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Human pathogenic streptococcal proteomics and vaccine development

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Abstract

Gram-positive streptococci are non-motile, chain-forming bacteria commonly found in the normal oral and bowel flora of warm-blooded animals. Over the past decade, a proteomic approach combining 2-DE and MS has been used to systematically map the cellular, surface-associated and secreted proteins of human pathogenic streptococcal species. The public availability of complete streptococcal genomic sequences and the amalgamation of proteomic, genomic and bioinformatic technologies have recently facilitated the identification of novel streptococcal vaccine candidate antigens and therapeutic agents. The objective of this review is to examine the constituents of the streptococcal cell wall and secreted proteome, the mechanisms of transport of surface and secreted proteins, and describe the current methodologies employed for the identification of novel surface-displayed proteins and potential vaccine antigens.

Keywords

Proteome, streptococcal, vaccine, *S. pyogenes*, *S. agalactiae*, *S. pneumoniae*, *S. mutans*

Disciplines

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Human pathogenic streptococcal proteomics and vaccine development

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Abbreviations: ABC, ATP-binding cassette; ADI, arginine deiminase; CBP, choline-binding protein; CPS, capsule polysaccharide; FBA, fructose-bisphosphate aldolase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GAS, group A *Streptococcus*; GBS, group B *Streptococcus*; OCT, ornithine carbamoyltransferase; PGK, phosphoglycerate kinase; PpmA, putative proteinase maturation protein A; QTOF, quadrupole time of flight; SAGP, streptococcal acid glycoprotein; Sec, general secretory; SPase, signal peptidase; SRP, signal-recognition particle; Tat, twin-arginine translocation

Keywords: Proteome, streptococcal, vaccine, *S. pyogenes*, *S. agalactiae*, *S. pneumoniae*, *S. mutans*

Abstract

Gram-positive streptococci are non-motile, chain-forming bacteria commonly found in the normal oral and bowel flora of warm-blooded animals. Over the past decade, a proteomic approach combining 2-DE and MS has been used to systematically map the cellular, surface-associated and secreted proteins of human pathogenic streptococcal species. The public availability of complete streptococcal genomic sequences and the amalgamation of proteomic, genomic and bioinformatic technologies have recently facilitated the identification of novel streptococcal vaccine candidate antigens and therapeutic agents. The objective of this review is to examine the constituents of the streptococcal cell wall and secreted proteome, the mechanisms of transport of surface and secreted proteins, and describe the current methodologies employed for the identification of novel surface-displayed proteins and potential vaccine antigens.

1 Introduction – Streptococcal infection of humans

The Genus *Streptococcus* consists of numerous Gram-positive, non-motile, chain-forming cocci commonly found in the normal oral and bowel flora of warm-blooded animals. Streptococci are a diverse group of bacteria capable of colonizing and infecting a broad spectrum of host organisms and tissues. The Genus is comprised of 49 species and 8 subspecies of which 35 have been isolated as a source of infection in humans [1]. Pathogenic streptococcal species fall into three broad categories: pathogenic species commonly causing infection in humans; commensal species and zoonotic species which under the right conditions cause opportunistic infection in humans. *Streptococcus pyogenes* (group A *Streptococcus*; GAS), *S. agalactiae* (group B *Streptococcus*; GBS), *S. pneumoniae*, and *S. mutans* are pathogenic streptococcal species commonly causing infection in humans (Table 1). GAS colonizes the mucosa of the respiratory tract and skin causing, most commonly, pharyngitis and pyoderma. When *S. pyogenes* colonizes normally sterile tissues severe invasive disease may result [2, 3]. GBS is a commensal bacterium colonizing the intestinal tract of a significant proportion of the human population; it is also a pathogen that invades a number of different host compartments and may cause severe neonatal infections [4-7]. *S. pneumoniae* is a commensal streptococcal species carried asymptomatically in up to 60% of the population. The pneumococcus is also responsible for a significant burden of disease in children under the age of 2 years, the elderly and patients with immunodeficiencies causing diseases including pneumonia, meningitis, and otitis media [8, 9]. *S. mutans* is the main causative agent of human dental caries, one of the most common infectious diseases afflicting humans [10]. Infection by commensal and zoonotic streptococcal species occur primarily

in immunocompromised individuals and the elderly [11-15]. Commensal organisms colonizing the oral mucosa, the surface of teeth (organisms of the groups *S. mitis*, *S. mutans*, *S. salivarius*, *S. anginosus*) and other mucosal niches (*S. anginosus* group), may cause infective endocarditis and have also been associated with serious suppurative infections (Table 1) [10, 16-19]. A few species of *Streptococcus* with reservoirs in animals have proven to be zoonotic, including species from horses (*S. dysgalactiae subsp. equisimilis*, *S. equi subsp. zooepidemicus*), cattle (members of the *S. bovis* group), dogs (*S. canis*), pigs (*S. porcinus*, *S. suis*), fish (*S. iniae*), and chickens (*S. gallinaceus*). Zoonotic species most commonly cause invasive disease and have been associated with toxin-mediated infections [12, 14, 20-24] (Table 1). Zoonotic streptococcal species are primarily food borne or transmitted through cuts and abrasions [12]. Table 1 highlights the significant burden of disease caused by human pathogenic streptococcal species.

The complete genome has been sequenced for multiple strains of *S. pyogenes* [25-30], *S. agalactiae* [31, 32], *S. pneumoniae* [33, 34] and *S. suis* [35] and a single strain of *S. mutans* [36]. The public availability of complete genome sequences coupled with adjunct technologies such as bioinformatics tools, proteomics, MS and N-terminal amino acid sequencing, has facilitated the compilation of comprehensive proteomic maps and the identification of promising target antigens for inclusion into vaccine formulations against human bacterial pathogens [37, 38]. This review will focus on the human pathogenic streptococci and cover 1) the secreted, surface and cell wall proteomes, 2) transport mechanisms for secreted, surface and cell wall proteins, and 3) advances in vaccine development and the role played by genomic and

proteomic data in informing the development of safe, efficacious, and broadly protective vaccines.

2 Streptococcal proteomics

Several different strategies are currently employed to identify bacterial surface proteins including: 1) an *in silico* genome-based strategy using algorithms such as PSORT to search complete genome sequences for putative proteins harboring cell wall anchor motifs and transmembrane domains; 2) the purification of surface-displayed proteins by chemical or enzymatic methods followed by 2-DE and identification by PMF; and 3) the cloning, expression and subsequent generation of antibodies against putative surface-displayed proteins for whole-cell assays and/or electron microscopic analysis to confirm surface-localization [39]. In the majority of cases, surface-localized streptococcal proteins are isolated either by sequential chemical extraction, or enzymatic digestion of the peptidoglycan-containing cell wall with lysozyme or mutanolysin in an isosmotic buffer to minimize cell rupture and contamination of the preparation with cytoplasmic proteins [40]. The extracts are then fractionated by 2-DE, the excised protein spots digested with trypsin, and the peptide fragments analyzed by MALDI-TOF MS or MS/MS [40]. While this strategy has the capacity to identify anchorless cell surface proteins potentially overlooked with a computational genome-wide screening approach [41, 42], it typically detects a relatively small number of membrane-associated proteins [43, 44], due either to their inherent hydrophobicity and aggregation during IEF, or their low abundance within the cell wall [40]. To circumvent these problems, streptococcal surface proteins have recently been mapped using the techniques of reverse vaccinology, which involve: 1) the identification of numerous putative vaccine candidates by *in silico* analysis of complete

genome sequences; 2) the cloning, over-expression and purification of each recombinant antigen; and 3) the subsequent active immunization of mice to test for immunological protection [45]. This strategy has been successfully used for identification of novel antigens in *S. pyogenes* [46-49] and *S. agalactiae* [50].

