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The *Streptococcus pyogenes* proteome: maps, virulence factors and vaccine candidates

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Abstract

Streptococcus pyogenes is an important cause of human morbidity and mortality worldwide. A wealth of genomic information related to this pathogen has facilitated exploration of the proteome, particularly in response to environmental conditions thought to mimic various aspects of pathogenesis. Proteomic approaches are also used to identify immunoreactive proteins for vaccine development and to identify proteins that may induce autoimmunity. These studies have revealed new mechanisms involved in regulating the *S. pyogenes* proteome, which has opened up new avenues in the study of *S. pyogenes* pathogenesis. This article describes the methods used, and progress being made towards characterizing the *S. pyogenes* proteome, including studies seeking to identify potential vaccine candidates.

Keywords

cell wall-associated protein; group A streptococcus; proteomics; secretome; *Streptococcus pyogenes*; vaccine; virulence

Streptococcus pyogenes is a major cause of human morbidity & mortality

Streptococcus pyogenes (group A streptococcus) is a cause of significant morbidity and mortality worldwide. This Gram-positive respiratory and skin pathogen can be carried by humans asymptotically or cause uncomplicated pharyngitis; however, *S. pyogenes* can also cause life-threatening diseases such as streptococcal toxic shock syndrome and necrotizing fasciitis. Each year, approximately 1.8 million people in the USA are diagnosed with streptococcal pharyngitis, with an associated cost of over \$100 million [1]. Worldwide, nearly 700,000 people acquire an invasive infection, which is defined as the presence of the organism in a normally sterile site.

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Prompt treatment of pharyngitis is important in decreasing the likelihood of dissemination of the pathogen and to prevent postinfection sequelae such as acute rheumatic fever, an autoimmune disease caused by antibodies directed to *S. pyogenes* that also react with human tissue [2]. A variety of pediatric neuropsychiatric syndromes such as Tourette's syndrome, tics and obsessive compulsive disorders, are also associated with *S. pyogenes* infections; although the precise role *S. pyogenes* plays in these manifestations remains unclear [3,4].

Streptococcus pyogenes is also an important contributor to the so-called excess mortality associated with influenza, which occurs when influenza infections are succeeded by secondary bacterial infections. Excess mortality contributes to human morbidity and mortality during both influenza epidemics [5,6] and pandemics [7]. *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae* and *S. pyogenes* are the typical bacterial causes of influenza-associated secondary infections [7]. During the influenza pandemic of 1918–1919, *S. pneumoniae* and *S. pyogenes* were the most frequently observed bacterial species in the lungs of infected soldiers [7] and together contributed to an estimated 90% of all deaths [7]. During the 2009 H1N1 swine-origin influenza pandemic, 29% of deaths have been attributed to secondary bacterial pneumonia, and 27% of these fatalities were caused by *S. pyogenes* [8].

Overall, *S. pyogenes* causes in excess of half a million deaths each year worldwide, which is similar to that of hepatitis B or *H. influenzae* [9]. There is no licensed vaccine to prevent disease and while the pathogen remains sensitive to β -lactam antibiotics, it has relatively recently acquired resistance to macrolide antibiotics [10,11]. Thus, the current burden of *S. pyogenes* on human health is significant and a better understanding of the *S. pyogenes* proteome – the molecules largely responsible for mediating host–pathogen interactions – is essential for developing new treatment strategies and vaccines.

Streptococcal genomics

The complete sequence of the *S. pyogenes* genome was first reported in 2001 by Ferretti *et al.* [12], which ushered in the genomic era of *S. pyogenes* research and facilitated the development of functional genomics, including DNA microarrays and proteomics. Shortly thereafter, the genome sequences of several additional isolates of *S. pyogenes* were reported [13–21]. Currently, the genome sequences of 13 isolates of *S. pyogenes* are publicly available. These strains represent ten different serotypes (M1, M2, M3, M4, M5, M6, M12, M18, M28 and M49) based on the sequence of the variable antiphagocytic surface protein known as the M protein [22]. The genomes encode between 1697 and 1987 predicted proteins [23]. The core genome refers to genes present in all isolates or strains, while the pan-genome refers to those present in only a subset of isolates. Genes in the pan-genome are typically associated with horizontally transmitted genetic elements such as temperate bacteriophage, transposons and insertion elements. Comparative analysis of the *S. pyogenes* genome sequences identified a core metagenome containing 1376 inferred proteins (encoded by approximately 1670 kb) and a pan-genome of uncertain size [15,23]. The wealth of genomic information enhances the ability to use functional genomic approaches in the study of *S. pyogenes*.

