a micro tube and centrifuged at 10,000×g for 10 min at 4 °C. One ml of PBS buffer was used to wash the cell plates. Cells were then resuspended in 100μl of 0.5M EDTA, pH 8.0 and boiled in hot water for 10 min at 100 °C. The sample was then centrifuged at 10,000×g for 10 min at 4 °C. The clear supernatant was transferred to a new micro tube. Boiling cells with 0.5M EDTA is the best method known to date for the isolation of crude PIA from staphylococcal cell surface [11]. The crude PIA quantification was performed by a colorimetric method as described elsewhere [12]. Briefly, 50 μl of the crude PIA was transferred to a micro tube and mixed with 25μl of 80% w/v Phenol solution (Sigma-Aldrich) and 1 ml of concentrated sulphuric acid was added. The solution was kept at room temperature for 10 min, and absorbance was read at 490nm. Normalization of the amount of PIA was performed by dividing by the number of cells used for extraction.

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 containingm10 mm Tris HCl, 1 mm EDTA, 5 mm MgCl2. Approximately 5×109 bacterial cells were resuspended in 1ml of digestion mixture containing 35% raffinose, protease inhibitor cocktail (1 tablet/ml of digestion buffer), lysostaphin (5units/ml) and then incubated at 37 °C for 30 min. Cell debris were removed by centrifugation at 8,000g for 20 minutes and the supernatant was collected. After digestion and centrifugation, the digest was kept at −20 °C overnight and then centrifuged at 8,000g for 20min precipitated raffinose was discarded. After digestion and centrifugation, the protein solution was subjected to ultrafiltration using the Millipore ultrafiltration tube and centrifuged as per manufacturer’s instructions. Protein concentration

in the solution was determined using 2 D Quant (GE) and the resulting solution will be stored at −80 °C for 2-DE.

Two dimensional gel electrophoresis

In preparation for 2-DE, 150 μg proteins was resolubilized by adding standard sample solubilization buffers containing urea (8M), thiourea (2M), ASB 14 (1%), DTT (1%), and Carrier ampholytes (0.08%).The resulting solution was diluted to the desired volume with destreak rehydration solutions. Rehydration of IPG strips with the sample was carried out in the Immobiline Dry Strip Re-swelling Tray (GE Healthcare) according to the manufacturer's instructions. IPG strips of pH 3-11 (NL 24 cm) were used. The rehydrated strips were subjected to isoelectric focusing (IEF), performed using IPGphor 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 50mm Tris-HCl, pH 8.8, 6 M urea, 30% w/v glycerol, 2% w/v SDS and traces of bromophenol blue with 100 mg/10 ml (w/v) of DTT.

A second equilibration was carried out for 15 minutes by adding iodoacetamide (250mg/10 ml) instead of DTT in equilibration solution. Second dimension vertical SDSPAGE 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 exits the gel. The gels was stained with Colloidal Coomassie brilliant blue (BioRad). Gels were scanned as 12-bit TIFF images using Biorad GS-800 densitometer and analyzed by Nonlinear Dynamics Same Spots (v.3.2). Spot volumes were normalized by the software to a reference gel. At least three gels (biological replicates) for each treatment was used for analyses.

Protein identification

For mass spectrometric identification, gel spots were excised, destained, and digested with sequencing grade trypsin (Promega). Peptide samples were analyzed by Nano ESI-MS/MS using LTQ (Finnigan, Thermo, USA). Nano LC was performed at reversed phase conditions using an Ultimate 3000 (Dionex corporation, USA) C18 column with a flow rate of 1–5 microliter/min in 70–90% acetontrile containing 0.1% formic acid. MS and MS/MS data was collected and interrogated using SEQUEST against the NCBI non-redundant protein database for S. aureus providing peptide tolerance of 1.4 amu. Searched results were filtered using three criteria: distinct peptides, Xcorr 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 Xcorr value of more than 50 and Sf score for individual peptide of more than 0.8.