Two-dimensional gel electrophoretic profiles, or reference maps, have been generated for numerous streptococcal spp. [38, 51-56]. Basic reference maps of soluble cytosolic proteins are routinely used to compare various strains of streptococci and identify changes in protein expression under physiological stresses [51, 57]. Pathogenic streptococci express a broad spectrum of multifunctional cell wall and surface-associated proteins which facilitate adherence to and invasion of host cells, evasion of the host immune response, and degradation of host proteins [2, 58]. Consequently, the surface proteins of these bacteria are often important targets for vaccine development. Several cell wall and surface-localized streptococcal antigens have been identified as promising vaccine candidates by immunoproteomic analyses [41, 43, 44, 59-62]. Many proteins are also secreted from the streptococcal cell surface into the extracellular milieu [38, 46, 56, 62-65]. These proteins influence host-pathogen interactions and may contribute to disease. Gram-positive proteins destined for transport across the cytoplasmic membrane frequently contain a hydrophobic N-terminal signal sequence [66, 67]. Curiously, a number of secreted streptococcal proteins lack apparent secretion signal sequences [46, 56, 66]; thus the mechanism by which these proteins are transported to the bacterial cell surface is yet to be elucidated. This section will describe the methodologies currently employed for the identification of streptococcal surface-displayed and secreted proteins, which represent potential vaccine antigens or therapeutic targets.

2.1 *S. pyogenes* cell wall and surface-associated proteome

The initial proteomics-based identification of group A streptococcal cell wall and surface-associated proteins was undertaken by Cole *et al.* [59]. Cell wall extracts were harvested by enzymatic digestion using a combination of lysozyme and mutanolysin. The muralytic enzyme mutanolysin cleaves the β -1,4 linkage of the *N*-acetylmuramyl-*N*-acetylglucosamine in the glycan backbone of the highly conserved peptidoglycan-polysaccharide polymer, and is routinely used for the preparation of streptococcal protoplasts [68, 69] and the solubilization of cell wall-associated proteins, with minimal leakage of cytoplasmic contents [70, 71]. Mutanolysin extracts harvested from *in vitro* stationary-phase cultures of three different *S. pyogenes* serotypes (M69, M53 and M6) were analyzed by 2-DE and the most abundant proteins identified by in-gel tryptic digestion and MALDI-TOF MS analysis. Interrogation of publicly available databases containing complete GAS genomic sequences identified a total of 74 distinct proteins, 66 of which had not previously been characterized as cell wall-associated in GAS. In order to identify the immunoreactive cell wall-associated proteins, 2-D western blots of mutanolysin extracts were probed with pooled antisera from GAS-infected humans. This technique identified 33 immunoreactive cell wall proteins which are presumably expressed during the course of human infection. To determine which cell wall-associated proteins were surface-exposed, and hence potential vaccine candidate antigens, the GAS cell surface was biotinylated prior to mutanolysin extraction, 2-D immunoblotting and detection of biotin-labeled proteins with a streptavidin HRP conjugate. In the aggregate, 23 cell wall-associated proteins were biotinylated and thus surface-exposed, illustrating the usefulness of high-throughput proteomics in elucidating novel vaccine candidate

antigens. Depending on the strain, *S. pyogenes* has 14-17 cell wall-associated proteins predicted from *in silico* analysis of completely sequenced genomes [39, 72, 73]. The study by Cole *et al.* [59] only identified 3/17 predicated cell wall-linked proteins. Furthermore, while this proteomic approach is quantitative, the enzymatic degradation of the bacterial cell wall may cause cell lysis and contamination of cell wall fractions with cytoplasmic proteins [40]. However, the use of mutanolysin, lysozyme and other biochemical methods for the extraction of surface-associated proteins followed by 2-DE, has a distinct advantage over *in silico* genome searches given that a significant number of such proteins lack N-terminal signal and C-terminal anchor sequences [43].

Rodríguez-Ortega *et al.* [39] identified the surface-exposed proteins of *S. pyogenes* SF370 (serotype M1) by using a combination of biochemical, proteomic and bioinformatic techniques. Whole cells were treated *in situ* with proteolytic enzymes (trypsin or proteinase K individually, or in tandem) to “shave” the protruding cell surface-associated proteins. The released peptide fragments were purified, analyzed by nano-LC/MS/MS, and identified by searching the publicly available genome sequence of SF370 [39]. This approach identified 68 putative surface-associated proteins, including 12 canonical cell wall proteins harboring the LPXTG cell wall-sorting motif, 11 lipoprotein family proteins, 37 transmembrane proteins, and 8 secreted proteins. Only 4 identified proteins were predicted cytosolic proteins, illustrating that digestion of bacterial surface proteins does not rupture the cells nor release contaminating cytoplasmic proteins [39]. PSORT topological prediction analysis and fluorescence-activated cell sorting with protein-specific antibodies raised in mice, confirmed the surface-localization of most identified proteins. Furthermore, the putative cell-envelope serine proteinase

Spy0416 identified in this study was protective against virulent DSM2071 GAS challenge in a mouse model of intranasal infection [39]. The methodology used in this study circumvents contamination issues associated with the preparation of cell wall proteins by conventional enzymatic methods, and provides a tool for the selective identification of bacterial surface-exposed proteins and new vaccine candidates. This approach is also cheaper and less labor-intensive than the *in silico* genome-based strategy employed by Maione *et al.* [50], which involved the cloning, over-expression, and purification of 312 surface proteins of *S. agalactiae*, only 4 of which were protective in a mouse infection model [74]. However, Rodríguez-Ortega *et al.* [39] only identified 6.5% of PSORT-predicted transmembrane proteins, suggesting that these proteins are either resistant to protease digestion, undergo complete proteolytic degradation, are poorly expressed, are differentially regulated, or are hidden within the bacterial cell membrane [39]. Furthermore, this investigation only utilized a single growth medium and harvested the bacteria at a single growth phase. It is well established that the proteome of *S. pyogenes* differs significantly according to growth conditions and the phase of growth, which may influence the composition of the *S. pyogenes* surfaceome [45]. Of note, the SF370 M1 serotype differs significantly genetically from the globally disseminated M1T1 clone - the serotype responsible for a substantial proportion of severe invasive diseases, such as necrotizing fasciitis and streptococcal toxic shock syndrome [56, 75-77].

