Mini Review
Current Proteomics

Title:

Understanding virulence mechanisms in *Staphylococcus aureus*: an update on proteomic and bioinformatic investigations

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Abstract: *Staphylococcus aureus,* one of the major pathogenic bacteria, is associated with substantial morbidity and mortality. The disease burden of staphylococcal infections is significant, which is primarily attributed to its adaptability and resistance to environmental stresses. *S. aureus* has the ability to develop multiple resistances to antimicrobial agents. These high resistances make pathogenicity of *S. aureus* one of the most complex mechanisms to understand and manage. Proteomic approaches show great potential in exploring microbial adaptation strategies, ability to cause disease by pathogenic bacteria and the development of diagnostic tools. A summary of the latest developments in the application of proteomic technologies to understand resistance mechanisms in *S. aureus* and their future role in anti-staphylococcal vaccine and/or drug discovery is given here.

Keywords: Bioinformatics, methicillin-resistant, pathogenicity, proteomics, *Staphylococcus aureus*, virulence

1. INTRODUCTION

Research in microbiological sciences has entered the post-genomic era more than a decade ago now. Proteomic and bioinformatics methods have emerged as powerful tools to investigate physiological conditions, mutations, changes in response to external factors and adaptation [1, 2]. Rapid developments in research tools used in proteome analysis i.e. one-dimensional polyacrylamide gel electrophoresis (ID-PAGE), two-dimensional electrophoresis (2DE), Matrix Assisted Laser Desorption/Ionization-Time of Flight-Mass Spectrometry (MALDI-TOF-MS) and Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS) has allowed the identification and quantification of global protein-protein and protein-ligand interactions named as the 'interactome'. The genome sequence only provides the blueprint of life whereas the proteome brings this genome sequence to real life [2]. Identification of proteins has substantial research applications in life and health sciences including bio-prospecting, detection and cause of diseases and drug discovery. Protein expression profiling is an excellent approach not only to expressed protein pattens,depending on environmental conditions, but also to show to what extent each single protein is expressed. This proteomic view of the cell's physiology provides global information on basic cell functionalities, on metabolism and/or on stress/starvation responses [3].

The pathogenesis of *S. aureus* is a complex process that involves the strongly coordinated synthesis of cell wall-associated proteins and extracellular toxins. Methicillin-resistant *Staphylococcus aureus* (MRSA) is at present the most commonly identified antibiotic-resistant pathogen in many parts of the world [4, 5]. Hospital-acquired MRSA infection increases morbidity, the risk of mortality and costs [6]. The societal costs accrue either directly or indirectly and financial repercussions include the costs for containment of outbreaks and changes to empiric antibiotic prescribing habits. Plowman *et al.* [7] estimated the costs of all hospital-acquired MRSA to the National Health Service in England to be ca.

£1 billion, including community costs of over £50 million. In Australia, as in most of the world, antimicrobial resistance in *S. aureus* is a major impediment to effective treatment. An Australian survey of *S. aureus* bacteraemia from 1999 to 2002 documented 3,129 episodes [8].

Pathogenicity of *S. aureus* is determined by the ability of the organism to express multiple virulence factors [9]. The existence of a great variety of virulence factors enables *S. aureus* to cause a broad spectrum of infections ranging from superficial abscesses, osteomyelitis, endocarditis, and toxic shock syndrome [10]. These illnesses cannot always be successfully treated with commonly used antibiotics and because MRSA isolates are becoming increasingly prevalent in the community, additional control strategies are urgently needed. A vaccine for *S. aureus* can offer such a mechanism which would boost the immune system to eradicate the infecting microbe. Whole genome analysis of *S. aureus* isolates have changed the way investigators approach the classical questions asked about the bacterial-host interaction. Proteomic studies have provided new insights into bacterial pathogenesis and vaccine discovery concepts. New opportunities are arising to use proteomic approaches to understand the regulatory and adaptation mechanisms of *S. aureus*. This review will discuss recent advances in *S. aureus* proteomics.