Results and Discussion

Blood serum affects polysaccharides intercellular adhesins

We have developed an experimental protocol for isolation and quantification of polysaccharides intercellular adhesins of S. aureus by boiling cells with 0.5M EDTA, digesting the PIA with concentrated sulphuric acid and phenol, and then measuring

absorbance at 490nm. Although isolation of crude PIA by 0.5M EDTA is a routine procedure for PIA purification [13], to our knowledge it has not been reported for crude PIA quantification. We combined the EDTA extraction [13] with determination of sugars and their derivatives by colorimetry [11]. Using this procedure, we were able to reproducibly quantify PIA from S. aureus. As evident from the Figure 1, significantly higher amounts of PIA were observed in presence of blood serum. Similar to these findings, we also observed increased level of PIA in elevated level of NaCl [14].

PIA biosynthesis is mediated by ica operon-encoded enzymes [15,16]. The icaA, D and C gene products are involved in translocation of the growing polysaccharide to the cell surface [17], while IcaB is responsible for deacetylation of the PIA I molecule (providing its positive charge) which is essential for biofilm formation [18]. In contrast, the icaR gene, located upstream of the ica ADBC operon, encodes a transcriptional repressor which plays a central role in the environmental regulation of the ica operon [19]. For example, exposure to NaCl activates the ica operon in an icaR-dependant manner [18–20]. We anticipate that blood serum might have similar effect on the ica operon in an icaR-dependant manner, which is yet to be explored.

Blood serum and fibronectin binding and collagen binding proteins

In the SDSPAGE (Figure 2) (Table 1), several virulence-associated surface proteins were identified such as fibronectin-binding protein (b2), collagen-adhesins precursor (b4, b7), trigger factor (b8). However, serum supplement significantly reduced the abundance of fibronectin binding protein, although the abundance of collagen binding protein was not affected. In a recent report, Shinji H et al. (2011) studied, Fibronectin-binding protein A (FnBPA) and FnBPB, by constructing constructed fnbA and/or fnbB mutant strains and reported that the serum levels of interleukin-6 and nuclear factor κB (NF-κB) activation have no significant reduction in fnbB mutant infection(18)s. It is probable that the NF-κB of serum we used might have reduced the fibronectin-binding protein.

Serum proteins in bacterial surface

We identified two serum proteins, apolipoprotein and globulin (Fc and Fab), in the membrane fraction of bacterial proteins. These results were confirmed from both 1D and 2-DE SDS PAGE. The presence of serum proteins in membrane fraction of bacterial protein has raised several questions. If we consider these proteins as a contaminant from the serum, why were we unable to wash out these proteins while we successfully washed out the most abundant serum protein such as albumin? If not a contaminant, what is causing these proteins to remain attached to the bacterial surface? It is known that Fc and Fab motifs of globulin interact with Spa C and Spa D domains of protein A. But the bacterial strain we used was a mutant of proteins A. In addition, by using a deletion mutant of Newman, we confirmed the presence of Fc and Fab with bacterial membrane associated protein (Figure 3). This raise another question of what components of bacteria are causing this Fc and Fab to remain attached with bacterial proteins.

Conclusion

Polysaccharide intercellular adhesins production was significantly increased due to the addition of blood serum in the media. We identified two serum proteins, apolipoprotein and globulin (Fc and Fab), remained attached with the membrane fraction of bacterial proteins even after several washing procedures, indicating that these proteins might play a critical role in bacterial processes of biofilm formation.

Acknowledgments

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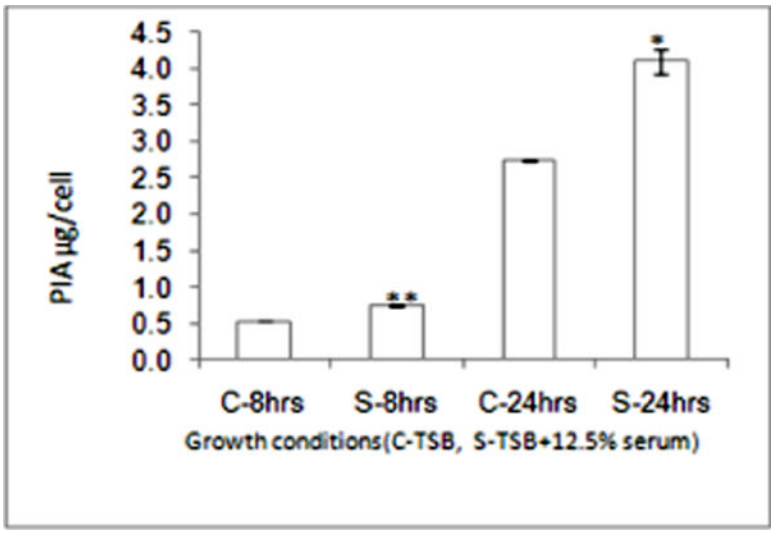

Figure 1.