The temporal progression of genome-scale studies of *S. pyogenes* has mimicked the so-called central dogma of molecular biology. The field enjoys an abundance of genomic data relating to various isolates of *S. pyogenes*, which has greatly enhanced our appreciation of the genetic diversity of the pathogen. Numerous studies using DNA microarrays to characterize the transcriptome have led to significant insight into the pathogen's response to changes in its environment and a realization that the genomic diversity observed in the species is compounded by strain-associated variation in regulatory networks. Proteomic

approaches have been used to address a very broad range of topics related to the virulence associated with *S. pyogenes*. With that in mind, the objective of this article is to review studies that have analyzed the *S. pyogenes* proteome with an attempt to emphasize those studies that illustrate the increase in complexity generated at the protein level, which is likely to contribute to phenotypic variation within the species and, at least to some extent, the diverse clinical outcomes associated with infection.

Methods used to study the *S. pyogenes* proteome

The proteome is defined as the entire complement of proteins expressed by an organism under defined conditions [24]. Proteomic studies typically involve the isolation, separation, quantitation and identification of proteins. Based on the predicted proteins encoded by the SF370 genome sequence [12], a hypothetical map of the proteome separated by 2D gel electrophoresis (Figure 1) is bimodal, with the majority of proteins migrating around isoelectric points (pIs) 5.5 and 9.0, which is similar to predicted proteome maps of *Escherichia coli*, *Bacillus subtilis* and *Listeria monocytogenes* [25–27]. In *S. pyogenes*, proteins with pI values between 4 and 7 represent approximately 60% of the predicted proteins in the genome. 63% of proteins with a pI outside the range of 4–7 had a pI greater than 9.0. Due to the difficulty associated with simultaneously separating such a biochemically heterogeneous mixture, investigators have typically focused on a subproteome, or a portion of the proteome.

Fractionation into subproteomes

Commonly studied subproteome fractions include proteins recovered from the culture supernatant (the secretome), cell wall-associated proteins and cytoplasmic proteins. The secretome has been the most studied, in part because the proteins can be isolated relatively easily and because there are fewer proteins compared with the cytoplasm, which simplifies the analysis. More importantly, several established virulence factors, including cytolytins, superantigens, DNases and proteases are present in this fraction. A cysteine protease secreted by many, but not all [28–31], isolates of *S. pyogenes* is SpeB, which degrades several streptococcal and host proteins. To minimize SpeB-mediated degradation of proteins in the secretome, some studies have used *speB* mutant strains. The use of *speB* mutants in the study of the secretome also aids in the detection of secreted proteins because in many isolates SpeB is so abundant it obscures the detection of other exoproteins.

Another subproteome associated with virulence consists of cell wall-associated proteins, which serve as the interface between the pathogen and host. Cell wall-associated proteins are generally isolated by incubating whole cells with lysozyme and mutanolysin in a hypertonic solution. The proteins are released from intact cells, along with hydrolyzed cell wall debris, and separated from protoplasts by centrifugation. A detailed protocol for the isolation and separation of cell wall-associated proteins with 1D or 2D gel electrophoresis (1-DE or 2-DE) is available [32]. Typically, the term applies to proteins covalently attached to peptidoglycan by an LPXTG motif present in the carboxyl terminus of the protein. Sortase recognizes and cleaves the polypeptide at the motif, allowing covalent attachment to the cell wall [33,34]. Important members of this fraction include the antiphagocytic M protein, C5a peptidase, which inhibits neutrophil migration to the site of infection by degrading the chemoattractant C5a component of complement, fibronectin binding-proteins involved in adherence (protein F, SfbI), the protein G-related α -macroglobulin-binding protein (GRAB), and serum opacity factor (SOF), among others [2]. Although the proteins localized to the cell wall are clearly important in virulence, analysis of this subproteome is hindered by the presence of the polysaccharide capsule and the susceptibility of many of these proteins to degradation by SpeB.

Characterization of cytoplasmic proteins is challenging given the relatively large number of proteins in the fraction (despite the relatively small genome) and the magnitude of differences in protein abundance, size, isoelectric points, solubility and other biochemical properties. Nonetheless, several studies, discussed below in more detail, have characterized cytoplasmic proteins during growth with different conditions, or between wild-type and mutated derivative strains [35–42].

