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Review

Proteomics in studies of *Staphylococcus aureus* virulence*

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Staphylococcus aureus **is a widespread, opportunistic pathogen that causes community and hospital acquired infections. Its high pathogenicity is driven by multifactorial and complex mechanisms determined by the ability of the bacterium to express a wide variety of virulence factors. The proteome secreted into extracellular milieu is a rich reservoir of such factors which include mainly nonenzymatic toxins and enzymes. Simultaneously, membrane proteins, membrane-cell wall interface proteins and cell wall-associated proteins also strongly influence staphylococcal virulence. Proteomics shows a great potential in exploring the role of the extracellular proteome in cell physiology, including the pathogenic potential of particular strains of staphylococci. In turn, understanding the bacterial physiology including the interconnections of particular factors within the extracellular proteomes is a key to the development of the ever needed, novel antibacterial strategies. Here, we briefly overview the latest applications of gel-based and gel-free proteomic techniques in the identification of the virulence factors within *S. aureus* secretome and surfacome. Such studies are of utmost importance in understanding the host-pathogen interactions, analysis of the role of staphylococcal regulatory systems and also the detection of posttranslational modifications emerging as important modifiers of the infection process.**

Key words: *Staphylococcus aureus*, proteomics, virulence factors, secretome, surfacome

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INTRODUCTION

Over the last few years whole genome sequences (WGS) of multiple *Staphylococcus aureus* strains become available (Price *et al.*, 2013). The genetic information coupled with proteomic techniques permit to study *S. aureus* virulence in previously unavailable details (Pocsfalvi *et al.*, 2008; Becher *et al.*, 2009; Ravipaty & Reilly, 2010; Ziebandt *et al.*, 2010; Gurung *et al.*, 2011; Hessling *et al.*, 2013; Kolar *et al.*, 2013; Smith *et al.*, 2013; Basell *et al.*, 2014; Islam *et al.*, 2014). It is widely accepted that staphylococcal pathogenicity is driven by a wide variety of virulence factors rather than single toxins as observed in certain other bacteria. The extracellular and cell wall-associated proteins known as secretome (Khoon & Neela, 2010) and surfacome (Dreisbach *et al.*, 2011a), respectively, are crucial factors enabling host colonization and, subsequently, acute infection (Zecconi & Scali, 2013). The detailed proteome analysis is a powerful tool to explore the interplay of factors responsible for the pathogenicity of bacteria. Indeed, proteomic approach provides a detailed overview of *S. aureus* cellular processes, helps to uncover the virulence determinants thus allowing to reveal the complexity of the mechanisms of pathogenicity.

The proteomic analysis uses various techniques which may be most generally divided into two groups: gel-based and gel-free. Recent reviews provide the current state of proteomic characterization of various microorganisms (Chao & Hansmeier, 2012). Other articles describe the impact on utilizing diverse proteomic methodology to understand the mechanisms of *S. aureus* virulence (Hecker *et al.*, 2010). This diverse technique also provides important insights into elucidation of mechanisms of *S. aureus* antibiotic resistance and found its role in anti-staphylococcal vaccine development and drug discovery (Hussain & Huygens, 2012). Here, we aimed to concisely present recent advances of proteomics in identification of novel virulence factors, understanding the host-pathogen interactions, analysis of the role of global regulatory systems and detection of posttranslational modifications of proteins which likely affect staphylococcal virulence.

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Abbreviations: 2-DE, two-dimensional gel electrophoresis; 2D-DIGE, two-dimensional fluorescence difference gel electrophoresis; AD, atopic dermatitis; *agr*, accessory gene regulator; Atl, bifunctional autolysin; CA-MRSA, community-associated methicillin-resistant *Staphylococcus aureus*; Can, collagen-binding protein; ClfA, clumping factor A; ClfB, clumping factor B; Coa, coagulase; Efb, fibrinogen-binding protein; ESI, electrospray ionization; ETs, exfoliative toxins; FnBPA, fibronectin-binding protein A; FnBPB, fibronectin-binding protein B; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HlgA, Gamma-hemolysin, component A; HlgB, Gamma-hemolysin, component B; IMAC, immobilized metal affinity chromatography; IsaA, immunodominant staphylococcal antigen A; Isd, iron-regulated surface determinant; iTRAQ, isobaric tags for relative and absolute quantification; LC-MS/MS, liquid chromatography tandem mass spectrometry; Luk, leukocidin; MALDI, matrix-assisted laser desorption/ionization; MDLC, multi-dimensional liquid chromatography; MS, mass spectrometry; MRSA, methicillin-resistant *Staphylococcus aureus*; MSCRAMMs, microbial surface components recognizing adhesive matrix molecules; MSSA, methicillin-susceptible *Staphylococcus aureus*; MVs, membrane vesicles; NO, nitrogen monoxide; PTM, post-translational modifications; PVL, Panton-Valentine leukocidin; *S. aureus, Staphylococcus aureus*; SarA, staphylococcal accessory regulator A; SasG, *Staphylococcus aureus* surface protein G; Sdrs, serine-aspartate repeat proteins; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SILAC, stable isotope-labeling by amino acids in cell culture; Spa, staphylococcal protein A; SspB, staphopain B; SSTIs, skin and soft tissue infections; Stk1, serological thymidine kinase 1; TOF, time of flight; VISA, vancomycin-intermediate *Staphylococcus aureus*; vWbp, von Willebrand factor-binding protein.

A RESERVOIR OF *STAPHYLOCOCCUS AUREUS* VIRULENCE FACTORS

S. aureus is a gram-positive bacteria and a component of human and animal microbial flora. *S. aureus* is transiently or persistently colonizing at least a quarter of the human population without causing any apparent adverse effects. In parallel, *S. aureus* is one of the major human and animal pathogens associated with substantial morbidity and mortality. *S. aureus* is one of the major causes of community-acquired infections. It is also responsible for an estimated 40% of all nosocomial infections (2004; Boucher & Corey, 2008; Becher *et al.*, 2009). The bacterium is able to infect almost every tissue or organ of the body. As such it is responsible for a broad spectrum of symptoms beginning from relatively harmless superficial abscesses and similar minor ailments, but also severe conditions such as endocarditis, osteomyelitis, toxic shock syndrome or sepsis (Waldvogel, 1995). This Janus face of *S. aureus* results in a fact that despite years of investigation, factors responsible for the commensal and pathogenic phenotype are still only partially distinguished and the underlying processes are poorly understood. It is currently postulated that immunomodulation within host defense system is the major event in the transition of *S. aureus* from an asymptotic commensal cohabitant to a lethal pathogen (Peres & Madrenas, 2013). *S. aureus* pathogenesis was linked with the production of a large variety of factors, including membrane proteins, membrane-cell wall interface proteins, cell wall-associated proteins and proteins actively secreted *via* different pathways into the extracellular milieu. All these factors enable and determine the pathogenicity of the bacteria, playing pivotal role in the colonization and invasion of host tissues, mediate cytotoxicity against the host, facilitate persistence and help in the evasion of the host immune system. As such, these proteins constitute a reservoir of virulence factors (Fig. 1) (Lowy, 1998; Engelmann & Hecker, 2009).

S. aureus utilizes various strategies to colonize and latter infect the host. These strategies are mediated by production of virulence factors (Krishna & Miller, 2012). In line with the above, the virulence factors are divided into two major groups (Zecconi & Scali, 2013). The first group is involved in early stages of infection and encompasses proteins engaged in bacterial adhesion to the host cells and extracellular matrix, the process known as colonization. At this stage peptides that enable intercellular communication are also of importance.