Polysaccharide intercellular adhesions production as affected by blood serum. The data for each treatment was derived from three replicates; the bars represent standard error. */** denotes tatistically significant difference compared to the controls.

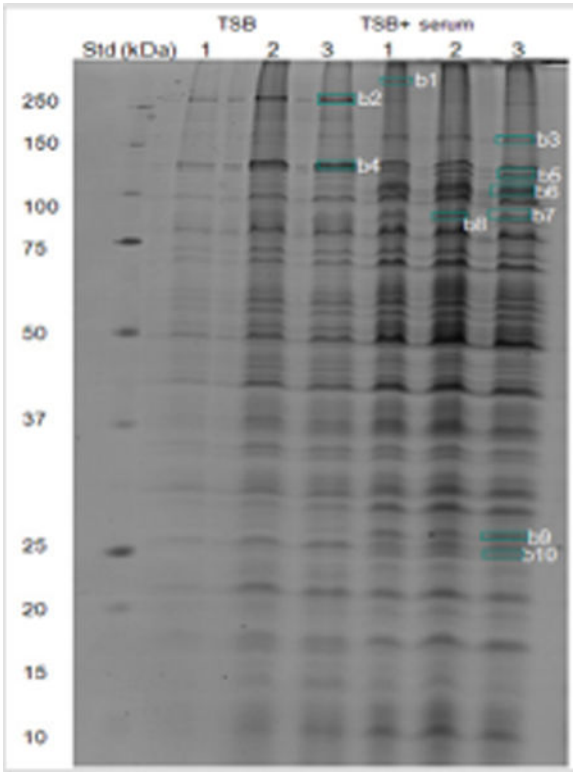

Figure 2.

SDS PAGE gel image of Staphylococcus aureus. 1, 2 and 3 indicate three biological replicates. Membrane protein fraction of bacteria grown in shake flasks was extracted by digesting with lysostaphin in presence of raffinose. After precipitation with cold acetone, proteins were resolved in sample buffer containing 0.5M Tris-HCl (pH 6.8), 2.5% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 10% (v/v) glycerol. 15ug of proteins were loaded in stalking gel (30% acrylamide and 0.8% bis-acrylamide) and separating gel (30% acrylamide and 0.14% bis-acrylamide). Ten bands were shown in the figure and identified by LC ESI.