Separation & identification of streptococcal proteins

Following the isolation of streptococcal proteins from one of the subproteomes, separation has most often employed 1-DE or 2-DE, followed by protein staining. Gel-based proteomics, particularly 2-DE, is a powerful tool to separate complex protein mixtures and the availability of immobilized pH gradients has simplified and standardized the procedure and made it accessible to more laboratories. The technique is particularly effective in resolving post-translationally modified proteins. Fluorescent protein stains are available that have sensitivity similar to silver staining but superior linear dynamic range. Despite significant technologic advances in recent years, the technique remains cumbersome and has several well reported limitations, including its inability to resolve proteins with very low or high pIs or molecular weights. And while fluorescent stains are comparatively sensitive, consistent detection of proteins present in the cytoplasm at low levels, such as regulatory proteins, remains a challenge with 2-DE.

Although early studies used Edman degradation to sequence the amino terminus of proteins, proteins are now typically identified by using either matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-MS) or electrospray ionization tandem mass spectrometry (MS/MS) coupled to liquid chromatography. The advantage of MS/MS in protein identification is that the masses of the intact tryptic peptides, and those derived from fragmenting the peptides, are determined and used to identify the corresponding protein in genomic databases. In contrast, identification with MS is accomplished by determining only the masses of each intact tryptic peptide and thus multiple peptides are necessary for robust protein identification. By this regard, MS/MS techniques have largely replaced those relying solely on MS for protein identification. Moreover, recent advances in MS facilitate MS/MS analysis using both electrospray ionization and MALDI [43].

Dynamics of the *S. pyogenes* proteome

Several studies have characterized changes in the *S. pyogenes* proteome when the pathogen is cultured under conditions designed to mimic growth within the human host. These include identifying changes in the proteome following exposure to antibiotics, human blood or plasma, and fluoride, as well as at different times during batch culture [37,38,40–42,44–47]. Changes among virulence and stress associated proteins identified in these studies are summarized in Supplementary Table 1 (www.futuremedicine.com/doi/suppl/10.2217/fmb.10.116).

Growth phase-associated changes in the proteome

Although clearly oversimplified, the exponential phase of growth is thought to be representative of early stages in colonization when the bacterial density is low and nutrients are abundant. By contrast, the postexponential phase is thought to represent later stages of infection when the bacterial density is high and nutrients are scarce [48]. Growth phase-associated changes in the abundance of cytoplasmic proteins were identified using a serotype M49 strain [36]. Cytoplasmic proteins from the exponential and stationary phases were isolated, separated by 2-DE, and proteins of interest identified using MS/MS. An average of 527 and 403 protein spots were detected in the exponential and stationary phases

of growth, respectively. 128 of the growth phase-regulated proteins were identified by MS/MS. Briefly, enzymes involved in glycolysis and pyruvate metabolism, as well as several stress-responsive proteins, were more abundant in the stationary phase of growth [36]. For example, the proteins AtpA and Nox1, which are essential for the growth of *Enterococcus faecalis* under acidic conditions [49,50], were more abundant during the stationary phase [36]. In addition, CysM, which contributes to the resistance of *Pseudomonas putida* to cold shock [51] and resistance of *Staphylococcus aureus* to tellurite, hydrogen peroxide and acidic conditions [52], were more abundant during stationary phase when grown in Todd-Hewitt yeast media and during growth in human plasma [36,38]. The study also used Affymetrix DNA arrays to measure transcript levels under ostensibly identical culture conditions [36]. While the array approach provided significantly more genome-wide information, the proteomics approach revealed extensive differences in protein modification associated with the two phases of growth, as inferred from differences in protein migration in 2-DE gels. Although the nature of the modifications is unknown, the results suggest that much remains to be learned at the protein level regarding the regulation of transitioning from the exponential to the postexponential phase of growth.

A related study revealed that long-term (over 3 months) persistence of *S. pyogenes* in batch culture is associated with the generation of variants with heterogenic metabolic activity [41,53]. In addition, the study identified a number of positional variants in 2-DE gels associated with the persistor cells. Such phenotypic variation *in vivo* may increase the likelihood of the pathogen successfully colonizing the host by allowing for selection the most successful phenotype.

