Surface analyses and immune reactivities of major cell wall-associated proteins of Group A Streptococcus

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Abstract
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Keywords
Group A Streptococcus, surface analysis, Streptococcus pyogenes, GAS cell-surface

Disciplines
Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

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Surface Analysis and Immune Reactivity of Major Cell-Wall Associated Proteins of Group A Streptococcus

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Running title: Proteomic analysis of GAS cell-wall extracts.
A proteomic analysis was undertaken to identify cell-wall associated proteins of *Streptococcus pyogenes*. Seventy-four distinct cell-wall associated proteins were identified, 66 of which were novel. Thirty-three proteins were immunoreactive with pooled *S. pyogenes*-reactive human antisera. Biotinylation of the GAS cell-surface identified 23 cell-wall associated proteins that are surface exposed.

The Gram-positive human pathogen *Streptococcus pyogenes* (group A streptococcus; GAS) is the etiologic agent of numerous suppurative diseases, ranging from mild skin infections such as pharyngitis, scarlet fever, impetigo and cellulitis, to severe invasive diseases such as septicemia, streptococcal toxic shock syndrome and necrotizing fasciitis (8). *S. pyogenes* express a range of multifunctional surface proteins which facilitate adherence to and invasion of host cells, resistance to phagocytosis and degradation of host proteins (8). Although many surface-exposed and secreted proteins have been identified and characterized in GAS, there has been no systematic analysis to identify the major cell-wall associated proteins.

To identify the major cell-wall associated proteins of GAS, a 2D sodium dodecyl sulfate polyacrylamide gel electrophoresis (PAGE) proteomic analysis (6) of mutanolysin cell-wall extracts (19) was undertaken for GAS strain NS931 (necrotizing fasciitis isolate; serotype M69) (11), NS13 (bacteremia isolate; serotype M53) (11) and S43 (bronchopneumonia isolate; serotype M6) (21). Proteins of interest were excised from 2D Coomassie stained PAGE gels, digested with trypsin and analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) as described by Cordwell *et al.* (6). Peptide masses were matched by searching the Swiss-Prot and TrEMBL databases at PeptIdent (http://us.expasy.org/tools/peptident.html). Representative 2D-PAGE gels from two independent
mutanolysin cell-wall extractions are shown in Fig. 1 (panel A-C). The protein profiles are similar across all strains, with molecular weights ranging from 14.4 to 77.5 kDa and a pI range of 4.4 to 7.9. A total of 155 protein spots (51 for NS931, Fig. 1A; 33 for NS13, Fig. 1B; and 71 for S43, Fig. 1C), corresponding to 74 unique proteins, were positively identified by MALDI-TOF MS (Table 1). Several proteins were detected as multiple isoforms in one or more strains. These results suggest that some proteins exist in different charge states or may have undergone post-translational modifications. It remains to be determined whether or not these modifications are physiological or an artefact caused by urea carbamylation, deamidation or IPG strip overload. With the exception of glyceraldehyde-3-phosphate dehydrogenase (30), enolase (31), manganese-dependent superoxide dismutase (26), collagen-like protein B (38), SpeM (36), FcrA (16), M protein (13) and cysteine protease SpeB precursor (18), all of the proteins identified in this study have not, to our knowledge, previously been reported as cell-wall associated in *S. pyogenes*. Thirty-six of the 74 cell-wall associated proteins have been previously identified in the cellular or extracellular GAS proteomes (2, 4, 22, 27, 37) (Table 2).

Western blot analysis (3) was used to ascertain the immunoreactivity of proteins in cell-wall extracts harvested from *S. pyogenes* NS931, NS13 and S43. Immunoreactive proteins were detected by probing the membranes with pooled human sera obtained from the Menzies School of Health Research, Darwin, Northern Territory, Australia. Serum samples were pooled from ten school-aged children residing in a remote community in northern Australia, where GAS infections are endemic and up to 70% of children have GAS-associated impetigo (9). To act as a negative control, two-dimensional mutanolysin extract blots for each strain were probed with goat anti-human IgG HRP only prior to development with DAB. The extracts were separated in two-dimensions over a linear pH range of 4 to 7, transferred to PVDF membrane and probed with the pooled human sera (Fig. 1 panel D-F). Reactive protein spots were identified according
to their relative positions (pl and molecular weight) (Fig. 1 panel A-C). Of the 74 cell-wall associated proteins identified in this study, only 33 (45%) were identified as immunoreactive (Table 1) and therefore are presumably expressed during the course of human infection. Multiple immunoreactive proteins situated near the pH 7 end of the NS13 2D immunoblot (Fig. 1E) could not be identified because the protein concentrations in the corresponding region of the Coomassie stained gel (Fig. 1B) were below the detection threshold. Interestingly, several proteins were identified as immunoreactive in only one or two of the GAS strains examined (Table 1). Given the use of pooled sera and the highly conserved nature of these proteins, an immunoreactive protein should presumably be detected in all three strains. However, strain-specific differences affecting protein expression levels, antigenic variation or the sensitivity of spot detection may account for this discrepancy.