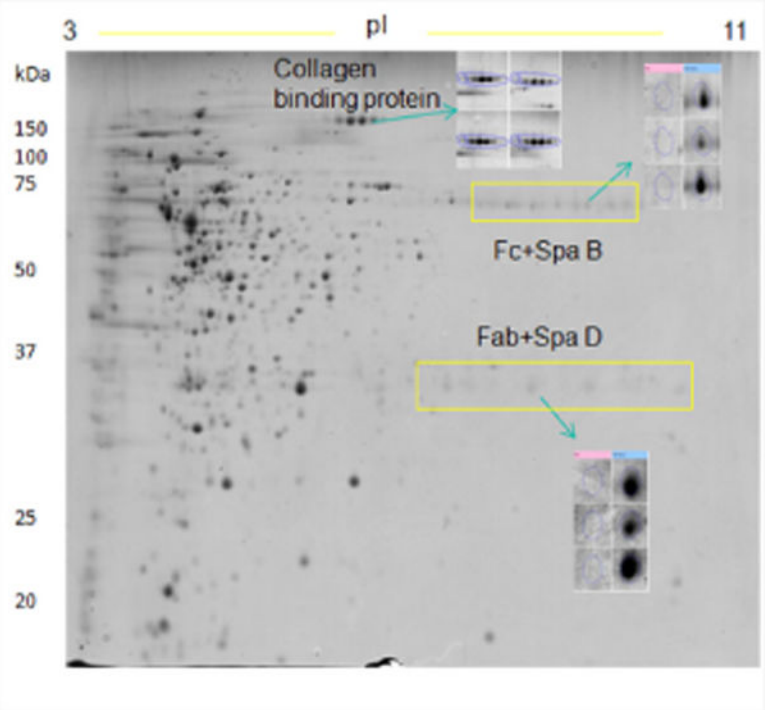

Figure 3.

2DE gel image of Staphylococcus aureus. Membrane protein fraction of bacteria grown in shake flasks was extracted by digesting with lysostaphin in presence of raffinose. Proteins shown on the gel were differentially expressed and identified by LC ESI MS. The in-set images show differentially protein expression in different biological replicates.

Table 1

Identification of proteins bands. Protein bands cut from the gels, digested by trypsin and identified via tandem mass spectrometry (MS).

Band ID	Protein Name/Gene	Functional Category	pliMW	Acce. No.
b1	Apolipoprotein (Homosapien)	Encodes lipoprotein	6_6/515	PO4114
b2	Fibronectin-binding protein (fnb)	Adhesin and anchor	4_4/113	NP_373027
b3	DNA-directed RNA polymerase(rpoB)	Transcription of DNA into RNA	4_7/127	AB 020472
b4	1. Aconitate hydratase (citB)	Carbohydrate metabolism	43/98	NP_371274
	2. Collagen adhesin precursor (MW2612)	Surface Adhesin	5.81133	NP_647429
b5	1. DNA-directed RNA polymerase(rpoB)	Transcription of DNA into RNA	6_5/135	NP_371067
	2.Pyruvate carboxylase (pycA)	Carbohydrate metabolism	5_0/128	NP_371638
	3.1soleucyl-tRNA synthetase (ileS)	Aminio Acid biosynthesis	5.2/104	NP_371717
b6	1. Alanyl-tRNA synthetase	RNA synthesis	4.9/93	NP_372142
	2. Hypothetical protein (SAV0219)	Not known	4.6/37	NP_370743
	3.Collagen adhesin precursor (MW2612)	Surface Adhesin	5.81132	NP_647429
b7	1 Alpha-keto acid dehydrogenase (pdhc)	Carbohydrate metabolism	4.8/46	NP_371619
	2. Preprotein translocase (secA)	Protein export	5_0/46	NP_371277
	3. Translation initiation factor (infE)	Protects of tRNA from hydrolysis	5.2/77	NP_371619
b8	1. Formate acetytansferase	Anaerobic glycolysis	52184	NP_370750
	2. CIpB chaperone-like protein	Stress proteins	4_2/98	NP_371499
	3. Translation initiation factor IF-2	Translation	5.0/17	NP_371793
	4. Trigger factor	Promotes protein folding	4.248	NP_372199
	5. Branched-chain alpha-keto acid dehydrogenase subunit E2	Carbohydrate metabolism	4.8/46	NP_371619
b9	1. A Chain A, Crystal Structure At 2.7a Resolution Of A Complex	Binding as a protein complex	8.0/23	1DEE_A
	Between A Staphylococcus Aureus Domain And A Fab Fragment Of A			
	Human lgm Antibody			
	2. 50s ribosomal protein	Expression of mRNA	9_5/24	NP_371062
b10	1. Triosephosphate isomerase	Glycolysis	4.7125	NP_371298
	2. Purine nucleoside phosphorylase	Purine metabolism	43/26	NP_372662
	3. Deoxyribose-phosphate aldolase	Pentose phosphate pathway	4.5/23	NP_370662
	4_Phosphoribosylaminoimidazole-succinocarboxamide synthase	Purine metabolism	5.2/26	NP_371590
	5. Putative translaldolase	Carbohydrate metabolism	5.6/26	NP_6465,38
	6. A Chain A, Crystal Structure At 2.7a Resolution Of A Complex	Binding as a protein complex	8.0/23	1DEE_A