Changes in the proteome in response to changes in the environment

Fluoridation is widely used in several countries to decrease dental caries; however, the effect on microbes of the upper respiratory tract is not completely understood. Cytoplasmic and secretome maps of *S. pyogenes* were created using cultures grown in chemically defined media [54] in the presence, or absence, of fluoride [40]. Although fluoride did not alter the viability of the pathogen, its presence decreased the abundance of 38 proteins, including stress-responsive proteins and proteins previously implicated in the development of postinfection sequelae, such as the M protein. Of note, the abundance of M protein decreased in the fluoride-exposed cultures, while that of SpeB increased. Because SpeB degrades the M protein, it remains unclear if the changes in M protein were due to SpeB-mediated degradation or if the differences were due to changes in expression. Nonetheless, the results led the authors to speculate that the lower incidence of poststreptococcal sequelae in many countries may be due, in part, to decreased expression of autoimmune-inducing streptococcal proteins; although the main factors thought to be responsible for less postinfection sequelae are primarily attributed to prevention by rapid antibiotic treatment of pharyngitis and the presence of fewer serotypes of *S. pyogenes* associated with rheumatic fever in these regions [55,56].

Changes in protein expression in *S. pyogenes* exposed to human plasma were investigated both with gel-based separation techniques and by a gel-free quantitative approach [38,57]. In the study by Johansson *et al.*, *S. pyogenes* was grown in the presence and absence of human plasma and cytoplasmic proteins were separated using 2-DE with two different pI ranges (4–7 and 6–11) [38]. Plasma was associated with increased abundance of 24 proteins, including metabolic proteins, and the virulence-associated M protein and C5a peptidase. Only two proteins were less abundant in the presence of sera; a hypothetical protein and a protein predicted to be involved in cell division. The study also identified multiple variants of two virulence factors: one M1 protein variant lacked 13 amino acids at the amino terminus, while six variants of C5a peptidase were identified in plasma-grown cells [38]. Again, the functional significance of these protein variants, if any, remains to be determined.

In the related study by Lange *et al.*, multiple (also referred to as selected) reaction monitoring assays were used to quantitate nine virulence-associated proteins when *S. pyogenes* was grown in the presence of 1–20% human plasma [57]. In general terms, the technique involves selecting a tryptic peptide and peptide fragments for each protein of interest that generate high intensity signals under optimized mass analyzer parameters. Using this quantitative approach, the abundance of a putative endopeptidase O, collagen-like surface protein A, C5a peptidase and SpeB were discovered to be responsive to the presence of plasma, consistent with results obtained by Johansson *et al.* [38]. Although the development of the quantitative assay is currently time consuming because proteotypic precursor peptides and MS conditions must be identified, once these ‘coordinates’, or parameters, are established, the technique can be used to quantitate streptococcal proteins with a label free-shotgun technique (Box 1). Thus, the study provides an example of how nonlabeled shotgun strategies can be used to quantitate streptococcal proteins.

Box 1

Shotgun proteomics

- A heterogeneous collection of proteins is digested (often with trypsin) resulting a large mixture of peptides, which are then separated by liquid chromatography and analyzed by tandem mass spectrometry to identify proteins in the original mixture.
- Proteins that are not amenable to 2D gel electrophoresis (DE), such as those that are not soluble, too small or large, or of low abundance, can be detected because the proteins are not separated with 2-DE prior to identification with mass spectrometry. The technique is effective, in part, because it converts biochemically heterogeneous proteins into relatively homogenous peptides, which are more amenable to analyses.
- Due to the large number of peptides typically present following digestion of complex protein samples, multidimensional chromatography is often employed.
- Technical advances in mass spectrometry, liquid chromatography and bioinformatics will accelerate the development of shotgun proteomics.

Additional studies designed to mimic various disease states have examined differences in protein expression in the presence of hyaluronic acid, which is a primary component of the human extracellular matrix [42], and changes in supernatant proteins in response to variations in atmospheric conditions (O₂, CO₂), temperature, osmolarity (NaCl), glucose and iron availability [45]. Among these conditions, iron supplementation appeared to have the greatest effect on the exoproteins and decreased the abundance of 17 exoproteins, including several established virulence factors (Sic, SpeF and Ska) [45]. The presences of hyaluronic acid increased the abundance of several virulence factors including M protein, glyceraldehyde-3-phosphate dehydrogenase and a collagen-like surface protein, as well as two hypothetical proteins [42].

High dose clindamycin and penicillin have been recommended for the management of streptococcal toxic shock syndrome, in part because clindamycin reduces the production of certain streptococcal exoproteins that contribute to disease, including the superantigen SpeA [58,59]. A re-examination of the influence of clindamycin on the secretome using 2-DE confirmed that the abundance of SpeB decreased in cultures exposed to clindamycin [47]. In addition, the abundance of several other exoproteins increased, many of which were previously reported to be degraded by the SpeB protease [60]; surprisingly, the increase appears to occur independently of SpeB-mediated degradation [46] and was recently

reported to occur at the transcript level for at least three of the exoproteins examined (SLO, NAD-glycohydrolase and SKA)[61].