A primary event in *S. aureus* colonization involves adherence to host components mediated by binding of fibrinogen, fibronectin and cytokeratins usually within nasal epithelium or epidermis. The binding is mediated by surface exposed proteins of *S. aureus* referred to as MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) which include: two related fibronectin-binding proteins (FnBPA, FnBPB), collagen-binding protein (Can), fibrinogen-binding proteins (Efb), clumping factors (ClfA, ClfB), staphylococcal protein A (Spa) a potent immunomodulatory molecule that binds

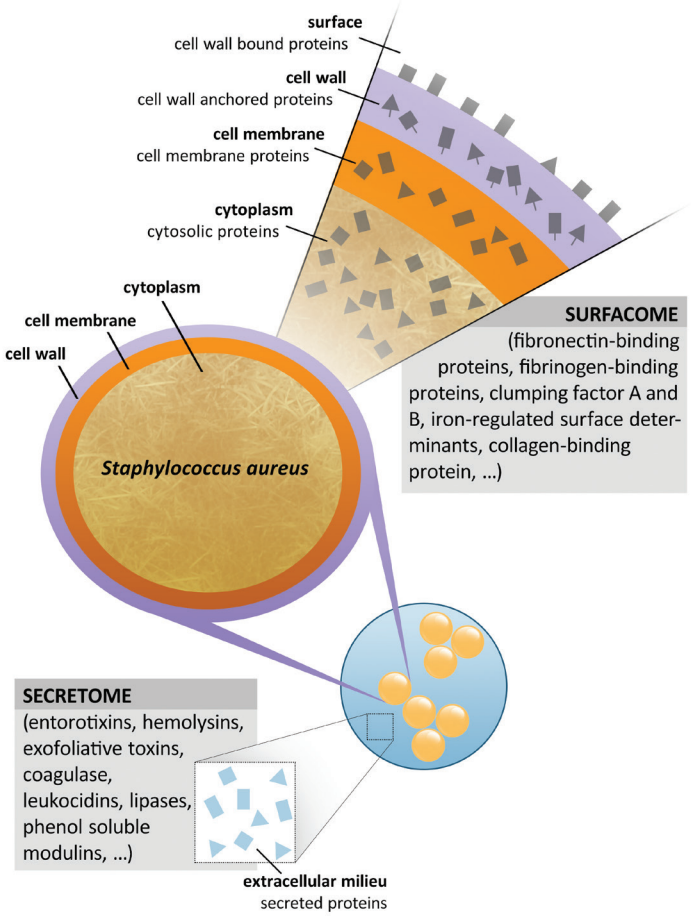

Figure 1. Localization of *Staphylococcus aureus* virulence factors in the context of proteomics.

immunoglobulins G and von Willebrand factor, and a set of iron-regulated surface determinants (Isd — IsdA, IsdB, IsdC, IsdH) implemented in resistance to phagocytic killing, promoting bacterial adhesion to squamous cells, binding lactoferrin and thus protecting *S. aureus* from bactericidal effects of host's factors (Foster, 2009). Elastin-binding protein (Ebp) and serine-aspartate repeat proteins (Sdrs — SdrC, SdrD, SdrE) also act as adhesins. Extracellular matrix protein-binding protein (Emp) is involved in biofilm formation. *S. aureus* surface (Sas) proteins, including for example protein G (SasG), promote bacterial adhesion to squamous cells (Foster & Hook, 1998; Foster, 2009; Khoon & Neela, 2010). Moreover, proteins released to extracellular milieu such as von Willebrand factor-binding protein (vWbp) and coagulase (Coa) also facilitate colonization. Coa and vWbp mediated activation of prothrombin leads to localized clotting which results in immobilization of the bacteria in a pseudocapsule providing protection against host immune cells (McAdow *et al.*, 2012; Dubin *et al.*, 2013).

The second group of virulence factors encompasses proteins becoming important upon successful host colonization which in turn mediate detachment and spread within the host. This group includes mainly enzymes and nonenzymatic toxins. Hemolysins (Hla, Hlb, Hlγ) permeabilize cell membranes and lyse macrophages and lymphocytes and alter platelet morphology. Leukocidins (LukD, LukE, LukM), and Panton-Valentine leukocidin (PVL) which often associates with severe infections (Cremieux *et al.*, 2009), form pores in leukocyte membranes which results in cell death (Watkins *et al.*, 2012). LukGH leukocidin enhances inflammatory response (Malachowa *et al.*, 2012) and contributes to the development of abscesses by killing the infiltrating neutrophils. Phenol soluble modulins (PSMs) are yet another type of cytolytic toxins (Laabei *et al.*, 2014). Exofoliative toxins (ETs — ETA, ETB, ETD) are in turn extremely specific serine proteases hydrolyzing desmoglein 1, a cadherin responsible for the integrity of cell-to-cell adhesive structures. Due to desmoglein-1 localization within the skin, the action of ETs results in skin exfoliation (Bukowski *et al.*, 2010). Enterotoxins (SEs — SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI) exhibit superantigen activity deregulating the immune system of the host.

Secreted enzymes belonging to group two mediate the conversion of host cells components and extracellular proteins enabling nutrient acquisition and deregulating the immune system in parallel to nonenzymatic toxins. The latter process involves among others the degradation of host antimicrobial peptides. These mechanisms negatively affect the host innate and adaptive immune response (Dinges *et al.*, 2000; Pocsfalvi *et al.*, 2008; Khoon & Neela, 2010). Defining the mode of action and interplay of virulence factors is crucial for understanding the process of pathogenesis and a key to identification of new drug targets and development of vaccines (Wu *et al.*, 2008). Localization and differential expression of virulence factors is most conveniently analyzed by proteomics. Investigation of *S. aureus* secretome brings us ever closer to the full understanding of host-pathogen interactions. The studies on protein expression, targeting, transmembrane crossing, cell wall anchoring, posttranslational maturation and release to the extracellular milieu provide an overview of how *S. aureus* secretome and surfacome are involved in colonization and pathogenesis and how host defense system evasion leads to the development of diseases and toxinoses (Khoon & Neela, 2010).

PROTEOMIC TOOLS IN EXAMINATION OF *S. AUREUS* VIRULENCE FACTORS

Genomic sequences provide only the "blue-print" of life whereas the proteome brings genome sequence to real life (Becher *et al.*, 2009). The name "proteome" coined by Marc Wilkins in 1994 describes the complete set of proteins expressed by an organism, cell or tissue at a given time (Wilkins, 1996). Opposite to the genome, the proteome is highly dynamic as proteins are continually synthesized, modified, distributed and finally degraded. Therefore, the proteome is a collection of proteins occurring under specific physiological circumstances and time. The proteomics is a set of techniques dedicated to the identification and extensive characterization of proteins including their interaction networks, functions and posttranslational modifications. Proteomics constitutes an excellent tool that permits visualization and quantification of the proteome. This allows to truly understand the functions of genes. Proteomics reveals the overall pattern of protein expression under given environmental conditions and its changes in response to various stimuli, including host-pathogen interactions. Moreover, it provides information on expression level and subcellular localization of individual proteins. Proteomics is currently employed in the massive exploration and evaluation of bacterial processes, *inter alia* provides a comprehensive insight into bacterial virulence.

The *S. aureus* proteomic era began in 2001 when the genome sequence of two methicilin resistant (MRSA) strains, N315 and Mu50 has been published (Kuroda *et al.*, 2001). Soon it became an obligatory tool for the global analysis of *S. aureus* physiology (Hecker *et al.*, 2010; Hussain & Huygens, 2012). The next year, another complete genome sequence was made available for MRSA strain MW2 (Baba *et al.*, 2002). Taking the advantage of the availability of genomic data the first reference maps of cytoplasmic proteins of *S. aureus* strains COL and 8325 were determined (Cordwell *et al.*, 2002). To provide comprehensive information source about virulence factors from various bacterial pathogens, including *S. aureus*, the virulence factors reference database (VFDB) was created and made available online (http://www.mgc.ac.cn/VFs/) (Chen *et al.*, 2005). Today, the complete genome sequences of a large number of *S. aureus* strains are available in the various databases (eg. www.tigr.org; www.ncbi.nlm.nih.gov). The number of open reading frames within a single genome ranges from 2600 to 2900 (Kusch & Engelmann, 2014). Despite dynamic development of recent years, the function of hundreds of predicted proteins remains unknown or poorly understood. Proteomics provides a starting point of more detailed studies on crucial aspects of bacterial physiology. It also allows to track global changes in protein repertoire upon alteration of particular gene expression. The proteomic approaches to understand staphylococcal physiology and pathogenicity include gel-based and gel-free ("shotgun"), methodologies (Fig. 2) (Hecker *et al.*, 2010).

GEL-BASED PROTEOMICS

Gel-based proteomics began in 1975 when the two-dimensional gel electrophoresis (2-DE) technique was invented (O'Farrell, 1975). 2-DE allows to simultaneously analyze hundreds of different proteins present in a single sample. It is a perfect technique for comparative analysis. Using gel-based proteomics it is possible to compare the proteomes at different stages of growth, the meta-