2. PROTEOMIC METHODS USED TO INVESTIGATE *S. AUREUS* PATHOGENESIS

New approaches to study pathogenic bacteria using combined technologies of genomics, proteomics and bioinformatics is rapidly increasing globally. The use of proteomic tools can lead to the identification of immunogenic proteins that may be potential vaccine targets as well as improving the understanding of antibiotic action. Proteomics has generated innovative and valuable information on bacterial pathogens and will continue to be an important source

of information in the coming years [11]. Proteomic investigations were initially developed based on ID-PAGE and 2DE. Recently, the 2DE gel approach combined with mass spectrometry for protein identification was used to create master gels of extracellular proteins of *S. aureus* in order to study the regulatory network involved in the pathogenicity of this organism [12]. Furthermore, cytoplasmic and membrane proteins of *S. aureus* were analyzed by gel-free proteomic approaches [13]. Graham *et al.* [14] reviewed a basic overview of the applications of mass spectrometry in protein identification and ways to reduce the protein complexity of microbial samples in gel-based and gel-free methodologies.

Protein sample preparation is a vital part of proteomic analysis. Considering the importance of MRSA, proteomic methods particularly focusing on the preparation, identification and analysis of different protein fractions have been developed [15]. Nandakumar *et al.* [16] described a method to lyse cells with lysostaphin, which lyses staphylococcal peptidoglycan, followed by solubilization with urea, thiourea, amidosulfobetaine 14 (ASB 14) and dithiothreitol (DTT) in order to generate membrane/cell wall proteome map of *S. aureus.* This method minimizes the contamination from cytosolic proteins. Globally research efforts have been made to correlate transcriptomic and proteomic data [13] to streamline the process of peptide identification [17]. A variety of proteomics techniques to study *S. aureus* infections are available now (see a review by Francois *et al.* [18]).

3. CURRENT STATUS OF PROTEOMIC AND BIOINFORMATIC TOOLS IN *S. AUREUS* VIRULENCE AND ANTIBIOTIC RESISTANCE MECHANISM INVESTIGATIONS

The numbers of different proteins produced by *S. aureus* are more than genes [19]. Though a great wealth of *S. aureus* genomic information is available now, yet information on

functional proteomics is limited. Therefore, for a better understanding of virulence factors and resistance mechanisms to antibiotics, it is important to know the functionalities of the proteins involved. An example of how important it is to find out the functional elements of an operative protein can be seen in Boisset *et al.* [20] study. It was suggested that Rot, a transcriptional regulatory protein, activated the synthesis of several exoproteins through the indirect action of RNAIII on many downstream genes. Thus emphasizing the regulatory role played by RNAIII and its 3′ domain in establishing a network of *S. aureus* virulence factors. A recent report based on transcriptomic and proteomic analysis of *S. aureus* showed that *RsaE* regulates the synthesis of proteins involved in various metabolic pathways of *S. aureus* [21]. A coordinated action of multiple regulators, transcriptional regulatory proteins, the alternative sigma B factor and the regulatory RNAIII are the major tools used by *S. aureus* for the expression of numerous virulence factors and causing a diverse range of infections [22, 23]. This complexity of regulatory networks is a vital weapon of this pathogen to integrate multiple external signals.

Many researchers are currently using tools to look for signal peptide sequences and structural motifs located within genomic sequences in order to develop vaccines. Genome sequences of ten *S. aureus* strains have been published to date (www.tigr.org; www.ncbi.nlm.nih.gov). There are 2600-2700 open reading frames for these strains. Despite the detailed availability of genome information, the function of at least one thousand proteins encoded by these genes is still unknown [19]. The availability of genomic information has enabled the parallel use of microarrays and proteomics in *S. aureus* resistance and pathogenicity studies. Mapping of membrane-associated proteins provides a benchmark for biological experimentation such as strain comparisons of pathogens with differing modes of pathogenicity and antibiotic resistance mechanisms, and can aid in the definition of vaccine and therapeutic targets [24]. Brötz-Oesterhelt *et al.* [25] reviewed the major achievements of

proteomic approaches to investigate bacterial survival and adaptation networks with a special emphasis on the stress induced by antibiotic treatment. They also explored the possibilities of applying bacterial proteomic studies directly for the antibacterial drug-discovery process.