Severin and associates [40] also directly digested the surface-exposed proteins of *S. pyogenes* SF370 (serotype M1) with trypsin and identified the resultant peptide fragments by multidimensional nano-LC-MS/MS. However, in contrast to Rodríguez-Ortega *et al.* [39], surface-associated proteins were harvested from cells grown to early-

and late-exponential phase to account for the well characterized growth phase-dependent differences in protein synthesis [52]. *In silico* genomic analysis was also employed to identify putative cell surface-associated proteins. This proteomic approach identified 79 surface-associated proteins, including 33 proteins not previously characterized as being surface-localized in *S. pyogenes*. These proteins included 4/17 predicted cell wall anchor motif-containing proteins, 12/27 predicted lipoproteins, 9 secreted proteins, 22 membrane-associated proteins, 1 bacteriophage-associated protein, and 21 proteins traditionally classified as cytoplasmic. In the aggregate, 58 of the 79 proteins identified in this study were predicted to be surface-localized by computer-assisted sequence analyses with well established algorithms. Cell surface-localization of some proteins (12 of 16 tested) was confirmed by whole-cell ELISA using mouse antisera raised against purified recombinant protein. One of these, putative translation elongation factor Tu, is a traditional cytoplasmic protein involved in protein synthesis. Three hypothetical proteins exhibited high antibody titres [40], two of which are known to elicit protection against GAS challenge in a mouse model of infection [39, 48]. These findings suggest the method of direct digestion of bacterial surface-exposed proteins is an efficient way to identify putative surface antigens, which may facilitate the development of a safe and efficacious human vaccine. To avoid potential contamination of surface-exposed proteins with cytoplasmic proteins, whole cell tryptic digestion was performed in a buffer supplemented with D-arabinose, which promotes the formation of protoplasts in the event of cell wall hydrolysis. Cryo-field emission scanning electron microscopic analyses, viable cell counts, and the absence of the abundant cytoplasmic proteins lactate dehydrogenase and pyruvate oxidase, confirmed the integrity of whole-cells following

trypsin treatment [40]. Surprisingly, only 33 surface-exposed proteins were common to this study and the work of Rodríguez-Ortega *et al.* [39]. The differences in algorithms used for bioinformatic analyses, the stringency of matched peptides and protein identification, as well as differences in growth-phase, may account for this disparity [40].

2.2 Secreted group A streptococcal proteome

Several studies of the *S. pyogenes* proteome have focused on proteins released into the extracellular environment [46, 56, 64, 65]. Streptococcal pyrogenic exotoxin B (SpeB) is an extracellular and surface-associated cysteine protease virulence factor produced by most GAS strains [78]. Genetic inactivation of the *speB* gene or chemical inactivation of SpeB with the cysteine protease inhibitor E64, reveals that SpeB can efficiently degrade the majority of proteins in the secreted GAS proteome [46, 63, 79]. Using 2-DE and amino-terminal amino acid sequencing, Lei *et al.* [46] identified forty-four distinct proteins in the culture supernatants of two isogenic *speB* mutant GAS strains of serotypes M1 and M3, grown *in vitro* at 37°C. Many of these proteins had not previously been described in GAS and only sixteen of these had apparent secretion signal sequences, which included virulence factors streptolysin O, M protein, SpeA, SIC, and streptokinase. The remaining twenty-seven extracellular proteins identified had no apparent secretion signal sequences and included traditional cytosolic proteins such as glycolytic enzymes, chaperonins, protein translation factors, urea cycle proteins, ribosomal proteins, a DNA binding protein, and general housekeeping enzymes. Following 2-D western blot analyses with sera from GAS-infected mice and humans, many of the secreted proteins were found to be immunogenic and thus expressed during course of human infection [46]. The

absence of a signal peptide and LPXTG motif suggests that an alternative secretory pathway may exist for many secreted GAS proteins.

2.3 *S. pneumoniae* cell wall and surface-associated proteome

An immunoproteomics-based approach was used to identify novel *S. pneumoniae* vaccine candidates [44]. Enriched cell wall protein extracts from *S. pneumoniae* WU2 were resolved by 2-D electrophoresis and identified by MALDI-TOF MS. Two-dimensional western blot analysis using sera from children frequently exposed to *S. pneumoniae* and healthy adults, identified 17 common immunogenic proteins, 13 of which exhibited an age-dependent increase in antigenicity. Two of these proteins, identified as glycolytic enzymes fructose-bisphosphate aldolase (FBA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), elicited cross-strain protective immunity against respiratory challenge with virulent pneumococci in a mouse model of intranasal infection [44]. However, this investigation only identified a relatively small number of streptococcal cell wall-associated proteins containing cell wall anchor motifs, despite bioinformatic predictions of 14-16 in *S. pneumoniae* [72, 80].

Overweg and coworkers [61] isolated hydrophobic, surface-associated proteins from *S. pneumoniae* strain FT231 using the *N*-tetradecyl-*N,N*-dimethylammonio-1-propanesulfonate (SB14) extraction procedure [81]. Following separation by 2-DE, individual proteins were identified by MS and N-terminal amino acid sequencing. Two of the three proteins identified as contributing to opsonophagocytic activity *in vitro* included the previously characterized pneumococcal surface protein A and the oligopeptide-binding lipoprotein. The third protein was identified as the putative proteinase maturation protein A (PpmA). The surface localization of PpmA was confirmed using

immunoelectron microscopy, and the opsonophagocytic antibodies raised against PpmA were cross-reactive against several pneumococcal strains. In addition, PpmA was required for full virulence in a mouse pneumonia model. These data suggest that PpmA may be a suitable candidate antigen for inclusion in future pneumococcal vaccine formulations [61]. Additionally, proteomic analysis of mutanolysin cell wall extracts from *S. pneumoniae* WU2 (serotype 3) identified immunogenic proteins which may represent future targets for pneumococcal vaccine development [41]. These include traditional housekeeping proteins such as FBA, enolase, trigger factor, L-lactate dehydrogenase, pyruvate kinase, and phosphoglycerate kinase (PGK).

2.4 Group B streptococcal cell wall and surface-associated proteome

A proteomic analysis was undertaken by Hughes *et al.* [43] to identify the most abundant surface-associated proteins of *S. agalactiae*. Mutanolysin extracts of outer cell surface proteins were separated by 2-DE and identified using MS and reverse genetic techniques. The absence of DNA in the protein extract confirmed the absence of intracellular contamination. In total, 27 major protein spots were identified, 6 of which were previously unidentified in *S. agalactiae*, including ornithine carbamoyltransferase (OCT), PGK, nonphosphorylating GAPDH, purine nucleoside phosphorylase, enolase, and glucose-6-phosphate isomerase. Two of the putative surface-displayed cytoplasmic enzymes, OCT and PGK, were cloned, over-expressed *in vitro*, and the purified recombinant proteins used to raise polyclonal antisera. The antibodies were used in subsequent whole-cell ELISAs to confirm the cell surface localization of each protein. Passive immunization with antibodies against OCT and PGK in a mouse model of neonatal intraperitoneal infection afforded some protection against lethal *S. agalactiae*

challenge (46 and 35% survival for anti-OCT and anti-PGK, respectively, versus 64 and 2% survival for whole-cell positive control and preimmune serum, respectively), suggesting these proteins warrant further investigation as potential vaccine candidates [43]. Many of the anchorless surface-associated proteins identified by this proteomic approach may not have been identified by the conventional whole-genome screening strategy [82]. However, this method identified a relatively small number of streptococcal wall-associated proteins containing cell wall anchor motifs [43], despite genomic predictions of 25-35 proteins in *S. agalactiae* [72, 80]. Although labor intensive, this approach is quantitative and identifies bona fide surface-associated proteins [39].