bolic pathways, the cellular responses to different environmental conditions, to analyse protein aggregates, or to identify protein posttranslational modifications. Gel-based proteomics is used to study cytosolic, membrane- and cell wall-associated as well as extracellular proteins. The large field of gel-based *S. aureus* proteomic covers the analysis of the surfacome and the secretome. The surfacome, also defined as surface-exposed proteins, includes all proteins present on a cell envelope or directly exposed to the extracellular milieu (Dreisbach *et al.*, 2010). These proteins are the first to interact with the host cells, therefore have a crucial role in colonization of host's tissues and latter invasion. The secretome, in turn, is a profile of proteins secreted into the extracellular environment. Secretome examinations identify many proteins that act directly as virulence factors. Cell surface exposed and extracellular proteins were broadly examined using 2-DE in order to investigate how the pathogenic bacteria are affected by the changes within their environment and how they interact with their host (Gatlin *et al.*, 2006; Pocsfalvi *et al.*, 2008; Dreisbach *et al.*, 2010; Dreisbach *et al.*, 2011a; Enany *et al.*, 2012). Gel-free techniques often face restriction in analysis, identification or quantification of protein components present in complex mixtures (Curreem *et al.*, 2012). In contrast, the gel-based proteomics is capable of resolving complex protein mixtures, is relatively simple and has good reproducibility. Therefore, this approach provided a significant contribution to the bacterial proteomics. Despite certain limitations, including time consuming experimental procedures, gel-based proteomics remains a powerful technique in analysis of bacterial protein expression patterns and continues to evolve. In the gel-based approach, separation of a complex protein sample in a gel is followed by cutting out the spots of interest of the gel, protease (routinely trypsin) digestion and further analysis including separation using ion exchange (IEX) chromatography or reverse-phase (RP) chromatography prior to identification by mass spectrometry (MS). 2-DE is routinely combined with fast, reproducible MALDI (matrix-assisted laser desorption/ionization) or ESI (electrospray ionization) identification techniques. Despite the most recent spectacular advances in gel-free techniques (see below), the gel-based proteomics is still considered an indispensable and irreplaceable technique in investigation of pathogenic bacteria (Wu *et al.*, 2008; Engelmann & Hecker, 2009; Francois *et al.*, 2010; Hecker *et al.*, 2010; Curreem *et al.*, 2012; Otto *et al.*, 2014).

Gel-based proteomics is a highly valuable tool in the exploration of microbial physiology. The comparison of *S. aureus* strain COL cytoplasmic proteome in exponential and stationary growth phases revealed differences in the amounts of enzymes involved in protein synthesis, transcription, glycolysis and gluconeogenesis (Kohler *et al.*, 2005). For *S. aureus*, gel-based analysis was important to analyse proteins that mediate interactions with host cells or components of the host immune system and proteins that play a role in pathogenesis when act as degradative enzymes and/or toxins. Gel-based approach was also used to obtain

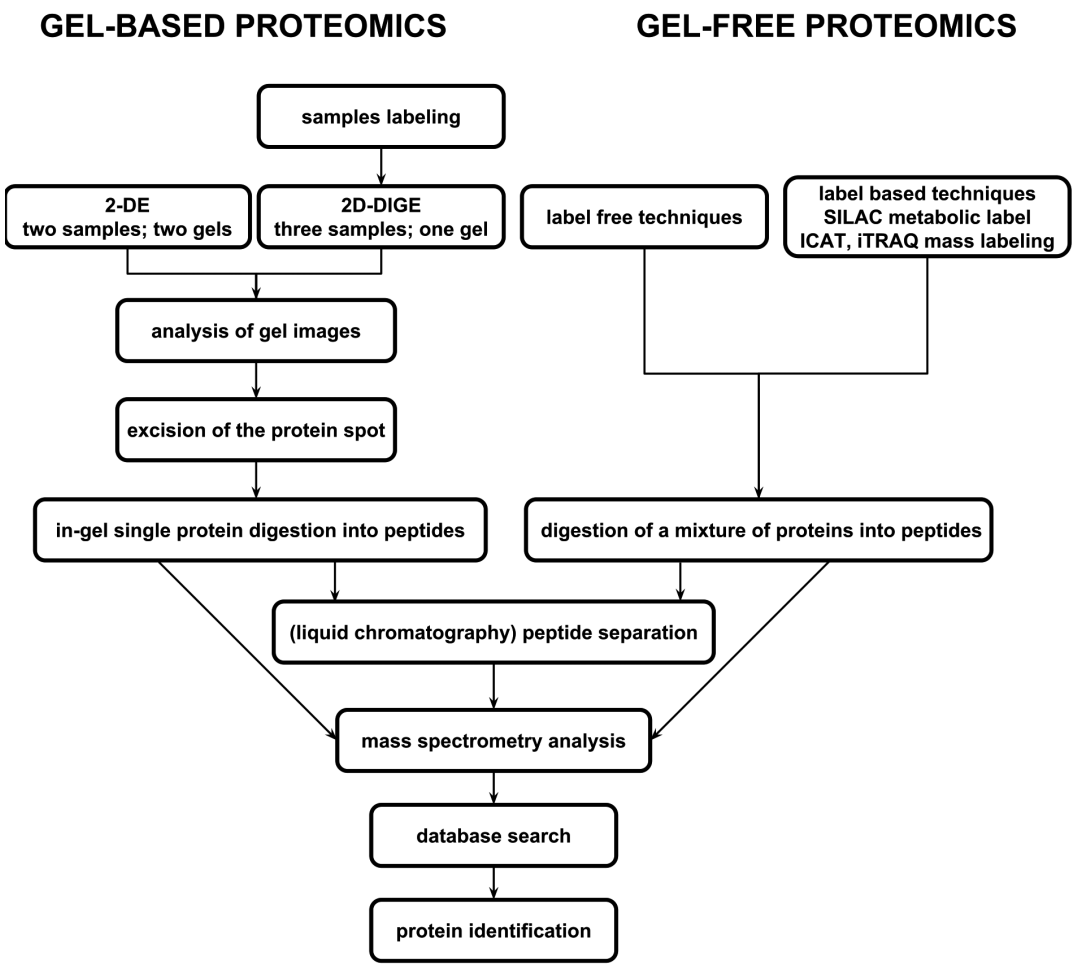

Figure 2. General workflow of the two main strategies commonly used in *Staphylococcus aureus* proteomic analysis.

qualitative and quantitative proteomic data in order to track and analyze bacterial mixed cultures of *Staphylococcus aureus* with *Burkholderia cepacia* and *Pseudomonas aeruginosa* (Kluge *et al.*, 2012) to obtain insights into microbial behaviors in such communities. The analysis of mixed cultures in the presence or absence of antibiotics revealed about 270 differentially expressed proteins related to bacterial interactions, antibiotic treatment response or metabolic shifts.

An extreme advance in proteome analysis was provided by 2D-DIGE (two dimensional fluorescence difference gel electrophoresis) (Unlu *et al.*, 1997; Marouga *et al.*, 2005). In 2D-DIGE the compared proteomes are labeled with different fluorescent dyes prior to analysis. The labeled samples are mixed together and separated on the same 2-DE gel. The power of the method lies in the fact that despite mixing different protein populations can be visualized and analyzed separately taking the advantage of distinct excitation and emission characteristics of the covalently bound dyes. Therefore, 2D-DIGE technology helps to overcome problems with reproducibility between gels typical to 2-DE (Fig. 2). The role of the *S. aureus* cytoplasmic ClpP protease (previously recognized as an essential factor in cellular stress tolerance) in virulence regulation was examined with the application of 2D-DIGE technique. ClpP deletion in thee *S. aureus* strains (Newman, COL, SA564) was analyzed by comparing the proteomes of deleted and wild type strains. Interestingly, despite the fact that the expression of global virulence regulators (RNAIII, *mgrA*, *sarZ*, *sarR*, *arlRS*) was similarly affected in all *clpP* mutants, the effect on expression of particular virulence related genes was strain-dependent (Frees *et al.*, 2012). A year later proteomics was used to identify directly the cellular substrates of the ClpP protease in *S. aureus*. From approximately 70 identified proteins, about one-third was previously known as substrates of ClpP in other bacteria. Novel ClpP substrates included global transcriptional regulators (PerR and HrcA), proteins involved in DNA damage repair (RecA, UvrA and UvrB) and proteins essential for protein synthesis (RpoB and Tuf). The study also documented the involvement of Clp-driven proteolysis in a number of pathways contributing to the success of *S. aureus* virulence (Feng *et al.*, 2013).

Gel-based proteomics is also suitable for analysis of particular fractions of *S. aureus* proteome. For instance 2-DE has been used in the investigation of VISA (vancomycin-intermediate *S. aureus*) cell envelope-associated proteins (Gatlin *et al.*, 2006). Similar approach has been applied to generate the initial 2-DE reference map of *S. aureus* membrane and cell wall associated proteins (Nandakumar *et al.*, 2005). Simultaneously, whole *S. aureus* proteome might be investigated by analyzing separately the cytosolic, membrane-bound, cell surface-associated and extracellular proteins. With this approach over 1700 proteins, covering around three-quarters of the known open reading frames, were identified as truly being expressed. The proteomes of the cells from exponential and stationary growth phases were also compared (Becher *et al.*, 2009). In summary, various gel-based proteomic workflows allowed analysis of whole cell proteome or particular subproteomes of *S. aureus* strains, e.g. N315, O11, O46, MW2 (USA 400), LAC (USA 300), Newman and other (Scherl *et al.*, 2005; Pocsfalvi *et al.*, 2008; Ziebandt *et al.*, 2010; Dreisbach *et al.*, 2011a; Le Marechal *et al.*, 2011a).