The failure rate of treating pharyngeal infections with β -lactam antibiotics is estimated to be as high as 35%, for reasons that are not known but may include the presence of intracellular bacteria sequestered from antibiotics [62], or the presence of a subpopulation of bacteria that are tolerant, but not resistant, to the antibiotic [63]. Changes among cytoplasmic proteins following exposure to penicillin were examined using 2-DE and MS/MS [37]. In general, penicillin induced changes in the abundance of proteins involved in fatty acid biosynthesis, glycolysis and various stress responsive proteins. The analysis also indicated that in the absence of penicillin, a penicillin-tolerant strain of *S. pyogenes* had a protein profile that was similar to that of a susceptible strain following exposure to penicillin [37]. The results suggest that the changes that occur in response to penicillin exposure may confer tolerance to the antibiotic when expressed prior to exposure, or may confer tolerance to a subpopulation of cells in the culture, which might contribute to treatment failure.

Overall, studies examining the proteome under various conditions designed to mimic certain aspects of the infectious process, reveal a highly dynamic proteome, particularly with respect to proteins localized to the cell wall and the secretome. The observation is consistent with the knowledge that the expression of these proteins is complex and involves multiple regulatory factors [48,64,65]. Not surprisingly, the studies reveal significant complexity at the protein level compared with the genome or transcript levels. In addition, they indicate that post-translational mechanisms of regulating protein function are likely to contribute to the ability of the pathogen to respond to changes in its environment.

SpeB protease-mediated remodeling of the extracellular proteome

The secreted cysteine protease SpeB cleaves several host proteins, as well as the M protein and other streptococcal surface proteins [66–68]. Aziz *et al.*, analyzed SpeB alterations of an M1 serotype secretome using a proteomics approach [60]. 2-DE was used to resolve the secretome isolated from both a wild-type strain and a *speB* mutant in the presence, or absence, of the cysteine protease inhibitor E64. SpeB was found to degrade nearly the entire secretome [60], which implies that SpeB is an important factor in post-translational regulation of streptococcal exoproteins. Moreover, the results contributed to the discovery that SpeB-mediated degradation of streptococcal proteins contributes to the transition from localized mucosal colonization to an invasive phenotype [60,66,69–71].

The HtrA (DegP) family of proteins are important in the processing secreted proteins. To determine the role of HtrA in processing of *S. pyogenes* proteins, cell wall and culture supernatant proteins were isolated from an *htrA* mutant strain and the parental strain and compared by using 2-DE [72]. Proteins were identified with MS and the results showed that HtrA contributes to the activation of SpeB; however, it does so indirectly [72].

Together, proteomic studies indicate that significant post-translational modifications of extracellular proteins occur, much of it mediated by the SpeB protease. While the significance of many protein modifications is not known, amino terminal truncation of the M protein alters binding to host cell ligands, such as IgG and fibrinogen [73], and virulence in murine models of disease [74], illustrating the potential impact of such modifications on host–pathogen interactions.

Characterization of regulons

Proteomics has been a useful tool to identify streptococcal regulons, particularly in assessing changes in the expression of cell wall-associated and secreted proteins. In general, studies

have compared protein fractions obtained from a wild-type parental strain to an isogenic strain in which a regulatory gene has been mutated. Some studies have used both proteomics and DNA microarrays to characterize regulons and to determine the potential contribution of post-transcriptional regulation in the control of gene expression.

Nra is a transcriptional regulator in the RALP family that represses the transcription of virulence factors localized primarily to the cell wall, including SfbX, SOF, C5a peptidase, M protein, SpeA, SclA, SLO, NAD glycohydrolase and surface adhesins (protein F2, PrtF.2), among others [39]. Proteomics was used, in conjunction with DNA microarrays, to identify other members of the regulon on a genome-scale. Both culture supernatants and cell wall-associated subproteomes were analyzed with 2-DE. In a modification of the protocol commonly used for obtaining cell wall-associated proteins, phage lysin C was used instead of mutanolysin. Following spot identification with MALDI-TOF MS, a total of 67 proteins, in both fractions, were identified as being differentially expressed in an *nra* mutant derivative of the parental strain (serotype M49) [39]. The largest differences in protein abundance were PrtF.2 in culture supernatants and SclA in the cell wall-associated fraction. With some exceptions, the changes identified with proteomics correlated with results obtained by DNA microarrays.