2.5 *S. mutans* cell membrane-associated proteome

Len *et al.* [38] generated proteomic reference maps for soluble cellular and extracellular proteins of *S. mutans* grown in continuous culture. However, this study only identified three proteins associated with the cellular membrane (WapA, Gbp and SpaP). Membrane-associated proteins are frequently under-represented in proteomic reference maps due either to their low-abundance or hydrophobicity, which leads to poor solubility, aggregation during IEF, and poor resolution on 2-D gels [83-86]. Recently, Hasona *et al.* [87] undertook a systematic proteomic analysis of cytoplasmic membrane-associated proteins in *S. mutans* strain UA159 by combining 2-DE and nanoelectrospray quadrupole time of flight (QTOF)-tandem MS/MS. Previously, only the cytoplasmic proteins of *S. mutans* had been characterized by 2-DE [88-92]. Zuobi-Hasona *et al.* [85] solubilized cellular membrane proteins from *S. mutans* UA159 using three different methods, arrayed the samples by 2-DE, and identified the proteins by QTOF MS/MS. Several known membrane- and surface-associated proteins were successfully identified, including

enolase, glucose 6-phosphate isomerase, PGK, GAPDH, and ribosomal protein L7/L12 [85].

2.6 *S. suis* cell wall and surface-associated proteome

Two temperature-induced surface-associated proteins of *S. suis* strain I9841/1 (serotype 2) identified by a proteomic approach are highly homologous to members of the arginine deiminase system of *S. pyogenes*, including an OCT protein and the virulence factor streptococcal acid glycoprotein (SAGP) [93]. Immunoelectron microscopic analyses using anti-*S. pyogenes* SAGP antiserum confirmed the localization of the *S. suis* SAGP homologue, designated AdiS, on the streptococcal cell surface [93]. The surface localization, ubiquitous distribution of the *adiS* gene among *S. suis* strains, temperature-induced expression, and high sequence homology with the SAGP virulence factor, suggest that AdiS may represent a temperature stress induced virulence factor in *S. suis* [93].

In an attempt to identify the cell wall-associated proteins of *S. suis* harboring a C-terminal LPXTG motif, guanidine-insoluble mutanolysin-released cell wall proteins from strain NCTC 10234 (serotype 2) deficient in *srtA*, a sortase-encoding gene, were analyzed by 2-DE. Comparison with the parent strain identified 15 missing proteins in the cell wall-linked protein file of the *srtA* mutant, which were restored upon complementation with cloned *S. mutans srtA*. Four of these proteins were identified by N-terminal amino acid sequence analysis and their putative genes were predicted to possess an N-terminal signal sequence and a C-terminal LPXTG motif. The proteomics-based strategy utilized in this study overcomes the lack of complete *S. suis* genome sequence data for *in silico* bioinformatic screening of novel cell wall-associated proteins [94].

3 Protein export

Surface-exposed and secreted proteins are key players during the infectious process of streptococci and may serve as protective antigens for vaccine development. The first step in exporting proteins to the cell surface is the trafficking of protein across the cellular membrane. Several distinct pathways have been identified in Gram-positive bacteria for protein export from the cytoplasm via controlled translocation channels such as the general secretory (Sec) pathway, twin-arginine translocation (TAT) pathway, a pili-specific secretion/assembly pathway, and the “ExPortal” system [95].

3.1 Sec pathway

The majority of Gram-positive bacterial proteins, including streptococcal proteins, are exported by the highly conserved Sec pathway (Figure 1). This pathway has been extensively characterised [95] and will not be further described in this review.

3.2 TAT pathway

The TAT pathway translocates folded proteins harbouring a distinctive twin-arginine motif in the signal peptide of the corresponding precursor proteins [95] and comprises the three functionally distinct membrane proteins, TatA, TatB, and TatC (Figure 1) [96].

While TatB and TatC are involved in the specific recognition of the substrate, TatA may be the major pore-forming component [96]. Homologs of TatA and TatC have been identified in *S. thermophilus* and these homologs appear to be absent in other streptococci [97].

3.3 Pili-specific secretion/assembly pathway

Pili in Gram-positive pathogens are formed by covalent polymerization of adhesive pilin subunits and are secreted through the cell membrane by the Sec machinery [67] (Figure

1). Sortase, as described below, is required for the polymerization of the pili subunits and attachment of pili to the bacterial cell surface. Pilin subunit proteins are synthesized as precursors carrying N-terminal signal peptide and C-terminal LPXTG motif [67]. The identification of pili in streptococcal species has only been recently published [98-100]. Pili of *S. pyogenes* serotype M1 promote adhesion to human tonsil epithelium and primary human keratinocytes and in the formation of biofilm [101, 102], while the pili proteins of *S. agalactiae* facilitate adherence and invasion of brain endothelium [103]. The pilus operons promoting synthesis and assembly of pili are found in genomic islands such as the FCT pathogenicity island in *S. pyogenes* [98], the *rlrA* pathogenicity island in *S. pneumoniae* [100, 104], and in novel genomic islands in *S. agalactiae* [99, 105].

3.4 Exportal

A distinct microdomain termed the ExPortal has been identified in *S. pyogenes* which contains a high concentration of the Sec translocon component SecA [106]. Immunogold electron microscopic analyses demonstrate the location of the ExPortal to a hemispherical position distal to either cell pole [106]. *S. pyogenes* secretes the cysteine protease SpeB exclusively through the ExPortal and its maturation protein HtrA co-localize with SecA at this domain (Figure 1) [106, 107]. However, in a recent study using SecA antiserum, it has been demonstrated that SecA was distributed throughout the periphery of the surface of *S. pyogenes* and was not confined to the single ExPortal as described in earlier studies [108]. Such differences may relate to the different M types used in each study.

4 Protein retention

Retention of streptococcal secreted proteins to the cell surface can be achieved by one of the following mechanisms: 1) covalent attachment to the cell wall by sortase enzymes; 2)

non-covalent association with other surface molecules such as teichoic acids and lipoteichoic acids; and 3) direct anchorage to the cytoplasmic membrane as lipoproteins.