GEL-FREE PROTEOMICS

Despite clear success, the gel-based proteomics faces certain limitations, among others speed and sensitivity of analysis or detection of proteins of low abundance. Therefore, alternative gel-free, solely MS-based approaches are used for efficient protein detection, quantification and analysis. In the gel-free proteomics the analyzed peptides or proteins are separated using various chromatographic techniques and directly subjected to mass spectrometry analysis.

Continued advances in MS techniques including the introduction of hybrid mass spectrometers such as QqQ (triple quadrupole), Q-TOF (quadrupole-time-of-flight), Q-Trap (quadrupole-ion trap) and TOF-TOF (time-of-flight time-of-flight) able to measure the peptide mass within ppm (parts per million) error, work at high speed and record several MS/MS spectra per second portend the decline of 2-DE based analysis. Current MS and nano-HPLC techniques are indeed able to resolve very complex peptide mixtures and generate meaningful data for thousands of peptides (Fig. 2) (Armengaud, 2013).

Label-free and label-based methods were developed for identification quantitative analysis. Label based techniques include SILAC (stable isotope-labeling by amino acids in cell culture), a metabolic based labelling of proteins with amino acids (lysine, arginine, methionine, tyrosine) with incorporated stable isotopes (^{13}C and ^{15}N) and multiple chemical labels such as ICAT (isotope-coded affinity tag) and iTRAQ (isobaric tags for relative and absolute quantification), based on isobaric reagents or phosphoproteomic techniques like MudPIT (multidimensional protein identification technology) (Yates *et al.*, 2009). SILAC was employed for the identification and quantitation of the proteome of *S. aureus* after internalization by human bronchial epithelial S9 cells (Pfortner *et al.*, 2013) or in comparative phosphoproteomic studies of macrophage response to *S. aureus* virulence effectors (Chen *et al.*, 2012). In order to correlate the transcriptome based observations showing that toxins production in *S. aureus* is negatively regulated by CodY (the transcription factor, important regulator of metabolism and virulence) with changes in protein synthesis, and in order to study toxin and virulence determinant production in CA-MRSA (community-associated methicillin-resistant *S. aureus*) iTRAQ-based proteomics was used. Obtained results revealed the importance of CodY in infection and disease development (Rivera *et al.*, 2012). FLAQ (fluorescence labeling absolute quantification) technique was developed as a chromatography based quantification method in combination with MDLC (multidimensional liquid chromatography), LIF (laser-induced fluorescence) detection and tandem mass spectrometry.

5-iodoacetamidofluorescein (IAF) labels proteins on all cysteine residues and is compatible with trypsin digestion and MALDI MS identification (Liu *et al.*, 2012). Label-based mass spectrometry is a powerful tool, but gel-free non-label techniques based only on MS/MS, or nano LC-MS/MS measurements provide the most unbiased approach. An efficient label-free protein identification and quantification method with LC-MS/MS resulted in quantification of 990 proteins within whole cell lysates from non-stressed and nitric oxide stressed *S. aureus* (Muntel *et al.*, 2012).

Gel-free proteomic approaches may be additionally divided as "bottom-up" and "top-down". The first refers to the identification of proteins by analysis of peptides obtained by specific proteolysis. Peptide masses and sequences obtained using tandem mass spectrometry are

used to identify corresponding proteins. The "top-down" proteomics uses masses of intact proteins and their fragments for identification and characterization (Yates *et al.*, 2009; Bergmann *et al.*, 2012; Zhang *et al.*, 2013). The top-down proteomic approach, using MALDI-based tandem mass spectrometry might be used in the identification of bacterial protein biomarkers and toxins even from unfractionated bacterial cell lysates (Fagerquist & Sultan, 2013).

The best way, however, to obtain the most complete results is to combine both gel-based and gel-free techniques. Quantitative analysis of *S. aureus* extracellular proteins, cell surface-associated, and cytoplasmic fraction by gel-free and gel-based (GeLC) approach was reported. In order to make this possible, prior to GeLC-MS/MS-based proteome profiling, supernatant precipitation, trypsin shaving and biotinylation have been applied. The cytoplasmic proteome of *S. aureus* COL strain was analyzed with gel-based (2-DE/MALDI-TOF MS) and gel-free (MDLC) techniques. 473 proteins implicated in variety of cellular functions such as transcriptional and translational machinery, tricarboxylic acid cycle, glycolysis, biosynthetic pathways of nucleotides, fatty acids and cell wall components were identified with gel-based system. In a gel-free approach additional 650 proteins were detected, including alkaline and hydrophobic proteins. Altogether, 1123 cytoplasmic proteins were identified which represents two-thirds of the cytoplasmic proteome (Kohler *et al.*, 2005). Recently, *S. aureus* secretome profiling using two parallel approaches was reported. Proteins were pre-fractionated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by in-gel and in-solution trypsin digestion, both coupled with LC–MS/MS (Enany *et al.*, 2012). As a result, 174 distinct proteins were identified with a high confidence. The high-resolution 2-DE and iTRAQ mass tagging were used to identify potential biomarkers useful for the detection of VISA strains (Drummelsmith *et al.*, 2007). Phosphorylation patterns of *S. aureus* cultured under diverse physiological conditions were analyzed by 2-DE with a phosphosensitive stain and using gel-free titanium dioxide based phosphopeptide enrichment. 103 putative phosphorylated proteins were identified including those related to virulence. Differences in phosphorylation patterns under different physiological conditions were also demonstrated (Basell *et al.*, 2014). In conclusion, the combination of gel-based and MS-based proteomics can be successfully employed to follow each individual protein in the cell. This greatly improves our understanding of staphylococcal pathogenicity providing a new quality in understanding cell physiology and virulence of *S. aureus* (Hecker *et al.*, 2010).

PROTEOMICS IN STUDIES OF SECRETOME, SURFACOME AND IDENTIFICATION OF VIRULENCE FACTORS

Classical biochemical methods, In Vivo Expression Technology (IVET) (Mahan *et al.*, 1993) and Signature-Tagged Mutagenesis (STM) (Hensel *et al.*, 1995) are widely used to identify virulence factors. Currently, however, more powerful methods of comparative proteomics combined with genomics and transcriptomics become more popular, not only for identification of virulence factors, but also for detection of proteins that indirectly contribute to pathogenesis. Analysis of secretomes and surfacomes allows comprehensive elucidation of the role of secreted, membrane and cell wall proteins in various aspects of the pathogenicity. This is usually accompanied by different strategies to prefractionate the whole proteome to enrich the low-abundance or membrane hydrophobic proteins, usually underrepresented or simply not detected during the entire proteome analysis (Yang *et al.*, 2012).

A reference proteomic map of membrane and cell wall associated proteins of VISA strain provided a great starting point for further investigation. This achievement required testing various lytic and solubilization methods. Finally, application of enzymatic digestion of cell wall by lysostaphin followed by solubilization of exposed membrane proteins in a solution composed of urea, thiourea, amidosulfobetaine 14 and dithiothreitol, followed by two dimensional electrophoresis and MALDI-MS analysis proved most effective, resulting in a sample containing minimum contamination of cytosolic proteins (Nandakumar *et al.*, 2005). Another successful analysis of cell envelope-associated proteins used 2-DE and LC-tandem mass spectrometry and identified 144 envelope-associated proteins. *In silico* analysis revealed that among identified proteins, 48 contain predicted export signal or lipoprotein motifs. The remaining part of proteins lacking cell wall or membrane immobilization signals was nevertheless repeatedly identified in the cell envelope fractions. For example, *S. aureus* surface protein G (SasG) appeared as a one of the most abundant protein in VISA surfacome with numerous posttranslational variants in 2-DE gels, in contrast to its low expression level in the cell envelope fraction of a vanomycin sensitive strain 8325-4. Accordingly, an important role of SasG in antibiotic resistance of VISA strains has been suggested. Interestingly, numerous proteins with predicted and/or known cytoplasmic localization were also detected at the bacterial cell surface. At first autolysis was suggested as the major cause (Gatlin *et al.*, 2006). Nevertheless, despite the use of different means to prevent lysis, further proteomic studies repeatedly confirmed the presence of cytoplasmic proteins in the extracellular subproteome (Burlak *et al.*, 2007; Ravipaty & Reilly, 2010; Dreisbach *et al.*, 2011b; Hempel *et al.*, 2011). This phenomenon was subsequently explored in detail and now it is known that a cell may form membrane vesicles (MVs) to excrete cytoplasmic proteins (Lee *et al.*, 2009). Autolysin (Atl) activity also results in excretion of interacellular proteins (Pasztor *et al.*, 2010). Henderson and coworkers defined a group of proteins, now known as „moonlighting proteins", which includes molecules with more than one cellular location and exhibit dual functions usually one within metabolic pathways or stress adaptation, and a second one associated with bacterial virulence (Henderson & Martin, 2011). One of the best described of those is enolase (2-phosphoglycerate dehydratase), the first cytoplasmic protein detected on the cell envelope. Apart from its hydrolytic function in the glycolysis pathway, enolase has a clearly defined additional role at bacterial surface in binding a laminin (a major component of the host extracellular matrix). Thus, elonase plays a role in *S. aureus* adhesion and colonization (Carneiro *et al.*, 2004). Moonlighting character of other staphylococcal proteins involved in basic metabolism was described, including glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which acts not only as glycolytic enzyme but also as a staphylococcal transferrin receptor (Modun *et al.*, 2000). Interestingly, the second function of known moonlighting proteins of *S. aureus* is usually related to virulence.