Proteome analyses of *S. aureus* to investigate cell physiology and regulatory networks have revealed valuable information. Gel-based proteomics was considered as an extremely valuable tool in microbial physiology that had the flexibility to be used with various visualization and quantitation software programs for rapid and high-output of data from multiple samples [19]. A proteomic comparison made on growing and non-growing *S. aureus* cells showed higher production of enzymes involved in protein synthesis, transcription, and glycolysis in exponentially growing cells than stationary-phase cells [2]. Pieper *et al.* [26] compared the proteome maps of three isogenic strains of *S. aureus* to determine differences in their resistance to vancomycin. Comparative protein abundance analysis showed high expression of enzymes involved in the purine biosynthesis pathway in a vancomycin-resistant strain. A more recent report verified the expression of proteins predicted from genomic ortholog comparisons among 17 environmental and pathogenic bacteria. These expressed proteins showed exclusive relationships among the content of phenotypically related bacteria, which is indicative of the specific lifestyles associated with these organisms. It was demonstrated that proteomic studies would be able to establish expressed lifestyle differences among conserved genes [27].

Bioinformatic analysis of the intergenic regions of several *S. aureus* strains showed the expression of 11 novel, stable and Hfq-independent, noncoding RNAs (RsaA-K) in the late-exponential phase of growth. The transcription was regulated by the alternative σ^B factor (RsaA, D and F) in the majority of cases and the expression of RsaE is *agr*A-dependent. This transcriptomic and proteomic analysis indicated that RsaE regulates the synthesis of proteins

involved in various metabolic pathways. Phylogenetic analysis conducted in this study showed that most of the novel ncRNAs carry the conserved C–rich motif which was linked to members of a class of ncRNAs that target mRNAs by a shared mechanism [21]. The ability of bioinformatics to characterize genomic sequences from pathogenic bacteria for the prediction of genes that may encode vaccine candidates, e.g. surface localized proteins, has been evaluated [28, 29]. A combination of genomic, proteomic and bioinformatics led to the identification of a putative vaccine candidate, the outer membrane lipoprotein P6, for *Haemophilus influenzae* [30, 31]. The application of proteomic and bioinformatic techniques for the identification of vaccine candidates for another pathogenic bacterium, *Helicobacter pylori*, has also demonstrated that utilization of bioinformatics to study genomes and proteomics is a useful approach for vaccine discovery [32]. Similar approaches would be useful to investigate *S. aureus* proteomics aiming vaccine candidate protein or group of proteins.

4. MRSA PROTEOMICS

Proteomics is an ideal tool to analyse differences in gene expression between bacterial strains with different phenotypes. Generally *S. aureus* strains are grouped on the basis of their sensitivity to methicillin; methicillin-susceptible *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA). Currently, the focus is on strains causing disease in the community, MRSA in particular (Table **1**). It is not always true that MRSA strains are more virulent than methicillin-susceptible *S. aureus* strains. However, some MRSA strains contain factors or genetic backgrounds that may enhance their virulence or ability to cause particular diseases [40]. The latest technological advances in proteomic tools would be helpful for researchers to elucidate the complex molecular mechanisms involved in virulence and the resistance of MRSA [17]. Cordwell *et al.* [33] used 2DE analysis combined with Mass-Spectrometry to