4.1 Covalently bound cell wall proteins

Sortase (SrtA) is a membrane-associated transpeptidase that recognizes the C-terminal sortase recognition motif LPXTG [109]. SrtA homologs are responsible for anchoring the majority of LPXTG containing streptococcal surface proteins and have been identified in the genomes of *S. pyogenes* [110], *S. gordonii* [111], *S. mutans* [112], *S. suis* [94], *S. pneumoniae* [113], *S. agalactiae* [114], and *S. sanguinis* [115]. More than one sortase gene has been identified in *S. pyogenes* [110], *S. pneumoniae* [116] and *S. suis* [117]. A class C sortase (SrtC2) has been recently identified in *S. pyogenes* which anchors a protein (Orf100) containing a QVPTGV motif to the cell surface [118].

4.2 Non-covalently bound cell wall proteins

The choline-binding proteins (CBPs) of *S. pneumoniae* and other Gram-positive bacteria all contain structurally similar choline-binding domains, which are composed of multiple copies of a repeat segment of around 20 amino acid residues referred to as the choline-binding domain [119]. The choline-binding motif has also been identified in other exported proteins, including the glucan-binding protein of *S. mutans* [120] and glycosyltransferases of *S. mutans* and *S. downei* [121, 122].

4.3 Lipoproteins

Lipoproteins contain a distinctive lipoprotein signal motif LXXC in the N-terminus region which is subsequently cleaved by the type II signal peptidase and covalently attached to the palmitic acid in the cell membrane [123]. Several members of this family of proteins have been identified in *S. pneumoniae* including PpmA, SlrA, PsaA, PiaA,

and PiuA [123, 124]. Further, putative surface-associated lipoproteins have also been identified by surface proteome studies in *S. pyogenes* [39, 40, 59].

4.4 Anchorless proteins

Proteomic analyses of the streptococcal surface have revealed a number of anchorless proteins that do not possess the classical features such as signal sequences or membrane-anchor domains [39, 40, 43, 59]. Remarkably, the analysis has identified a significant number of traditional cytoplasmic proteins commonly thought to be intracellular in streptococci. Many of these proteins have been identified as virulence factors in streptococci, such as the glycolytic pathway enzymes enolase and GAPDH, which have been identified as plasminogen-binding and cell wall-associated in *S. pyogenes* [125-127] and in *S. pneumoniae* [128].

The mechanism of translocation and retention of these proteins is not yet known. It could be hypothesized that these proteins are either passively released during autolysis, or that an alternative secretory pathway may exist for these proteins. Recently it has been demonstrated that α -enolase of *S. pneumoniae*, which is one of the key enzymes in the glycolytic cycle, re-associates to the cell surface by interacting with receptors on encapsulated and unencapsulated pneumococci [129]. This type of re-association mechanism may be utilized by the numerous anchorless proteins of streptococci, but further investigation is required. The enolase and GAPDH of *Lactobacillus crispatus* bind to the lipoteichoic acids on the bacterial surface in a pH-dependent manner, demonstrating for the first time a mechanism of glycolytic enzyme binding to the bacterial cell wall [130].

5 The development of vaccines against streptococcal infection

Traditionally, vaccines designed to prevent bacterial infection have been based on strategies including: 1) whole-cell vaccines containing heat-killed pathogens; 2) live attenuated vaccines constructed via inactivation of bacterial genes responsible for essential metabolic functions or pathogenicity; 3) toxoid-based preparations; 4) polysaccharides conjugated to suitable carrier proteins; and 5) subunit vaccines containing either a) whole proteins (purified or recombinant), b) minimal peptide epitopes, or c) cell wall and capsule antigens identified using biochemical, serological, microbiological, or genetic techniques. Such approaches were time-consuming, often taking many years or decades, and often failed when pathogens were difficult to culture *in vitro* [131]. However, a new era of vaccine development technology has emerged, aided by whole genome sequencing, bioinformatics, proteomics, and DNA microarrays. Such technologies have allowed rapid discovery and identification of putative virulence factors, surface-associated proteins and vaccine candidates.

The development of human streptococcal vaccines is challenging, facing obstacles such as the occurrence of many unique serotypes, antigenic variation within the same serotype, differences in geographical distribution of serotypes, and the production of antibodies cross-reactive with human tissue which can lead to host autoimmune disease. Streptococcal disease is a continuing worldwide problem, occurring in both developed and developing regions; thus the imperative for efficacious vaccines to prevent streptococcal disease is high. Furthermore, whilst antibiotics continue to be used to control streptococcal infection, there is increasing concern that such use, and particularly over-use, leads to the emergence of resistant strains [58, 132, 133]. Additionally, streptococci may avoid the effect of antibiotics via intracellular invasion

[134]. Although there are many candidate antigens and vaccine preparations under investigation, currently, for all streptococcal species, there are only two licensed human vaccines for use against pneumococcal infection.

5.1 Pnuemococcal vaccines

Although the two licensed *S. pneumoniae* vaccine preparations, comprising a conjugate heptavalent and a 23-valent vaccine, have been successful in reducing the incidence of streptococcal pneumonia and invasive pneumococcal disease caused by serotypes incorporated in the vaccine preparation [135-139], these have no protective effects against heterologous serotypes. Additionally, the 23-valent vaccine elicits a T-cell independent immune response and is thus not suitable for use in children under 2 years old or immunocompromised individuals. Comprehensive reviews of the different conjugate pneumococcal vaccine formulations and human clinical trials have been published by Wuoiimaa & Kayhty [140] and Oosterhuis-Kafeja *et al.* [135].

Pneumococci are genetically very flexible and can undergo natural transformation in order to obtain new phenotypic traits. Recombination events at operon(s) encoding capsule components can result in serotype switching, conferring the ability to evade the host immune system [141-143]. Consequently, high levels of diversity and evidence of capsule switching is observed in invasive pneumococcal isolates [144]. Given that the current capsule-based vaccines, both those licensed and those in pre-clinical and clinical development only protect against included serotypes, and that the administration of such vaccines can result in serotype switching, it is thought that protein-based vaccines may result in broader protection against *S. pneumoniae* infection [145].

Licensed pneumococcal vaccines and current vaccine candidates under investigation are outlined in Table 2. Whilst several protein-based vaccine preparations have been observed to protect in pre-clinical animal studies, and show promise as vaccine formulations, very few have reached human clinical trials. In other attempts to circumvent current problems associated with capsule polysaccharide (CPS)-based vaccines, several groups have used proteomic and bioinformatic based reverse engineering approaches to identify potential protein vaccine candidates. One proteomic study identified two immunogenic proteins, FBA and GAPDH, which were observed to protect against heterologous mucosal murine challenge [44]. A bioinformatics based study utilized an *in silico* whole genome screening approach to identify six vaccine antigens observed to protect against systemic challenge [146]. Such techniques show promise for the identification of efficacious vaccine candidates.