In an attempt to determine which cell-wall associated proteins are surface exposed, the cell-surface of GAS strain NS931, NS13 and S43 was biotinylated (1) prior to mutanolysin extraction and subsequent 2D western blot analysis. Biotin-labeled cell-surface proteins were detected using a streptavidin-horseradish peroxidase (SA-HRP) conjugate (Sigma). Two-dimensional blots containing non-biotinylated mutanolysin extract were used as negative controls for all strains (result not shown). Biotinylated spots were identified by MALDI-TOF MS from the corresponding Coomassie stained biotinylated cell-wall extract 2D gel. Only 23 (31%) of the identified cell-wall extract proteins were biotinylated and therefore surface exposed in at least one strain (Fig. 1 panel G-I) (Table 1).

Gram-positive proteins destined for transport across the cytoplasmic membrane frequently contain a hydrophobic N-terminal signal sequence and a conserved C-terminal membrane anchor motif of Leu-Pro-X-Thr-Gly (LPXTG) (5). Following protein translocation across the
cytoplasmic membrane, the signal peptide is proteolytically removed by signal peptidase. Proteolytic cleavage of the LPXTG motif by sortase facilitates the covalent cross-linking of the protein to the cell-wall (28). In this study, many GAS cell wall-associated proteins lack apparent secretion signal sequences and the LPXTG membrane anchor sequence (Table 1). Similarly, a number of secreted GAS proteins lack secretion signals (2, 22, 27). The absence of a signal peptide and LPXTG motif suggests that these proteins are either passively released during autolysis, or that an alternative secretory pathway may exist for many secreted GAS proteins. Although the mechanism by which these proteins are transported to the cell-surface is unknown, internal signal sequences, post-translational acylation or an association with a secreted protein may be involved (31). Recently, asymmetric protein secretion of GAS SpeB was shown to occur at distinct cytoplasmic membrane microdomains termed ExPortals (35). The role that this structure plays in the secretion of other GAS proteins is currently unknown. The authors also note the possibility that cytosolic proteins passively released by autolysis may have adsorbed to the GAS cell-surface.

SpeB is an extracellular and surface-associated cysteine protease virulence factor produced by most GAS strains (18) that can efficiently degrade the majority of proteins in the secreted GAS proteome (2). Twenty-six of the cell wall-associated proteins described in this communication have previously been identified in the extracellular proteomes of GAS SpeB mutants (2, 4, 22, 27, 37). The identification of these proteins in the cell-walls of SpeB-positive strains suggests that whilst these proteins are associated with the GAS cell-wall during late stationary phase, they are efficiently protected from SpeB-mediated degradation. Future studies may be performed with biochemically or genetically inactivated SpeB to test this hypothesis.
A significant number of traditional cytoplasmic proteins were also identified as cell-wall associated immunogens in this work. Several cytosolic proteins, such as the glycolysis pathway enzymes, have been reported as cell-wall associated in GAS or other prokaryotic species. The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), also referred to as the plasmin receptor protein (Pr), is a well-characterized GAS cell-surface protein with plasminogen-binding (40) and ADP-ribosylating activity (29). This multifunctional protein binds fibronectin, lysozyme, myosin and actin (30) and elicits signal transduction events in human pharyngeal cells (32). Streptococcal enolase (SEN) is a glycolytic and major plasminogen-binding protein located on the cell-surface of most GAS strains (12). SEN has been implicated in GAS adherence to and invasion of human pharyngeal cells (33) and is a highly immunogenic autoantigen with a possible role in the initiation of post-streptococcal sequelae (15). Phosphoglycerate kinase is a glycolytic and major outer surface protein of Streptococcus oralis (39) and Streptococcus agalactiae (group B streptococcus) (17).

Consistent with our findings, the normally cytoplasmic chaperonins DnaK and GroEL have been identified as immunoreactive antigens of S. pyogenes (23, 24). Although these chaperones have not previously been characterized as GAS cell-wall constituents, homologs of DnaK and GroEL are located in the cell-walls of S. agalactiae (17). Elongation factor Tu (Ef-Tu) is localized in the cell-walls of S. oralis (39). Other factors involved in protein synthesis, such as ribosome recycling factor and protein translation elongation factors G, Ts and P are expressed on the cell-surface of S. oralis (39).