Rgg, also known as RopB, was first identified as a transcriptional regulator of *SpeB* expression [75]. To identify additional changes in the expression of exoproteins associated with *rgg* inactivation, the culture supernatant proteins of an *rgg* mutant strain were compared with those of a *speB* mutant strain by using 2-DE and MALDI-TOF MS [76]. The *speB* mutant strain was used to avoid detecting changes in protein abundance due to *SpeB*-mediated proteolysis of culture supernatant proteins. The results showed that Rgg regulates the expression of at least two secreted nucleases and provided the first evidence that Rgg controlled the expression of more than *speB* in strain NZ131. Cytoplasmic proteins were also compared between a wild-type and an *rgg* mutant strain at three time points during batch culture [35]. A total of 125 proteins were identified corresponding to 78 genes. The most abundant cytoplasmic proteins were enolase and glyceraldehyde-3-phosphate dehydrogenase. The analyses also revealed the extent to which *rgg* inactivation disrupted growth phase-associated gene regulation. Specifically, the proteome of the *rgg* mutant in the exponential phase of growth resembled that of the wild-type strain in the postexponential phase of growth. To test this observation further, the abundance of each protein spot detected in the mutant strain during exponential growth was compared with the abundance of the matched protein spot in the wild-type strain during postexponential growth. The comparison revealed a correlation between protein levels in the two different phases of growth (Figure 2), illustrating that *rgg* inactivation uncoupled growth phase-associated regulation in this strain. Specific examples of the loss of growth phase-dependent regulation were also identified. For example, the arginine deiminase pathway, consisting of enzymes encoded by the ArcABC operon, is only expressed post-exponentially in wild-type bacteria; however, each enzyme was detected in the mutant strain during the exponential phase of growth. The results also correlated with metabolic studies that showed the *rgg* mutant catabolized arginine during this time [77]. Thus, *rgg* inactivation uncouples growth phase-dependent control of gene expression, which is of interest, in part, because many virulence factors are regulated in a growth phase-dependent manner. The finding is particularly important because growth phase-dependent regulation of gene expression in *S. pyogenes* does not appear to be dependent upon an alternative σ factor, such as RpoS. The only characterized alternative σ factor in *S. pyogenes* induces genes associated with genetic competence; although *S. pyogenes* is not known to be naturally competent for DNA transformation [78]. Thus, growth phase-dependent regulation appears to be coordinated by a handful of transcriptional regulatory factors, including Rgg [48,64].

Protein localization to the cell wall & culture supernatant

As noted in previous reviews [79,80], significant discrepancy exists between the proteins predicted to be cell wall-associated and supernatant proteins and the proteins actually identified in these fractions. Several explanations can account for the inability to detect predicted extracellular proteins in these fractions including a low level of expression, degradation by streptococcal proteases and the lack of transcription under the culture conditions employed. More intriguing, is the presence of canonical cytoplasmic proteins, such as glycolytic proteins, in extracellular fractions. In studies characterizing the cytoplasmic proteins [36,37,41], the most abundant proteins identified are also those typically identified in cell wall and extracellular fractions, suggesting that lysis of a subpopulation of the culture releases cytoplasmic proteins into the culture supernatant. In addition, some of these proteins are likely to interact with cell wall proteins, resulting in localization to the cell wall. *S. pyogenes* isolates are typically polylysogenized with temperate bacteriophage. Thus, one possibility is that prophage induction and lysis of a subpopulation of cells in culture, results in the release of cytoplasmic proteins. Moreover, because the genome typically encodes several extracellular nucleases, genomic DNA released into the culture medium may be quickly degraded and thus cell lysis is not detected by an increase in viscosity. If streptococcal prophages are involved in the release, and subsequent localization, of ostensibly cytoplasmic proteins to the cell wall, then strain-associated variation in prophage composition may generate additional diversity within the species in the number and types of proteins localized to the cell wall. Alternatively, the possibility exists that novel translocation processes are involved and remain to be discovered. Regardless of the mechanism whereby canonical cytoplasmic proteins are localized to the surface or culture supernatant, some have well characterized functions as extracellular proteins. Perhaps the best example is glyceraldehyde-3-phosphate dehydrogenase, or streptococcal plasmin receptor, which is localized to the surface of *S. pyogenes* and contributes to pathogenesis, in part, by binding plasmin [81–83]. Thus, proteomic analysis has revealed surprising patterns of protein localization, which has enhanced our understanding of streptococcal pathogenesis.