5.2 Group A streptococcal vaccine development

Currently there is no licensed vaccine to prevent group A streptococcal infection. Many surface-associated proteins have been identified and are under investigation as vaccine candidates (Table 3). GAS vaccinology has primarily focussed on the major virulence factor, the M protein. However, several factors have hampered the development of M protein-based vaccines such as the large number of unique M serotypes, the potential antigenic variation within a serotype due to the continual evolution of M protein [147], and the presence of cross-reacting epitopes which may trigger post-infective immune sequelae [148]. Furthermore, circulating GAS strains can rapidly be replaced by a new set of strains [149, 150]. As a consequence, N-terminal multivalent M protein vaccine preparations may need to be continuously reformulated

in order to protect against current circulating strains. On the other hand, non-M protein GAS vaccine candidates are generally highly conserved and have not been observed to result in the generation of cross-reactive antibodies. Several non-M protein vaccine candidates have been extensively investigated and shown to be protective in pre-clinical animal studies (Table 3), although, to our knowledge, none of these have reached human clinical trials.

M proteins are an obvious target for vaccine development as they are known to be a major virulence factor in GAS and have elicited protective immunity in several studies. Early studies involving vaccination of humans with crude M protein preparations followed by administration of live GAS to the pharynx effectively protected against GAS pharyngeal colonization [151-153], but one study noted an increased incidence of rheumatic fever in vaccinated versus unvaccinated control children [154]. This significantly hampered the development of whole M protein-based vaccines. Since then, a number of vaccine development studies have targeted the C-terminal repeat region peptides, conserved amongst all serotypes [155, 156]; whilst other studies have focussed on polypeptides derived from the serotype specific N-terminal repeats [157-162]. One group has developed a hexavalent vaccine containing protective N-terminal M protein fragments from six serotypes. The included serotypes were selected due to a frequent association with pharyngitis and acute rheumatic fever [162, 163]. This hybrid vaccine has been observed to generate high titre opsonising antibodies in rabbits [162] and to protect against murine mucosal challenge [164]. Recently, the vaccine was tested in phase I clinical trials and was found to produce a statistically significant increase in antibody titre for all six M protein fragments, with

five of the six serotypes being opsonised by the resulting anti-sera [163]. Additionally, there was no evidence the antibodies were cross-reactive with human tissue [163]. Although this vaccine was successful in phase I clinical trials, the major shortcoming of this hexavalent vaccine is that it only offers protection against six of an estimated 120 GAS M-types. In an attempt to broaden the protection, a multivalent vaccine containing variable amino terminal fragments of 26 different M proteins was produced using recombinant techniques [165]. Following immunisation of rabbits, type-specific antibodies raised against 25 of the 26 M protein fragments in the vaccine were detected and none of these antibodies cross-reacted with host tissue [165]. Additionally, this vaccine preparation was observed to be safe and immunogenic in phase I clinical trials [166]. Although this vaccine preparation shows promise for the prevention of GAS infection, it is likely a vaccine protective against all serotypes will be necessary for the eradication of GAS infection and disease.

In recent times, several proteomic studies of GAS have been carried out with the aim of identifying putative vaccine candidates. One such proteomic study, based on 2-DE and MALDI-TOF MS, identified 66 novel cell wall-associated proteins in three different strains of GAS [59]. Thirty-three proteins were observed to be immunoreactive with human antisera and 23 were suggested to be surface-exposed. Due to this surface localization and immunoreactivity data, such proteins may be suitable vaccine candidates. A proteomic trypsin digest of the cell-surface was utilised by Severin *et al.*, in which a total of 79 surface-proteins were identified, 33 of which were novel surface-associated proteins in GAS [40]. These proteins are yet to be tested as vaccine candidates in pre-clinical animal studies. Rodríguez-Ortega *et al.* identified

14 surface-exposed proteins which were tested for protection in a murine disease model. One of these antigens, Spy0416, elicited protection following heterologous mucosal challenge [39]. Such proteomics-based screening approaches may result in the identification of vaccine candidates efficacious against GAS infection.

5.3 Group B streptococcal vaccine development

Like GAS, there is no commercial vaccine available to prevent *S. agalactiae* (GBS) infection. Whilst several vaccine candidates, both CPS and protein-based, are currently under investigation (Table 4), one of the biggest hurdles for GBS vaccinology is the legal liabilities of immunizing pregnant women in clinical trials, in which any adverse outcomes could be linked to the experimental vaccine [167]. Of the current vaccine candidates under development, conjugated CPS-based vaccines have been found to be protective and immunogenic for vaccine strains [168-175], but are costly due to individual conjugation of each CPS to the carrier protein. Protein-based vaccines are a potentially cheaper alternative and may offer broader protection against all nine circulating serotypes.

Candidate GBS protein antigens have been identified using various proteomic and genomic screening methods. In a proteomic study of a GBS surface extract, 27 proteins were identified [43]. Two of these proteins, OCT and PGK, were observed to confer a degree of protection in a neonatal murine infection model, and thus warrant further investigation as GBS vaccine candidates. More recently, the same research group used an alkaline phosphatase-based genetic screening method to identify outer surface proteins. Two of the identified proteins, Pho2-2 and Pho3-1 (a *yutD* homologue and an ABC transporter subunit, respectively), protected against

heterologous subcutaneous challenge in a rat neonatal disease model [60]. Multi-genomic screening studies have also been utilized to identify potential GBS vaccine candidates. One group identified and tested a combination of four proteins, comprising surface immunogenic protein and three other putative surface-proteins, which were found to elicit protection in a neonatal murine disease model [50]. Furthermore, the combination of these proteins was found to protect against representative strains of all nine serotypes.

5.4 *S. mutans* vaccine development

Several surface antigens of *S. mutans*, the chief causative agents of dental caries, are also under investigation as vaccine candidates (Table 5). One of the limiting factors in the development of a vaccine to prevent dental caries is that it is a non-life-threatening disease and thus has a lower financial priority [176]. To date, limited proteomics studies have been performed on oral pathogens such as *S. mutans* and *S. sobrinus*, and those conducted primarily focus on growth under differing conditions rather than the identification of putative vaccine candidates. Refer to Macarthur & Jacques [177] for a review on the proteomic analysis of oral pathogens.

5.5 Streptococcal vaccine development: future prospects

Whilst there are many current vaccine candidates and newly emerging proteomics-based technologies leading to the identification of new candidates for *S. pneumoniae*, *S. pyogenes* and *S. agalactiae*, tested vaccine candidates for invasive zoonotic streptococcal strains such as *S. suis* or *S. dysgalactiae* subsp *equisimilis* and *zooepidemicus* are rare. Proteomic approaches may yield candidate vaccine antigens suitable for protection against human infection of these species. Although there are several streptococcal

vaccine preparations undergoing human clinical trials, the majority are designed predominantly to protect against strains in US and European populations. As the geographical distribution of strains is highly variable [178, 179], such preparations are often unsuitable for use in regions such as Asia, Africa and Oceania. If safe and protective universal vaccine candidates for streptococcal species can be trialed that elicit protection against all serotypes in humans, this would represent a major advance in human health.