Vancomycin is one of the few remaining treatment options for methicillin-resistant *S. aureus* infections. The

global analysis of the vancomycin induced *S. aureus* stress response proteome was carried out by *in vivo* 14N/15N labeling, 1-D SDS-PAGE and hybrid mass spectrometer analysis. Upon treatment with the drug, not only the anticipated adaptive processes induced by limited growth was observed. In parallel specific cellular responses were noted, such as an increased production of enzymes involved in production of amino acids essential for peptidoglycan synthesis, decreased production of proteins related to virulence and changes in the expression of two-component regulatory system VraSR and global regulons including SigB and SaeR (Hessling *et al.*, 2013).

Proteomic analysis of surface and secreted proteins of CA-MRSA mutant lacking 10 extracellular proteases provided a unique insight into the progression of CA-MRSA infection, and the role of secreted proteolytic enzymes. Increased abundance of secreted (e.g. a-toxin, LukAB, LukE, PVL, γ-hemolysin) and surface-associated (e.g. ClfA+B, FnBPA+B, IsdA, Spa) proteins was observed upon protease deletion. The study revealed that secreted proteases are key mediators of *S. aureus* virulence *via* the global modulation of virulence-determinant stability. The proteases are required for growth in peptide media, serum, human blood and for resisting phagocytosis by immune cells. Moreover, the extracellular proteases play a role in resistance against antimicrobial peptides, contribute to skin abscess formation and play a key role during systemic CA-MRSA infections (Kolar *et al.*, 2013; Pustelny *et al.*, 2014).

High throughput proteomics (2D-PAGE, iTRAQ, LC-MS/MS) was used to compare exoproteomes of a strain isolated from a nasal carrier with that of a genetically similar non-persistent strain. Moreover, the biofilm exoproteome of a former strain was compared against its planktonic equivalent. The results demonstrated that the strain isolated from a carrier secretes a larger number of proteins including cell attachment and immunoevasive proteins compared to the non-persistent strain. The exoproteome of a carrier strain biofilm contains a larger number of stress and immunoevasive proteins than its planktonic counterpart. The study allowed to conclude that differentially expressed proteins, such as staphylococcal Spa, present at significantly higher levels in persistent than in non-persistent strain may be considered a putative determinant of nasal carriage (Muthukrishnan *et al.*, 2011). Recently, using extracellular and intracellular quantitative NMR profiling of *S. aureus* grown as biofilm and planktonic cultures additional small-molecule biomarkers that distinguish between these two phenotypes has been indicated. These include, among others, amino acids, glycerol and malonate, which could be linked with adaptive energy production and cell wall components turn over in biofilm-forming bacteria. Moreover, the differences in metabolic strategies between planktonic and biofilm phenotype are reflected by, respectively, a drop and an increase of the pH level during cultivation (Ammons *et al.*, 2014).

A significant progress in profiling the cell wall-associated proteins was made by optimizing the trypsin surface shaving method. Analysis of cell surface-exposed proteins of four *S. aureus* strains with different genetic backgrounds resulted in identification of 96 different proteins and demonstrated significant heterogeneity in the expression patterns and a low percentage (below 10%) of common cell-surface proteins among the investigated strains. This indicates high heterogeneity of proteins exposed at the cell surface *S. aureus* among different strains (Dreisbach *et al.*, 2010). Trypsin surface shaving and subsequent MS analysis of liberated peptides was used to profile interactions between human serum proteins and *S. aureus* at the cell surface. This study identified constituents of the complement system, the platelet factor 4 and the isoform 1 of the inter-alpha-trypsin inhibitor heavy chain H4 on the staphylococcal cell surface revealing that surface shaving may constitute a valuable tool in profiling interactions of foreign proteins at the bacterial cell surface (Dreisbach *et al.*, 2011a). Another proteomic study reported identification of the previously uncharacterized leukotoxin as the critical factor promoting virulence of methicillin-resistant *S. aureus* USA300. The application of tandem mass spectrometry (LC-MS/MS) allowed to identify and quantify more than 100 proteins in surfacome of this staphylococcal strain (Ventura *et al.*, 2010). Subsequent analysis of wild-type and isogenic *lukGH* USA300 mutants allowed to conclude that the most abundant cell wall-associated protein (LukGH) acts as a leukotoxin and is responsible for the lysis of neutrophils. *In vivo* investigations confirmed the cytotoxic role of LukGH which is reflected in inflammatory response during infection (Malachowa *et al.*, 2012). Using genomic, transcriptomic and proteomic approach Marechal *et al.*, 2011 compared two clonally related *S. aureus* strains causing severe (O11) and milder (O46) mastitis to identify responsible factors. Protein samples of whole-cell lysate, cell wall and extracellular fractions of both strains were compared. The analysis of extracellular proteins revealed differences in toxin and protease production which indicates that this factors may contribute to the severity of mastitis (Le Marechal *et al.*, 2011b). Recently, a comprehensive overview of proteomic strategies in profiling of *S. aureus* surfacome with in depth discussion of pros and cons became available (Glowalla *et al.*, 2009; Dreisbach *et al.*, 2010; Solis *et al.*, 2010; Ventura *et al.*, 2010; Hempel *et al.*, 2011). The detailed comparison of the amounts of the identified proteins, their predicted sub-cellular localizations and different approaches to uncover surface proteome was presented. Five independent studies contributed to identification of the total 449 different proteins present in the surfacome (Dreisbach *et al.*, 2011b).

Several studies have examined the composition of the exoproteome only as a major reservoir of virulence factors (Pocsfalvi *et al.*, 2008; Khoon & Neela, 2010; Ravipaty & Reilly, 2010; Ziebandt *et al.*, 2010; Muthukrishnan *et al.*, 2011; Enany *et al.*, 2012). 2D-SDS-PAGE and LC-MS/MS was used to evaluate the changes in *S. aureus* proteome upon exposure to silver, an agent present in antibacterial ointments and adhesive bandages used to minimize wound infections. Such exposure resulted in the release of a range of proteins associated with stress response, virulence and metabolism. At the same time the release of cytotoxins, such as alpha-hemolysin, was decreased, but an increase was noted in the levels of antigenic proteins able to modulate the inflammatory response at the site of infection, thus delaying healing (Smith *et al.*, 2013). *In silico* analysis of the genomic sequences of 15 *S. aureus* reference strains revealed that around one third of the encoded proteome might in fact be secreted. Within the analyzed strains 1354 proteins were predicted as members of *S. aureus* pan-secreteome, that is proteins encoded within the core genomes of all species but also within dispensable genome (genes present in several or a single strain only). 580 of the identified proteins belonged to a "core secretome". Multi-transmembrane proteins were most conserved with more than 60% of all members present in the core secretome. The highest variability was observed among the extracellular, LPxTG cell-wall-anchored proteins and lipid-anchored proteins (only 22–34% of these

proteins are encoded by all *S. aureus* strains) (Kusch & Engelmann, 2014). Methicillin-resistant *S. aureus* (MRSA) strains attract special attention due to the threat posed on the healthcare system. Proteomics has been extensively utilized for characterization of MRSA secretomes. Characterization of exoproteins of MW2 (USA400) and LAC (USA300) strains using 2-DE followed by automated direct infusion-tandem mass spectrometry (ADI-MS/MS) resulted in identification of 250 proteins in two analyzed growth phases. Interestingly, only 20% of proteins identified at mid-exponential growth phase and 15% identified at early stationary growth phase have been previously associated with virulence (Burlak *et al.*, 2007). Another analysis of extracellular proteins in MRSA (strain COL) obtained using 2-D LC combined with MS complemented by MS/MS resulted in identification of only 59 secreted proteins. Nevertheless, using this approach it was possible to identify post-translational modifications and verify signal peptide cleavage sites (Ravipaty & Reilly, 2010). Enany *et al.* applied two proteomic approaches: SDS-PAGE and SCX (strong cation exchange) fractionation, both coupled with tandem mass spectrometry, in order to analyze the secretomes of ER13 and ER21 strains of CA-MRSA. Each of these studies identified above a hundred proteins. Another proteomic analysis combined gel electrophoresis and LC-tandem mass spectrometry to explore the virulence determinants of two clinical isolates, the methicillin susceptible *S. aureus* (MSSA) and MRSA. 168 extracellular proteins were identified in MSSA and 261 in MRSA. 117 identifications were identical, although in MRSA these proteins were identified with higher confidence possibly suggesting overexpression. Of 144 proteins unique to MRSA at least some are most probably virulence determinants of this strain (Enany *et al.*, 2014).