Surface binding characterization

Streptococcus pyogenes surface proteins bind several host–cell factors to mediate adherence, internalization and to evade the host immune response. Because the interactions are largely, but not solely, mediated by proteins, proteomics is a useful tool in characterizing these interactions. Protein chips were used to simultaneously analyze multiple proteins for the ability to bind various ligands. A protein chip consisting of 106 recombinant streptococcal proteins predicted to be surface localized was created by expressing the corresponding genes in *E. coli*, purifying the epitope tagged proteins and arraying the proteins on nitrocellulose-coated slides. Using the chip, Margarit *et al.*, identified proteins with the ability to bind to fibrinogen, fibronectin and the C4 binding protein [84]. In addition to confirming previously described interactions, the study discovered new interactions. Specifically, two membrane proteins were discovered to interact with C4 binding protein and a protein capable of binding fibrinogen was discovered [84]. The study illustrates how proteomics can aid in the characterization of protein–ligand interactions and in the discovery of protein function.

Discovering immunoreactive proteins

Vaccine candidates

Identifying immunogenic streptococcal proteins is important in the discovery of protein correlates of immunity, to identify cross-reactive epitopes involved in the pathogenesis of postinfection sequelae and in the identification of potential vaccine targets. *S. pyogenes*

secretes many proteins into the surrounding environment, which in nature is its human host. The genome sequence of strain SF370 is predicted to encode 83 proteins with a putative type II secretion signal peptide sequence [85]. In a study by Lei *et al.*, culture supernatant proteins were obtained during both the exponential and stationary phases of growth of strains MGAS5005 (serotype M1) and MGAS315 (serotype M3). The proteins were separated with 2-DE, and the amino terminal of proteins of interest were determined by Edman degradation [86]. The results showed significant interstrain differences in the secretomes, including differences in the abundance of important virulence associated proteins such as mitogenic factor (a DNase), NAD-glycohydrolase and streptolysin O, among others. In addition, the immunogenicity of culture supernatant proteins was determined by using human sera and sera obtained from experimentally infected mice [86]. In total, 44 proteins corresponding to 66 protein spots were identified [86]. Most (61%) did not have a signal peptide and were thought to be cytoplasmic proteins. These included many metabolic enzymes, ribosomal proteins, chaperones and proteins representing other functional classes. In addition, several isoforms of individual gene products were detected, often as a cluster of protein spots with similar molecular weights but having different isoelectric points. Because Edman degradation was used, the authors were able to show that some of the isoforms were derived from truncation of the polypeptide at the amino terminus, presumably by proteolysis. For example, two forms of mitogenic factor were identified. The amino terminus of one isoform corresponded to amino acid position 43 of the inferred polypeptide, while the other corresponded to at amino acid position 42. It is unclear if there are functional differences among the isoforms. Similar differences in the amino terminus of the M protein were reported. In addition, isoforms of NADase had identical amino terminus sequences but different pIs; perhaps indicating the presence of post-translational modifications, or truncation at the carboxyl terminus. Most of the exoproteins possessing signal peptides reacted with human sera, indicating the proteins are expressed during the course of human infection.

A related study also used the enzymatic extraction (lysozyme and muramic acid) to isolate cell wall-associated proteins from serotypes M69, M53 and M6. The proteins were separated by 2-DE and 155 proteins were identified by MALDI-TOF MS [87]. In addition, Cole *et al.* used pooled sera from ten adolescents living in a region of Australia where 70% of the children have impetigo due to *S. pyogenes* [87]. A total of 45% of the isolated proteins identified were immunoreactive with pooled sera. As an additional tool to identify surface exposed proteins, streptococcal cells were biotinylated, cell wall extracts obtained, separated by 2-DE, and biotinylated proteins detected by western blotting using anti-streptavidin. The results indicated that approximately 30% of the extracted proteins reacted with biotin, which implies they are surface exposed. In general, the biotinylated proteins were also immunoreactive. Consistent with other studies, several metabolic enzymes were identified in the cell wall-associated fraction, many of which had also been previously detected in the exoprotein fraction [45,76,86,88,89].