The three components of the arginine deiminase pathway, which consists of ornithine carbamoyltransferase, arginine deiminase and carbamate kinase, were identified as cell-wall associated in this study. The enzymes of this system catalyze the breakdown of arginine to
ornithine, CO₂ and two molecules of ammonia, with the concomitant production of ATP (7). Ornithine carbamoyltransferase is a bona fide cell-wall protein of *S. agalactiae* (17), *Streptococcus sanguis* (14) and *Streptococcus suis* (41). GAS arginine deiminase, also known as the streptococcal acid glycoprotein (SAGP), is thought to play a role in virulence factor expression and GAS internalization into epithelial cells (10, 25).

Although an association between biotinylated proteins and immunoreactivity was established, some biotinylated cell-surface proteins were not immunoreactive. To account for this, we suggest that these proteins are either poor immunogens or are expressed at low levels during GAS infection. Conversely, some immunoreactive proteins were not found to be biotinylated, which may indicate the absence of surface-exposed lysine residues for biotinylation. Alternatively, proteins with only a small number of surface-exposed lysine residues may have been below the limit of detection used in this study. For example, the M protein of NS13 exhibited immunoreactivity against the human antiserum (Fig. 1E), but was not found to be biotinylated. Cleavage of surface exposed M protein by SpeB (20, 34) may explain the apparent lack of M protein biotinylation in this study. Lack of M protein immune reactivity in GAS strains NS931 and S43 may suggest that the individuals from which the endemic serum was derived had not been exposed to these GAS M types. M protein fragments were detected in each of these strains (Fig. 1A and C; spots 73 and 74).

Numerous surface-exposed cell-wall proteins have been identified as vaccine candidates in GAS (8). However, a safe and efficacious commercial GAS vaccine has yet to be developed. In this study, we have undertaken a systematic proteomic analysis to extend the range of proteins known to associate with the GAS cell-wall. In summary, a total of 74 distinct proteins were identified in the cell-wall extracts of three GAS strains. Thirty-three of these proteins were
immunoreactive against pooled human sera and twenty-three were identified as surface-exposed. Further characterization of these proteins is required to elucidate their precise role in GAS pathogenesis. Taken together, these data illustrate the usefulness of proteomics in analyzing the cell-surface topology of GAS.

Acknowledgements

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References


**Figure Legend**

FIG. 1. Two-dimensional gel electrophoresis profiles of GAS mutanolysin cell-wall extracts. The extracts were harvested from GAS strain NS931 (A, D and G), NS13 (B, E and H) and S43 (C, F and I) after growth to late stationary phase (37°C for 16 h) in THBY medium without shaking. The protein extracts (170 μg) were isoelectric focused over a linear pH gradient of 4 to 7 and resolved with a 12.5% SDS-PAGE gel. (A-C) The gels were stained with colloidal Coomassie and destained in 1% (v/v) acetic acid. (D-F) The proteins were transferred to PVDF membrane and probed with a 1:100 dilution of pooled endemic human sera. Bound antibodies were detected using a goat anti-human IgG horseradish peroxidase (HRP) conjugate (Bio-Rad). Negative control blots probed only with goat anti-human IgG HRP conjugate contained no immunoreactive proteins (result not shown). (G-I) The cell-surface of each strain was labeled with biotin before the mutanolysin extract was harvested. The proteins were transferred to PVDF membrane and probed with a streptavidin-horseradish peroxidase (SA-HRP) conjugate prior to development with diaminobenzidine (DAB). Negative control blots of non-biotinylated extracts contained no labeled proteins (result not shown). Protein spots identified by peptide mass fingerprinting are denoted by numbered arrows, which correspond to the proteins in Table 1. Molecular mass markers are given in kilo-Daltons (kDa).
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* Identified proteins are indicated by a plus sign (+).
* Swiss-Prot/TrEMBL accession number.
* Theoretical values obtained from Swiss-Prot/TrEMBL database.
* Number of tryptic peptides detected by MALDI-TOF MS that could be matched to the protein.
* Percentage of the protein sequence covered by the matched peptides.
* Contains a putative secretion signal sequence identified by SignalP3.0 signal peptide prediction server (http://www.cbs.dtu.dk/services/SignalP/).
* Contains a C-terminal LPXTG membrane anchor motif identified by Pfam motif search (http://pfam.wustl.edu/hmmsearch.shtml).
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<td>Peptide deformylase</td>
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\[\text{a} \text{ Data obtained from Thongboonkerd et al. (37).} \]
\[\text{b} \text{ Data obtained from Aziz et al. (2); Chaussee et al. (4); Lei et al. (22); Nakamura et al. (27); and Thongboonkerd et al. (37).} \]
\[\text{c} \text{ This study.} \]