map the proteins of methicillin-resistant (COL) and methicillin-susceptible (8325) strains of *S. aureus.* A total of 377 proteins were identified including 266 distinct ORFs corresponding to proteins and 14 potential virulence factor proteins. Strain COL showed deferential expression of 12 protein spots, which included alkaline-shock protein 23 (Asp23) and cold-shock proteins CspABC. These proteins either were not present or were in significantly lower intensity in the methicillin-susceptible strain 8325. These comparative maps were used to characterize the *S. aureus* response to treatment with Triton X-100 (TX-100). It was reported that 44 protein spots showed altered levels of abundance, and 11 of these spots were found only in COL in the presence of the detergent (TX-100). Significantly altered levels of the gene products regulated by σ^B (the alternative sigma factor), including Asp23 and three proteins of unknown function, and *SarA* (a regulator of virulence genes) were observed in this study. The production of the essential methicillin-resistance factor FemA was not affected by growth of both strains in the presence of TX-100. This study suggested that proteins such as σ^B, *sarA* regulons along with other factors could be involved in methicillin resistance in *S. aureus*.

A comparative proteomic analyses of the expression of SAV2095 mRNA levels between 25 MRSA and vancomycin-intermediate *Staphylococcus aureus* (VISA)/heterogeneous VISA clinical isolates revealed high induction of SAV2095 mRNA in all VISA isolates relative to all MRSA strains ($p < 0.001$), and in several potential heterogeneous VISA strains. Increases in levels of SAV2095 expression in four unrelated clinical MRSA isolates displayed increasing levels of resistance to vancomycin. The results of this study suggested that SAV2095 expression levels could serve as a molecular diagnostic marker for the rapid detection of VISA [34]. This demonstrates the applications of proteomics in differentiating pathogens with varying levels of virulence abilities.

Immunological assays in conjunction with proteomics analysis were used in a study by

Glowalla *et al.* [35], which attempted to identify novel vaccine candidates. This approach used intravenous immunoglobulin (IVIG) preparation as a source of antibodies directed against anchorless *S. aureus* surface proteins and a subtractive proteome analysis (SUPRA) technique was employed to identify these proteins. Three candidate proteins (out of 40 proteins preselected on these basis of MALDI-TOF analysis), enolase (Eno), oxoacyl reductase (Oxo), and hypothetical protein hp2160, were expressed as glutathione S-transferase fusion proteins, purified, and used for enrichment of corresponding immunoglobulin Gs from IVIG by affinity chromatography. It was suggested that anchorless cell wall proteins along with identification of more target proteins in future evaluation by the SUPRA technique would be valuable in the formulation of a multivalent vaccine.

5. APPLICATION OF PROTEOMICS IN *S. AUREUS* VIRULENCE MECHANISM INVESTIGATIONS AND VACCINE DISCOVERY

To date the role of different virulence factors of this versatile pathogen in the development of staphylococcal infections remains poorly understood. Moreover, genomic approaches to vaccine development, termed “reverse vaccinology”, has not resulted in the successful development of an anti-staphylococcal vaccine [41, 42]. This approach entails the mining of genomic data *in silico* to help identify proteins encoded by a microorganism. However, proteomic technologies can serve as an important complement to the reverse vaccinology approach to antigen discovery. Proteomic techniques have the ability to identify protein expression by the pathogen to infect a host as well as the surface associated protein of the pathogen. These two groups of proteins could play a vital role in vaccine antigen selection to boost host immune response for a better protection against the pathogen [43]. A schematic illustration of such a proteomic approach to develop a vaccine is shown in Figure **1**.