6 Conclusion

The growing body of streptococcal proteomic data illustrates the usefulness of a proteomics-based approach in analyzing the cell surface topology of Gram-positive cocci, which lays the foundation for the future identification of novel virulence determinants, therapeutic agents and vaccine candidate antigens.

Figure legend

Figure 1. Protein export pathways in *Streptococcus*. Ribosomally synthesized proteins can be sorted to various destinations. A) via the Sec pathway (proteins containing N-terminal Sec-type signal peptide), B) via the TAT pathway (proteins containing twin arginine (RR/KR) signal peptides), C) via the pili-specific pathway, D) via the ExPortal, or E) via unknown export mechanisms (anchorless proteins).

Table 1 Clinical manifestations of human infection by pathogenic streptococcal species

Species group	Lancefield Group	Species	Clinical manifestations of human infection	Infection group	REFS
None	Group A	<i>S. pyogenes</i>	pharyngitis, pyoderma, erysipelas, bacteremia, septicemia, necrotising fasciitis, meningitis, pneumonia, septic arthritis, streptococcal toxic shock syndrome, scarlet fever, rheumatic fever ^b , glomerulonephritis ^b , reactive arthritis ^b , autoimmune neuropsychiatric disorders ^b	HP	[2, 3]
None	Group B	<i>S. agalactiae</i>	<u>Invasive disease in neonates (<6 days old):</u> septicemia, pneumonia, meningitis, cellulites, osteomyelitis, septic arthritis <u>Invasive disease in neonates (6 to 90 days old):</u> meningitis, bacteremia, necrotising fasciitis <u>Perinatal disease:</u> mild urinary tract infection, puerperal sepsis, chorioamnionitis, endometritis, cystitis, pyelonephritis, bacteremia, may trigger pre-term labour	HP	[4-7, 180]
<i>S. mitis</i>	Viridans	<i>S. pneumoniae</i>	otitis media, pneumonia, meningitis, septicemia, pleural empyema, septic arthritis, acute conjunctivitis	HP	[8, 9, 181, 182]
<i>S. anginosus</i> ^a	Viridans	<i>S. anginosus</i> <i>S. constellatus</i> <i>S. intermedius</i>	bacteremia, endocarditis, septicemia, meningitis (particularly <i>S. anginosus</i>), abscess (particularly <i>S. constellatus</i>), respiratory infections (particularly <i>S. intermedius</i>) including pneumonia, pleural empyema and pulmonary abscess	CO	[16, 18, 19, 183-186]
<i>S. mutans</i>	Viridans	<i>S. mutans</i> <i>S. criceti</i> <i>S. rattus</i> <i>S. sobrinus</i>	dental caries, bacteremia, endocarditis, septicemia	CO	[10, 11, 187, 188]
<i>S. salivarius</i>	None	<i>S. salivarius</i> , <i>S. vestibularis</i>	bacteremia, endocarditis	CO	[189-191]
<i>S. mitis</i> or <i>S. sanguis</i>	Viridans	<i>S. mitis</i> <i>S. oralis</i>	throat infection, bacteremia, endocarditis (primarily caused by	CO	[11, 17,

		<i>S. sanguis</i> <i>S. gordonii</i> <i>S. cristatus</i> <i>S. parasanguis</i>	<i>S. sanguis</i>), septicemia		189, 191- 196]
None	None	<i>S. sinensis</i>	bacteremia, endocarditis	CO	[184, 197, 198]
<i>S. anginosus</i> ^a	Viridans	<i>S. constellatus</i> subsp. <i>pharyngis</i>	pharyngitis	CO	[199]
<i>S. dysgalactiae</i>	Group C and G	<i>S. dysgalactiae</i> subsp. <i>equisimilis</i> , <i>S. equi</i> subsp. <i>zooepidemicus</i>	pharyngitis, bacteremia, septicemia, septic arthritis, endocarditis, meningitis (<i>S.</i> <i>zooepidemicus</i> only), pneumonia (<i>S. zooepidemicus</i> only), toxic shock syndrome, glomerulonephritis ^b	ZO	[14, 200- 205]
<i>S. bovis</i>	Group D	<i>S. bovis</i> <i>S. equinus</i> <i>S. infantarius</i> <i>S. gallolyticus</i> <i>S. lutetiensis</i> <i>S. macedonicus</i> <i>S. pasteuriana</i>	endocarditis, bacteremia, meningitis, septicemia <i>S. bovis</i> and <i>S. infantarius</i> are associated with severe inflammatory gastrointestinal disease and with a potential role in colon carcinogenesis	ZO	[206- 220]
None	Group G	<i>S. canis</i>	soft tissue infection, urinary tract infection, bacteremia, septicemia, bone infection, pneumonia	ZO	[24]
None	Group E, P, U, V	<i>S. porcinus</i>	wound infection, urogenital tract infections in women, implicated in pregnancy complications	ZO	[22, 221, 222]
None	None	<i>S. suis</i>	meningitis, sepsis, toxic shock syndrome, arthritis, enteritis, pneumonia, endocarditis, deafness ^b	ZO	[20, 223, 224]
None	None	<i>S. iniae</i>	bacteremic cellulites, arthritis, endocarditis, bacteremic osteomyelitis	ZO	[21, 225]
None	None	<i>S. acidominimus</i>	endocarditis, meningitis, pericarditis, abscess, pneumonia	ZO	[226- 229]
None	None	<i>S. gallinaceus</i>	bacteremia	ZO	[23]

Footnotes and abbreviations: a) the *S. anginosus* group was previously known as the *S. milleri* group; b) as sequelae; REFS, References; HP, common human pathogen; CO, commensal species causing opportunistic infection in humans; ZO, zoonotic streptococcal species causing opportunistic infection in humans.

Table 2 Licensed pneumococcal vaccines and vaccine candidates under investigation

Antigen	Function	Comment	Phase	REFS
Pneumococcus (<i>Streptococcus pneumoniae</i>)				
<i>Polysaccharide-based</i>				
7-valent (Prevnar)	Anti-phagocytic virulence	T-cell-dependent response (immunological memory). Shown to induce serotype redistribution. Costly.	L (2000)	[136, 143, 230-232]
9-valent and 11-valent	factor, most CPS conjugated to	Reduces incidence of disease. Safe and immunogenic. Only protects against limited serotypes.	II /III	[233-236]
23-valent (Pneumovax)	DT mutant	Non-conjugated, thus cheaper. T-cell-independent, not effective in children less than two years.	L (1983)	[138, 139]
<i>Protein-based</i>				
Surface protein A (PspA)	Complement inactivation	Protects against systemic infection. Safe and immunogenic. May induce cross-reactive antibodies.	P I	[237-239]
Surface adhesin A	Metal binding	Protects against mucosal and systemic challenge.	P	[240-243]
Surface protein C	Adherence, colonization	Protects against mucosal and systemic challenge. Not present in all strains. Highly variable.	P	[244, 245]
Protective protein A	Surface-exposed	Intranasal immunization of mice reduced colonization following heterologous mucosal challenge.	P	[246]
6PGD	Adhesin	Protection against heterologous mucosal challenge.	P	[247]
PiuA and PiaA	Iron uptake	Protection against systemic homologous challenge.	P	[248, 249]
Multi-antigen PspA, PsaA, PdB	As stated	Enhanced protection following mucosal and systemic challenge, multiple antigens may broaden protection.	P	[250, 251]
Pneumolysin (PdB)	Hemolysin	Highly conserved sequence. Toxic for humans. Non-toxic mutants best as a vaccine carrier molecule.	P	[252-257]
Histidine triad proteins	C3-degrading, unknown	Varying protection against heterologous mucosal or systemic challenge.	P	[258-260]
Neuraminidase	Glycoside hydrolase	Increased survival time and decreased colonization following mucosal challenge.	P	[261, 262]
Autolysin	Degrades cell wall	Protection following systemic challenge. Limited protection following mucosal challenge.	P	[263, 264]
Pilus subunits	Adhesion	Active and passive protection against challenge.	P	[265]