A highlight of studies aimed at identifying surface-exposed proteins, includes those using the so-called 'shaving' approach [85,89]. An alternative to enzymatic extraction, the approach involves treating washed streptococcal cells with a protease, such as trypsin. The tryptic peptides are then collected by centrifugation and directly analyzed with MS/MS [85,89]. Using this approach, 95% of the 72 proteins identified were predicted by bioinformatics to be surface exposed, based on the presence of various domains characteristic of transmembrane and secreted proteins, lipoproteins and proteins containing an LPXTG motif [85]. Surprisingly, only 37 of the estimated 524 membrane proteins were identified, possibly because they are cloaked from proteolytic cleavage by the cell wall and/or polysaccharide capsule [85].

A similar approach was used to analyze strain SF370 grown to both the exponential and post-exponential phases of growth [89]. In this case, 79 surface-exposed proteins were identified, including 14 proteins containing an LPXTG motif. A total of 26% of the proteins identified are generally considered to be cytoplasmic proteins, similar to results described using cell wall hydrolases to extract proteins. Together, the complementary studies identified 118 surface-exposed proteins of *S. pyogenes*; however, despite using the same strain and a similar approach, only 33 proteins were detected in both studies.

Subsequently, both studies expressed and purified several streptococcal surface proteins from *E. coli* and assessed their ability to elicit specific antibodies in mice. One surface-exposed protein conferred protection against a lethal dose of *S. pyogenes*, illustrating the feasibility of the method in identifying protective antigens [85].

Secreted proteins can also be considered as vaccine candidates. For example, Lei *et al.* analyzed the extracellular proteome and identified several previously uncharacterized proteins [86]. Additional studies using mouse models of infection identified five lipoproteins as potential vaccine candidates [90]. Outstanding reviews of studies using proteomics in streptococcal vaccine development are available [79,80].

Discovering proteins associated with autoimmune sequelae

Significant morbidity and mortality results from the postinfection sequelae associated with *S. pyogenes*. While studies have identified auto-antigens potentially involved in the pathogenesis of postinfection sequelae [91], Bombaci *et al.*, assessed the reactivity of sera from 335 children, including sera from 61 patients with neuropsychiatric symptoms, to 102 recombinant *S. pyogenes* proteins arrayed on glass slides [92]. The study revealed that patients suffering from neurological tics typically responded robustly to a wide range of streptococcal antigens and indicates that the continued development of protein arrays is likely to accelerate the discovery of immune-responsive antigens.

Conclusion & future perspective

A hallmark of *S. pyogenes* is the diversity of clinical outcomes that may result following human colonization, presumably a result of complex host–pathogen interactions, most of which are mediated by proteins. Future studies of the *S. pyogenes* proteome will undoubtedly benefit from continued advances in instrumentation, work-flow strategies, protein and peptide separation techniques, and other innovations [43]. Although beyond the scope of this review, new approaches involving shotgun proteomics, quantitative protein determination, and advances in MS that fragment proteins without destroying post-translational modifications (so called top-down proteomics), will enhance our knowledge of the *S. pyogenes* proteome.

Proteomics is perhaps best suited to address four areas related to *S. pyogenes* pathogenesis. First, is the potential disparity between transcript levels, as readily measured on a genome-scale with DNA microarrays, and protein abundance. Such differences may result from a variety of post-transcriptional mechanisms of gene regulation, including differences in mRNA stability [93,94], and proteolysis. In this regard, relatively little is known about protein turnover in *S. pyogenes*, particularly in response to changing environmental conditions. A second area of importance is protein localization. As discussed above, significant progress has been made in characterizing cell wall-associated proteins and those of the secretome; however, the composition and dynamics of proteins localized to the cytoplasmic membrane remains largely unexplored. Gel-free protein separation techniques are likely to be important in characterizing these proteins, many of which are relatively insoluble in aqueous solutions. Third is the characterization of post-translational

modifications. This will be important to not only to identify potential functional changes associated with structural protein modifications, such as proteolytic processing, but also to characterize regulatory protein modifications, such as the phosphorylation state under various culture conditions. Finally, the development of protein chips will provide tremendous insight streptococcal host–cell–ligand interactions [84], the identification of additional correlates of immunity, and in the discovery of potentially pathogenic cross-reactive antigens associated with the variety of postinfection sequelae associated with *S. pyogenes*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Predicted 2-DE map of the *Streptococcus pyogenes* SF370 proteome
The mass and pI of the 1697 predicted open reading frames in strain SF370 were determined using ExPASy [101].

Figure 2. Comparison of protein abundance between the wild-type and *rgg* mutant strains in postexponential and exponential phases of growth, respectively

The amount of each protein spot identified in the wild-type strain during the postexponential growth was compared to the matched spot in an *rgg* mutant strain during the exponential phase of growth ($r = 0.9$).