In summary, the above findings demonstrate that proteomic studies provide significant progress in the analysis of the surfacome and secretome, especially in identification of factors involved in staphylococcal virulence.

PROTEOMICS STRATEGIES IN THE STUDIES OF HOST-PATHOGEN INTERACTIONS

The infection is a constant struggle between the pathogen producing deleterious virulence factors and host's defense responses. Proteomic techniques allow to identify and catalog the virulence-associated proteins and also to monitor the adaptive changes in the bacterial proteome during infection, thereby defining novel vaccine targets. Such experimental *in vitro* and *in vivo* proteomic studies of host-pathogen interactions contribute to deeper understanding of the mechanisms involved in virulence determination. In response to bacterial infection, host innate immune cells, such as granulocytes, monocytes and macrophages, generate substantial quantities of oxide anion, hydrogen peroxide and nitric oxide all of which have strong cytotoxic effect against bacteria. To provide detailed insight into the mechanisms providing resistance against reactive oxygen species as well as to identify other factors required for *S. aureus* survival, analysis of the response to different oxidants was conducted. The proteome patterns stimulated by peroxide, superoxide and disulfide stress stimuli were analyzed by 2-DE combined with MALDI-TOF-MS. Not surprisingly, proteins involved in the detoxification of peroxides, including peroxiredoxins Tpx and AhpC, were mainly upregulated. Additionally, hydrogen peroxide resulted in irreversible oxidation of cysteine residues to sulphonic acid (Wolf *et al.*, 2008). This resulted in inactivation of certain enzymes including GAPDH (Weber *et al.*, 2004). Changes the cytosolic protein profile after nitrogen monoxide (NO) challenge were also analyzed using a gel-based approach. Beside proteins involved in anaerobic metabolism, flavohemoglobin (flavoHb) was intensively synthesized after NO stress (Hochgrafe *et al.*, 2008). It is not surprising since FlavoHb was demonstrated to efficiently detoxify $NO^{\cdot}$ by catalyzing its oxidation to nitrate (Gardner *et al.*, 1998; Forrester & Foster, 2012). Within the host the bacteria encounter not only oxidative stress but also growth restricting conditions which require special adaptations. Iron is one of the most limiting nutrients due to its sequestration by heme-containing proteins (hemoglobin, myoglobin) and iron-binding proteins (Skaar & Schneewind, 2004; Hempel *et al.*, 2011). The proteomic survey of adaptations within cytoplasmic, extracellular and cell surface-associated proteome upon iron-depletion identified 845 proteins, including 158 surface-exposed proteins, in *S. aureus* strain COL. Quantitative analysis demonstrated that iron limitation results in significant changes in the abundance of 29 proteins. Expectedly, iron-regulated surface proteins involved in iron-uptake (IsdA, IsdB, IsdC, IsdD) and iron-acquisition lipoproteins were highly upregulated (Hempel *et al.*, 2011). Importantly, however, new iron-regulated proteins were identified and these identifications confirmed previous transcirptomic data (Allard *et al.*, 2006). Hence, "-omics" techniques provided new insight into *S. aureus* adaptation to iron-starvation.

The above mentioned studies exposed the bacteria to selected factors in an *in vitro* setup which only partially mimics a complex environment encountered within the host. *In vivo* studies are much more experimentally demanding but if successful provide most relevant information on the host-pathogen interactions as a whole. Recently, such analyses became possible. For example the arsenal of extracellular proteins likely contributing to virulence was analyzed in two CA-MRSA strains during *in vitro* and *in vivo* growth (Burlak *et al.*, 2007). To investigate exoproteins produced *in vivo* during the infection, a mouse abscess model was used to generate immune sera from mice infected with MW2 (USA 400) and LAC (USA 300) strains. The exoproteins of those two strains were separated on 2-DE gel, transferred to nitrocellulose membrane and incubated with convalescent serum from previously infected mice. Immunoproteomic analyses demonstrated that bifunctional autolysin (Atl), collagen binding protein (Can), 1 phosphatidylinositol phosphodiesterase (Plc), lipase (Lip), staphylokinase (Sak), enterotoxin type 3 (Sec3) and staphopain B (SspB) were immunogenic produced by CA-MRSA during *in vivo* infection. The analysis of CA-MRSA proteome in infection provides a step forward in the development of vaccines (Burlak *et al.*, 2007). Possibilities of proteomic analysis of bacterial pathogens in *in vivo* conditions are usually limited because of the lack of appropriate methods to separate bacterial proteins from the host material. This challenge has been recently successfully faced using Orbitrap, a novel mass analyzer. The estimation of time-resolved changes in the metaproteome profile upon internalization of staphylococci by human bronchial epithelial S9 cells was possible using pulse-chase SILAC labeling (for quantification of proteome changes after internalization of bacteria), fluorescent particle sorting (for purification of green fluorescent protein-labeled *S. aureus* from host cells) and on-membrane tryptic digestion of the sorted bacteria followed by shotgun LTQ-Orbitrap-MS analysis (Schmidt *et al.*, 2010; Schmidt & Volker, 2011; Pfortner

et al., 2013). Presented workflow can be applied to describe host-pathogen pairs, to detect and quantify hundreds of proteins during several hours post infection. Integration of data obtained from *in vivo* and *in vitro* models largely broadens our understanding of host-pathogen interactions. Recently, another comparative proteome analysis presented the common and specific traits of the adaptation of *S. aureus* to certain host cell milieus. Using as little as two million internalized *S. aureus* bacteria by different types of human non-professional phagocytic cells (S9, A549 and HEK293) a quantitative data for about half of staphylococcal proteins have been provided. The decreased amount of ribosomal proteins or proteins belonging to the *de novo* purine biosynthesis as well as the increase of proteins belonging to stress responses were observed regardless the internalizing cell. Hence, these changes appear as conserved adaptations reactions to the intracellular environment of human host cells in general. In contrast, levels of enzymes involved in threonine degradation as well as those in fermentation and tricarboxilic acid cycle were different between *S. aureus* cells internalized by the epithelial cell lines and the kidney cell line HEK indicating the importance of the selection of appropriate model in studies of host-pathogen interactions (Surmann *et al.*, 2014). Bacteria are capable of delivering proteins and lipids directly to host cells by means of membrane vesicles (MVs). These strategy was first described in Gram-negative bacteria (Mayrand & Grenier, 1989) and extensively studied since (Kondo *et al.*, 1993; Khandelwal & Banerjee-Bhatnagar, 2003; Dutta *et al.*, 2004; Kuehn & Kesty, 2005). Not only gram-negative bacteria use MVs. These were also demonstrated in *S. aureus*. *S. aureus* (ATCC14458) derived MVs were analysed using quantitative proteomics coupling 1-D PAGE with nano-LC-MS/MS. Among 90 identified vesicular proteins, extracellular and surface-associated factors including toxins, adhesins, hemolysins and penicillin-binding proteins, were identified. This may indicate a direct role of *S. aureus* derived MVs in bacterial adhesion, colonization, tissue invasion and antibiotic resistance (Lee *et al.*, 2009). Moreover, another evidence obtained by the same research group indicates that *S. aureus* derived MVs are involved in the pathogenesis of atopic dermatitis (AD). The application of *S. aureus* derived MVs increased production of pro-inflammatory mediators, such as interleukin-6, thymic stromal lymphopoietin, macrophage inflammatory protein-1α and eotaxin, and in consequence induced AD-like inflammation in the skin (Hong *et al.*, 2011). After testing two type *S. aureus* strains and two clinical isolates, Gurung and colleagues concluded that MVs production is common to all the strains both *in vitro* and *in vivo* and that MVs are significant vehicle for transfer of bacterial effector molecules to host cells. The dose-dependent manner high cytotoxic potential of MVs towards human epithelial cells was demonstrated. As such, the MVs may significantly contribute in the pathogenesis of *S. aureus* infections (Gurung *et al.*, 2011).