Protein expression in bacteria has been extensively documented using different proteomic techniques. This has resulted in datasets reporting up to 40 to 60% of the theoretical proteome of *S. aureus* [13]. Inherently, there are some limitations with respect to the proteome coverage of 2DE-based maps. An analysis of the identified proteins using Transmembrane Hidden Markov Model (TMHMM) 2.0 [44] shows that proteins with helical trans-membrane domains are highly under-represented. While around 700 proteins or 25% of the theoretical proteome of *S. aureus* are predicted to contain at least one helical trans-membrane domain, of which only 16 proteins have been identified [45]. This is in accordance with many previous observations that 2DE is not an adequate approach for the profiling of membrane proteins. Conventional proteomic methodologies use 2DE gels to separate bacterial cell components followed by the identification of immunoreactive spots by mass spectrometry. These methods are only able to sample proteins which are produced by the bacterium in broth culture in the laboratory. Different proteins that are only expressed in an animal or human host will not be available for analysis. Although bioinformatic tools can predict the cellular location of bacterial proteins using homology searches, they are not capable of providing a perfect indication of protein subcellular localization. It is of utmost importance to characterize membrane, surface-associated and secretory proteins of *S. aureus* because these sub-proteomes substantially determine its virulence, and can be used as diagnostic targets or serve as vaccine candidates.

Use of proteomic approaches to study *S. aureus* is limited to metabolic studies and identification of drug targets. A comprehensive proteome map is an essential tool for a better understanding of the cell physiology of this human pathogen [2]. Thus progress in bacterial proteomics will be highly beneficial for medical research in particular to evaluate virulence in resistant strains in a single experiment [46]. There is still a long way to go for fine-tuning

proteomic applications in this area before we start to understand infection, pathogenesis and adaptation of *S. aureus* [18].

6. CONCLUSION

A pan-proteome concept can be instrumental in providing a novel selection criterion for the identification of potential diagnostic, therapeutic and vaccine targets. An emerging field of study is population proteomics. This discipline tries to address the spatial and/or temporal structure of populations (meta-populations) at the protein level. It is anticipated that such studies will overcome the apparent separation between population genomics and proteomics. The knowledge gained from a "pan-proteomic" approach will enable the discovery of targets that are essential to the bacterial organism to cause an *in-vivo* infection or to express virulence factors. A single proteome sequence is not entirely representative of a given bacterial species. As a consequence, an effective vaccine or diagnostic is only possible by including a combination of antigens based on the information retrieved from the pathogenic population structure. At the level of the expressed proteins, this diversity is even more spectacular. Differences in protein expression demonstrate the importance of combining information at different levels before determining the most relevant targets for inhibitors or vaccines.

We are getting closer to a time when vaccines will not be based on a single protein from a single organism, but rather from a collection of conserved proteins that together protect against a group of organisms. Therefore further advances are needed in the current knowledge of the disease-causing ability of the major hospital- and community-associated MRSA clones found around the world.