and Expression restricted to a subset of strains.
colonization

Abbreviations: CPS, polysaccharide capsule; DT, diphtheria toxin; 6PGD, 6-Phosphogluconate dehydrogenase; PspA, Pneumococcal surface protein A; PsaA, Pneumococcal surface adhesin A; PdB, pneumolysin toxoid; P, Pre-clinical; I, Phase I clinical trial; II, Phase II clinical trial; III, Phase III clinical trial; L, Licensed REFS, references.

Table 3 Group A Streptococcal vaccine candidates under investigation

Antigen	Function	Comment	Phase	REFS
Group A <i>Streptococcus</i> (<i>Streptococcus pyogenes</i>)				
<i>Protein-based</i>				
Whole M protein		Mucosal immunization reduces homologous colonization in humans. Increase of ARF in vaccinated subjects.	I	[151-154]
M protein C repeat	Surface adhesin, anti-phagocytic	Peptides protect against mucosal heterologous challenge. Systemic protection not investigated.	P	[155, 266-269]
M protein multivalent		6- and 26-valent immunogenic and safe in humans. Designed for US, may not protect in other regions.	P I/II	[160-166, 270]
C5a Peptidase	Cleaves C5a (complement)	Reduced colonization following mucosal challenge. Serotype independent protection.	P	[271-273]
FbaA	Fibronectin-binding	Protects against homologous systemic infection. Expression restricted to a subset of strains.	P	[274]
FBP54	Fibronectin-binding	Protection following systemic challenge. Expression restricted to a subset of strains.	P	[275]
Group A Carbohydrate	Anti-opsonisation	Protects against mucosal and systemic challenge. Cross-reacts with host tissues.	P	[276-278]
Protein F1/SfbI	Fibronectin-binding	Mucosal immunization protects against heterologous mucosal (not systemic) infection.	P	[279-281]
R18 Protein	Surface-associated	Passive protection against systemic infection. Expression restricted to subset of strains.	P	[282]
Serum Opacity Factor/SfbII	Serum opacification	Protection against systemic (not mucosal) challenge. Expression limited to subset of strains.	P	[283, 284]
SpeA and SpeC	Pyrogenic exotoxins	Rabbits protected against challenge. Expression restricted to a subset of strains.	P	[285, 286]
SpeB	Protease	Mice protected against systemic heterologous challenge.	P	[287]
Sib35	Ig-binding	Protection against homologous systemic challenge.	P	[288]
Spa	Surface-associated	Passive protection against systemic challenge.	P	[289]

Abbreviations: ARF, acute rheumatic fever; FbaA, Fibronectin-binding protein A; FBP54, Fibronectin-binding protein 54; Spe, Streptococcal pyrogenic exotoxin; Sib35, Streptococcal Ig binding protein; Spa, Streptococcal protective antigen; P, Pre-clinical; I, Phase I clinical trial; II, Phase II clinical trial.

Table 4 Group B Streptococcal vaccine candidates under investigation

Antigen	Function	Comment	Phase	REFS
Group B <i>Streptococcus</i> (<i>Streptococcus agalactiae</i>)				
<i>Based on Polysaccharide Capsule (CPS)</i>				
Type III polysaccharide		Safe and well tolerated but only 63% recipients responded. Immunogenicity must be improved.	I	[173]
CPS-TT conjugate	The capsule is an important virulence factor and there are nine distinct capsule types identified.	Vast improvement in immunogenicity. Only provides type-specific protection.	P I	[168, 169, 171, 172, 174, 175, 290-295]
Multivalent CPS-TT		Protective in murine neonatal model. Well tolerated in humans, but preparing individual conjugates is costly.	P I	[170, 296]
Non-TT conjugate CPS		Alternative carrier proteins include: C protein, C5a peptidase, diphtheria toxin, hepatitis B core antigen.	P	[297-300]
<i>Protein-based</i>				
C protein (α and β)	Surface-associated	Passive protection against systemic infection. β protective in neonatal mice. Limited expression.	P	[301-303]
BPS	Surface-associated	Mice protected from heterologous challenge. Murine neonatal immunization model not explored.	P	[304]
C5a peptidase	Cleaves C5a (complement)	Highly conserved. Immunization resulted in enhanced clearance following mucosal challenge.	P	[272, 300, 305]
Fbs protein	Surface-associated	Active and passive protection against systemic challenge. Expression restricted to a subset of strains.	P	[306]
Leucine-rich repeat protein	Surface-associated	Found in strains representing all nine serotypes. Protects against homologous systemic challenge.	P	[307]
Pho2-2 and Pho3-1	Surface-associated	Limited knowledge of proteins function. Protection against subcutaneous challenge in neonatal rat model.	P	[60]
Sip	Surface-associated	Found on all serotypes. Mice protected against heterologous systemic challenge.	P	[308, 309]
R28 (Alp3)	Surface-associated	Protection against systemic challenge following passive and active immunization.	P	[282, 310]
Rib protein	Surface-associated	Protection following mucosal challenge and systemic heterologous challenge.	P	[311-313]

Abbreviations: CPS, Polysaccharide capsule; TT, Tetanus toxoid; BPS, Group B protective surface protein; Sip, Surface immunogenic protein; Rib, Resistance to proteases, immunity, group B; P, Pre-clinical; I, Phase I clinical trial.

Table 5 *Streptococcus mutans* vaccine candidates under investigation

Antigen	Function	Comment	Phase	REFS
<i>Streptococcus mutans</i>				
Antigen I/II	Surface adhesin	Active and passive immunization of animals and humans resulted in decreased oral colonization.	P I	[314-321]
Glucosyltransferase	Synthesis of glucans	Reduced colonization following active and passive immunization. Immunogenic, well tolerated in humans.	P I	[322-331]
Glucan-binding proteins	Receptor for glucans	Active and passive immunization reduced dental caries in rats.	P	[332-335]
GTF and Antigen I/II	As above	Significant reduction in colonization following oral challenge of mice.	P	[336]

Abbreviations: GTF, Glucosyltransferase; P, Pre-clinical; I, Phase I clinical trial.

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