Biofilm formation is an important determinant of staphylococcal virulence. The biofilm matrix provides attachment to the host tissue facilitating persistent infection. Biofilm also constitutes a protective barrier against host defenses and antimicrobials (Costerton *et al.*, 1995; Costerton *et al.*, 1999; Stewart & Costerton, 2001). High-resolution 2-DE followed by tandem mass spectrometry allowed to compare *S. aureus* proteomes from biofilm and planktonic cells. This allowed to identify proteins involved in biofilm formation. Fibrinogen-binding proteins (Efb) and the accumulation-associated protein (Aap) were demonstrated essential for adhesion and biofilm formation. Moreover, increased production of staphylococcal accessory regulator A (SarA) indicated its impact on biofilm formation (Resch *et al.*, 2006). In another study, *S. aureus* was cultured under increasing fluid shear rates imitating the conditions encountered within the veins and heart. Biofilm associated proteins were identified using nano-LC-ESI-MS/MS. The experiment revealed significantly altered expression of 16 proteins in the membrane-enriched fraction and 8 proteins in the cytosolic fraction all of which are associated with various metabolic functions. Simultaneously, increased fluid shear stress did not influence the expression of important surface binding proteins (Islam *et al.,* 2014). The potential of extracellular proteins of the biofilm matrix to induce protective immune response against *S. aureus* was examined by gel-free proteomics (Gil *et al.*, 2014). The authors characterized the exoproteomes of exopolysaccharide-based and protein-based biofilm matrices produced by two clinical *S. aureus* strains and demonstrated that independently of the nature of the biofilm matrix, the mutual core of secreted proteins remains identical. These classifies biofilm matrix exoproteins among promising candidates for multivalent vaccination against *S. aureus* infections associated with biofilm production. Proteomics also allowed to reveal that serine protease (Esp) produced by commensal *S. epidermidis* degrades at least 75 proteins expressed by *S. aureus*, including 11 proteins crucial for biofilm matrix construction and host-pathogen interaction, such as extracellular adherence protein (Eap), bifunctional autolysin (Atl), extracellular matrix protein-binding protein (Emp), fibronectin-binding protein A (FnBPA) and Spa. Simultaneously Esp selectively degrades several human extracellular matrix and plasma proteins important for host-pathogen interaction, e.g. fibronectin (Fn), fibrinogen (Fg) and vitronectin (Vn) which are used for host colonization by *S. aureus*. Thereby Esp, produced by *S. epidermidis*, a major constituent of healthy skin flora, impairs *S. aureus* colonization (Sugimoto *et al.*, 2013) which likely contributes to the protective role of commensal *S. epidermidis* colonization.

Finally, apart from skin and soft tissue infections (SSTIs), *S. aureus* may causes a severe bacteremias. Using 2-DE, immunoblots, and MS analysis Liew and coworkers conducted comparative analysis of extracellular proteins profiles and its antigenicity in *S. aureus* bacteremia and SSTIs in comparison to healthy carriage. Despite exoproteome heterogeneity the authors identified twelve proteins from bacteremia isolates constantly present in more than 50% of the bacteremia isolates and in none of the SSTIs and healthy carrier isolates. Interestingly, although very few antigenic protein spots were observed in bacteremia patients in comparison with SSTIs patients and healthy carriers, two antigenic protein spots (chaperon protein DnaK and hypothetical protein SSP0002) were found to be selectively present in the bacteremia group. This result constitutes the step forward to identify diagnostic biomarkers for of *S. aureus* infections (Liew *et al.*, 2015).

PROTEOMIC ANALYSIS OF GLOBAL REGULATORY SYSTEMS

The expression of staphylococcal virulence factors including extracellular toxins, enzymes and cell surface proteins is growth phase and growth conditions dependent being regulated by various global regulatory systems. Two major groups of such regulons were described. The first group encompasses two-component signal transduc-

tion pathways (*agrA/C, sae*R/*S, srrA/B, arl*R/*S, rap/traP)*, whereas the second comprises proteins belonging to the SarA (staphylococcal accessory regulator) family (Cheung *et al.*, 1992; Bronner *et al.*, 2004). Accessory gene regulator *(agr)* is most thoroughly characterized (Morfeldt *et al.*, 1988; Peng *et al.*, 1988). In *agr*, the effector molecule RNAIII is produced in a mid-exponential to post-exponential phase of growth which results in global changes in gene expression. Surface-associated proteins, such as protein A, collagen binding protein and fibrinogen binding protein are down-regulated by RNAIII and as such are mainly expressed at the early stages of bacterial culture growth. Secreted molecules including toxins α, β and extracellular enzymes are up-regulated by RNAIII and thus are expressed in greater abundance during late phase of growth (Arvidson & Tegmark, 2001; Ziebandt *et al.*, 2004). SarA, a second important regulon affects gene transcription in a direct, *agr*-independent manner and via *agr*-dependent mechanism. Binding of SarA to promoter regions of genes encoding the virulence factors either repress or activates their expression. Interaction within intergenic region of *agr* results in indirect, *agr* mediated activation of virulence gene transcription (Arvidson & Tegmark, 2001). Using multiple global regulatory systems the bacterium coordinates the expression virulence genes during growth and infection.

Multiple studies compared proteomes of wild-type and regulon deficient strains to disclose complicated protein expression patterns. For instance, a comparative proteomic analysis of σ^B (SigB) and SarA dependent regulation was performed by comparing proteomes of *S. aureus* clinical isolate COL with laboratory strain RN6390 and their isogenic *sigB* and *sarA* mutants. This approach identified *sarA*-dependent adhesions and hydrolytic enzymes (Chan & Foster, 1998; Dunman *et al.*, 2001), as well as glycerolester hydrolase and autolysin. Concerning σ^B, its involvement in the coordinated regulation of infection related proteins was demonstrated. The finding, that σ^B is involved in controlled temporal expression of virulence factors was supported by a consecutive study. Ziebandt et al. applied the 2-D DIGE technique, instead of traditional 2-DE to define and quantify how the loss of *agr* or *sigB* locus affects the extracellular proteome of *S. aureus*. In addition to previously reported *agr*-dependent virulence factors they identified a new set of *agr* dependent proteins, including lipase, glycerophosporyl diester phospodiesterase (GlpQ), HlgB, HlgC, LukD, and surface protein D (SasD) produced in post-exponential growth phase. Proteins, synthesized in the exponential growth phase such as probable transglycosylase (IsaA), secretory antigen A (SsaA) and hypothetical protein SA2097 were down-regulated by *agr* (Ziebandt *et al.*, 2001; Ziebandt *et al.*, 2004). Few years later, the same research group has completed the analysis of extracellular protein fraction of 25 clinical MRSA isolates, divided into 17 clonally divergent strains. Coupled 2-DE and MALDI-TOF-MS identified a total of 63 distinct extracellular proteins. Interestingly, only limited overlap in protein profiles between different strains was observed. The authors demonstrated that high exoproteome heterogeneity was triggered not only by genetic background, but also by differences in regulation at transcriptional and post-transcriptional level. For example, lack of *agr* effector molecule, RNAIII, in one isolates correlated with a dramatically reduced expression level of late virulence factors. In addition, lipase transcript level did not correlate with the expression of respective protein and the authors concluded that post-transcriptional regulation is involved (Ziebandt *et al.*, 2010). In yet another study quantitative proteomic approach (1D-PAGE followed by nano LC-MS/MS) was used to analyze the contribution of global regulatory loci *agr* and *sarA* on protein profile during the exponential growth phase. Proteomes of wild *S. aureus* strain UAMS-1 and its isogenic *sarA*, *agr*, and double *sarA agr* mutants were analyzed. Results confirmed the previously determined role of *sarA* and *agr* on protein expression. The cell wall-associated proteins including immunodominant staphylococcal antigen A (IsaA), IgG-binding protein A (Spa) and heme-iron-binding protein (IsdA) were most abundant in the *agr* mutant. Extracellular enzymes including cysteine proteases staphopain A (ScpA) and staphopain B (SspB), serine glutamylendopeptidase (SspA) and metalloprotease aureolysin (Aur) were more abundantly expressed in *sarA* and double *sarA agr* mutants compared to the wild strain UAMS-1 (Jones *et al.*, 2008). *agr* interplay with CodY repressor was studied by Majerczyk et al., who suggested that the transcription factor CodY acts as a repressor of RNAIII synthesis, thereby inhibiting the synthesis of a number of virulence factors, as well as polysaccharide intercellular adhesin (PIA) (*icaADBC*), a major component of staphylococcal biofilm structure (Majerczyk *et al.*, 2008). To provide further insight into CodY mediated regulation of gene expression, secretome analysis was performed. The iTRAQ-based proteomics has been applied to define the quantitative changes in the post-exponential secretomes of *codY* mutant compared with wild-type methicillin-resistant *S. aureus* strain USA300. CodY deficiency resulted in overexpression of several secreted, *agr*-regulated proteins, particularly proteases, leukocidins and hemolysins in both post-exponential and stationary growth phases (Rivera *et al.,* 2012). In this case the proteomic analysis provided identification of new proteins regulated by CodY complementing previous genetic studies.