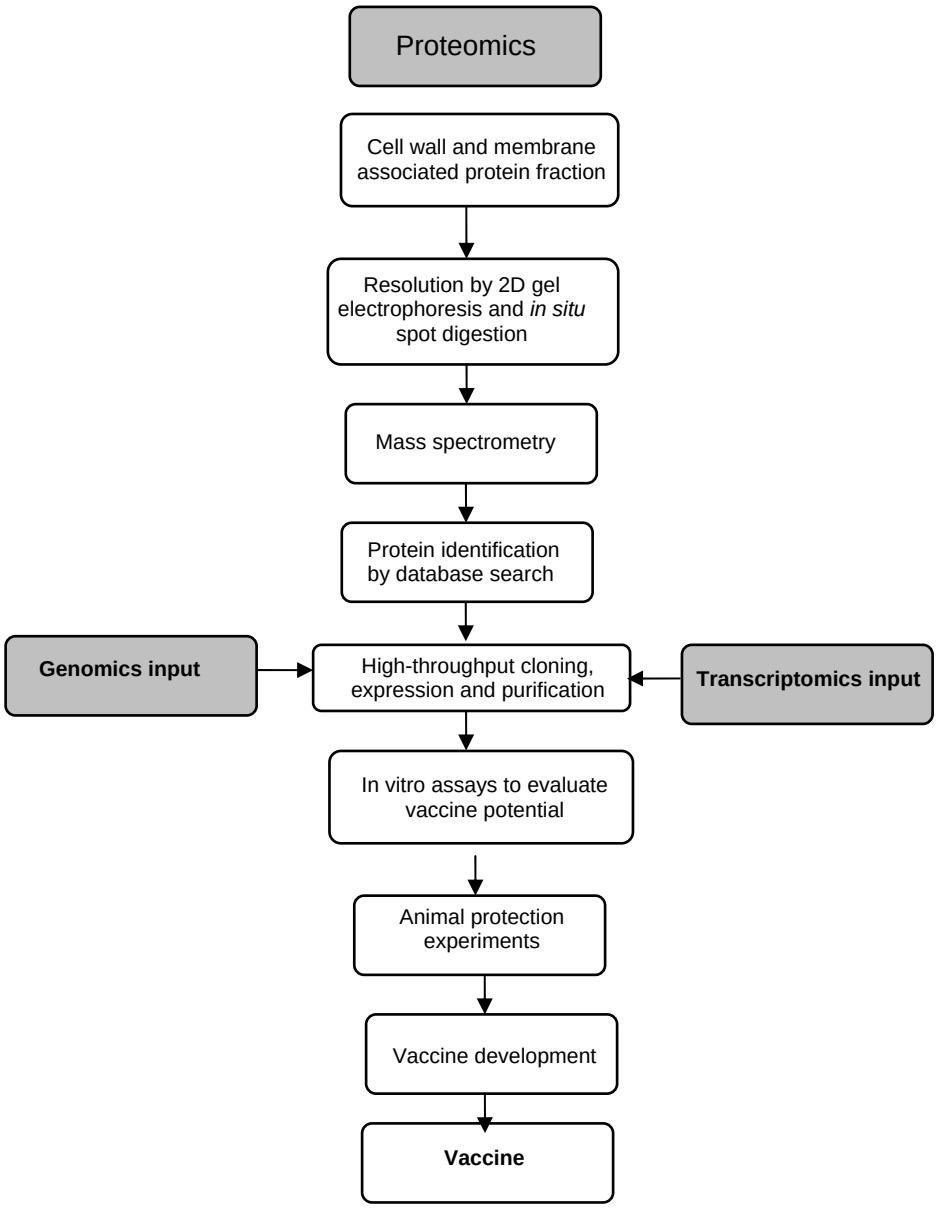

Fig. (1). A Schematic Overview of Proteomics Approaches Commonly Used in Vaccine Discovery Investigations.

Table 1. Proteins Identified in Methicillin-resistant *Staphylococcus aureus* Strains Linked to Their Virulence

Proteins/regulators identified	MRSA strains/isolates	Functionality/predicted role	References
σ^B, *sarA* regulons	*S. aureus* COL	Involved in methicillin resistance	[33]
SAV2095 mRNA	25 MRSA clinical isolates	Resistance to antibiotic vancomycin	[34]
enolase (Eno), oxoacyl reductase (Oxo), and hypothetical protein hp2160	*S. aureus* USA300	Anchorless cell wall proteins	[35]
cytotoxins, enterotoxins, proteases, lipolytic enzymes, peptidoglycan hydrolases	Methicillin resistance *S. aureus* COL	Virulence factors	[36]
63 Sec-dependent extracellular proteins	25 *S. aureus* isolates from human infections. *S. aureus* (RN6390, COL and Newman) used as reference strains.	*S. aureus*-associated virulence	[37]
Surface exposed proteins (the membrane protein FtsL, the elastin-binding protein EbpS, the amidase LytH and the glycerol phosphate lipoteichoic acid synthase LtaS)	*S. aureus* (Newman, COL, RN6390, RN4220, NCTC8325 and USA300)	The targets for novel drugs, therapeutic antibodies or vaccines.	[38]
VraR	*S. aureus* RN1HG	Vancomycin resistance-associated sensor/regulator	[39]

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