The *sae*R/*S* system is essential for *in vivo* expression of virulence genes in *S. aureus* (Goerke *et al.*, 2005). The combined transcriptomics, 2-D DIGE and MALDI-TOF-MS allowed for characterization of extracellular proteins regulated by this two-component system. Well-known SaeRS-dependent proteins were identified, but also several previously unknown ones including important virulence factors, such as HlgA, HlgB, HlgC, LukF, LukM and several proteins of unidentified function (SACOL0479, SACOL0480, SACOL0859 and SACOL1169). Identification of hypothetical proteins within virulence-associated regulon suggests their putative function as virulence factors (Rogasch *et al.*, 2006).

PROTEOMIC ANALYSIS OF POSTRANSLATIONAL MODIFICATIONS

Post-translational modifications (PTM) provide essential regulation of protein function in the bacterial cells. A notable advantage of proteomic techniques is their ability to define post-translational modifications at a global scale (Wu *et al.*, 2008). PTMs were first thought to be restricted to eukaryotes until early observations provided evidence of PTMs in *E. coli* (Garnak & Reeves, 1979). PTMs affect enzyme activity, stability, subcellular localization, signal transduction, protein-protein interactions and numerous other processes (Cohen, 2000). Therefore, many proteomic studies investigate not only the protein content of a cell, but also analyze global PTMs to discover mechanism of bacterial virulence (Rajagopal *et al.*, 2003; Shah *et al.*, 2008). To date, several bacterial phosphoproteomes were established using developments

in pre-enrichment methods and combined tandem mass spectrometry techniques (Levine *et al.*, 2006; Eymann *et al.*, 2007; Soufi *et al.*, 2008a). Due to high complexity and low abundance of phosphorylated proteins, the application of prefractionation, including antibody-based phosphotyrosine enrichment, hydrophilic interaction liquid chromatography (HILIC), metal oxide affinity chromatography (MOAC), immobilized metal affinity chromatography (IMAC) and strong anion exchange chromatography (SAX) provides major advances in phosphoproteome studies (Cantin *et al.*, 2008; McNulty & Annan, 2009; Ge & Shan, 2011). Proteomic research allowed to conclude that serine, threonine and tyrosine residues are the major phosphorylation sites in bacteria. PTMs were shown to play a role in pathogenicity (Ge & Shan, 2011), particularly in host-pathogen interactions (Schulein *et al.*, 2005; Lin *et al.*, 2009; Ravichandran *et al.*, 2009). Post-translational modifications of *S. aureus* have been also widely studied. Relatively recent proteomic analysis of MRSA strain enabled detection of 127 proteins, including 59 extracellular ones. The analysis identified multiple PTMs including methylation, oxidation and formylation. Moreover, proteolytic processing of secreted proteins has also been observed (Ravipaty & Reilly, 2010). Phosphorylation and dephosphorylation of serine and threonine residues takes place in regulation of cellular functions that determine metabolic activity and virulence of *S. aureus* (Ohlsen & Donat, 2010). Protein phosphorylation in *S. aureus* plays a role in the infection process, adhesion to the host cells, regulation of pathogenic functions and evasion of the host defense mechanisms (Soufi *et al.*, 2008b; Debarbouille *et al.*, 2009). Phosphorylation on serine and threonine residues by the interplay of corresponding eukaryotic-like serological thymidine kinase 1 (Stk1) and phosphatase (Stp1) is becoming widely recognized in bacteria. To determine the impact of Stk1/Stp1 on *in vivo* virulence potential of *S. aureus* an animal model experiment and histopathological analysis with *stk1* deletion mutant was carried out. The *stk1* knock-out showed significantly reduced colonization in a murine model of kidney infection. This was reflected by reduced bacterial loads and reduced level of renal lesions in kidneys in comparison to a wild *S. aureus* strain 8325-4 suggesting an important role of Stk1 in *S. aureus* virulence (Debarbouille *et al.,* 2009). The role of Stk1 and Stp1 in regulation of hemolysin expression and in consequence virulence of *S. aureus* clinical isolates was also demonstrated. Transcriptomic analysis revealed that Stk1 decreased transcription of hemolysin gene, while the phosphatase Stp1 increased its expression. The employment of the soluble nanopolymer (PolyMAC) and high resolution liquid chromatography coupled with tandem mass spectrometry led to enrichment and identification of phosphoprotome fraction of proteins isolated from *S. aureus* Newman and the respective *stk1* knock-out. Comparative analysis of phospoproteome revealed that DNA binding histone-like protein (HU), serine-aspartate rich fibrinogen/bone sialoprotein binding protein (SdrE) and hypothetical protein (NWMN_1123) were potential substrates of eukaryotic-like kinase. Based on the obtained results the authors suggested that Stk1 alters *S. aureus* gene expression and virulence (Burnside *et al.*, 2010). With the use of 2-DE, phosphosensitive stain and gel-free titanium dioxide based phosphopeptide enrichment the physiological dynamics of *S. aureus* phosphoproteome was examined. 103 putative phosphorylated proteins were identified and 68 phosphorylation sites were successfully mapped. Moreover, eight proteins phosphorylated on arginine residues have been identified.

Proteins related to pathogenicity (SarA, FbaA and EbpS) were found among other phosphorylated proteins. Significant changes in phosphorylation under diverse physiological conditions, including nitrosative stress were noted in 10 proteins (Basell *et al.*, 2014). Surface shaving combined with high resolution LC-MS/MS was applied to investigate PTMs within surface proteins in *S. aureus* strain Newman, cultivated in different conditions. Hydroxymethlyation of asparagine and glutamine residues was observed in 15 proteins at 41 sites. Interestingly 35 sites appeared location specific. These results suggested the observed hydroxymethylation is selective, dependent on growth conditions and therefore an authentic post-translational modification, rather than an unspecific process. Although the authors speculated that the modification may modulate staphylococcal virulence there is no direct data in favor of this hypothesis and the function of this modification remains unknown (Waridel *et al.*, 2012). Glycosylation of *S. aureus* proteins was also recently studied. SRRPs (serine-rich repeat proteins) adhesions that mediate attachment to host and bacterial surfaces were found to be glycosylated (Lizcano *et al.*, 2012). Moreover, glycosylation pathways have been investigated. Glycosyltransferases A and B have been identified as responsible for glycosylation of surface glycoprotein SraP (serine-rich adhesin for platelets) with a proven role in staphylococcal virulence (Li *et al.*, 2014).

Studies on other bacteria confirm that post-translational modifications, including glycosylation of surface-exposed proteins, may promote adhesion and invasion, and thus are directly involved in pathogenesis (Upreti *et al.*, 2003; Zhou & Wu, 2009; Reid *et al.*, 2010). Such modifications are also important in determining the antigenic properties of bacteria. Proteomic techniques, particularly high resolution tandem mass spectrometry, allow efficient global investigation of PTMs in bacteria. Various post-translational modifications of *S. aureus* surface or extracellular proteins have been identified. However, the detailed explanation of their function in staphylococcal pathogenesis requires further studies.

CONCLUDING REMARKS

In this review we demonstrated that proteomics became an irreplaceable tool in the investigation of multiple aspects of *Staphylococcus* physiology, including virulence. The constant dynamic development of proteomic methods and equipment guarantees continued utility in the coming years and promises multiple important new findings. The gel-based and gel-free methods widen our understanding of how virulence factors interact with host cells and how the pathogen responds during infection to the ever-changing environment. Novel virulence factors will yet undoubtedly be identified and even more interestingly a complicated interplay between the known factors is yet to be understood. The genomic studies demonstrate high plasticity within staphylococcal genomes, especially manifesting in variable mobile genetic elements content. This provides an overall explanation of the observed differences in virulence and host specificity of different strains but does not allow to conclude on the specific mechanisms. Only proteomics provides data to confirm and extend the assumptions based on genetic studies by providing information on virulence gene expression, interaction and modifications. Bacterial genome sequencing becoming a routine procedure rather than a scientific challenge stresses the importance of proteomics in explaining the genomic findings. The

further developments of proteomics and its combination with genomic studies will certainly have an unprecedented impact on understanding the virulence of bacteria in the years to come. Another challenge for future analysis is to fully describe and determine the role of post-transcriptional modifications in modulation of virulence factors potential. To date, proteomics significantly widened our knowledge on the virulence factors of *S. aureus* and allowed to identify potential targets for drug or vaccine development. Nevertheless, the current findings also demonstrate significant lack of understanding of many important aspects. Integration of proteomics with other 'omics' studies such as transcriptomics and metabolomics will certainly contribute novel valuable insights. Further technical developments and extensive studies will slowly allow us to grasp the most significant interconnections within the repertoire of staphylococcal virulence factors and understand the major traits in pathogenesis. Even though we are still far from a complete understanding of the determinants of staphylococcal virulence and their role in pathogenesis, the current findings are already highly motivating and contribute to the development of better strategies to combat the health threats associated with this dangerous pathogen.

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Conflicts of interests

The authors declare that they have no conflicts of interests.

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