INFECTION AND IMMUNITY, May 2005, p. 3137–3146 Vol. 73, No. 5
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.5.3137–3146.2005

Surface Analyses and Immune Reactivities of Major Cell Wall-Associated Proteins of Group A Streptococcus

Jason N. Cole,[1] Ruben D. Ramirez,[1] Bart J. Currie,[2] Stuart J. Cordwell,[3] Steven P. Djordjevic,[4] and Mark J. Walker[1]*

School of Biological Sciences, University of Wollongong, Wollongong, NSW,[1] Menzies School of Health Research, Charles Darwin University, Darwin, NT,[2] Australian Proteome Analysis Facility, Macquarie University, Sydney, NSW,[3] and NSW Agriculture, Elizabeth Macarthur Agricultural Institute, Camden, NSW,[4] Australia

Received 9 December 2004/Accepted 27 December 2004

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* expresses 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 in GAS have been identified and characterized, 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 two-dimensional (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 blue-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. 1A to C. The protein profiles are similar across all strains, with molecular masses 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 posttranslational modifications. It remains to be determined whether or not these modifications are physiological or an artifact caused by urea carbamylation, deamidation, or immobilized pH gradient strip overload. With the exception of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (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-five 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 immunoreactivities 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 10 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, 2D mutanolysin extract blots for each strain were probed with goat anti-human immunoglobulin G (IgG) horseradish peroxidase (HRP) only prior to development with diaminobenzidine. The extracts were separated in two dimensions over a linear pH range of 4 to 7, transferred to a polyvinylidene difluoride (PVDF) membrane, and probed with the pooled human sera (Fig. 1D to F). Reactive protein spots were identified according to their relative positions (pI and molecular weight) (Fig. 1A to 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 blue-stained gel (Fig. 1B) were below the detection threshold. Interestingly, several proteins were identified as immunoreactive in only one or two of

* Corresponding author. Mailing address: School of Biological Sciences, University of Wollongong, Wollongong, NSW 2522, Australia. Phone: 0061-242-213439. Fax: 0061-242-214135. E-mail: mwalker@uow.edu.au.

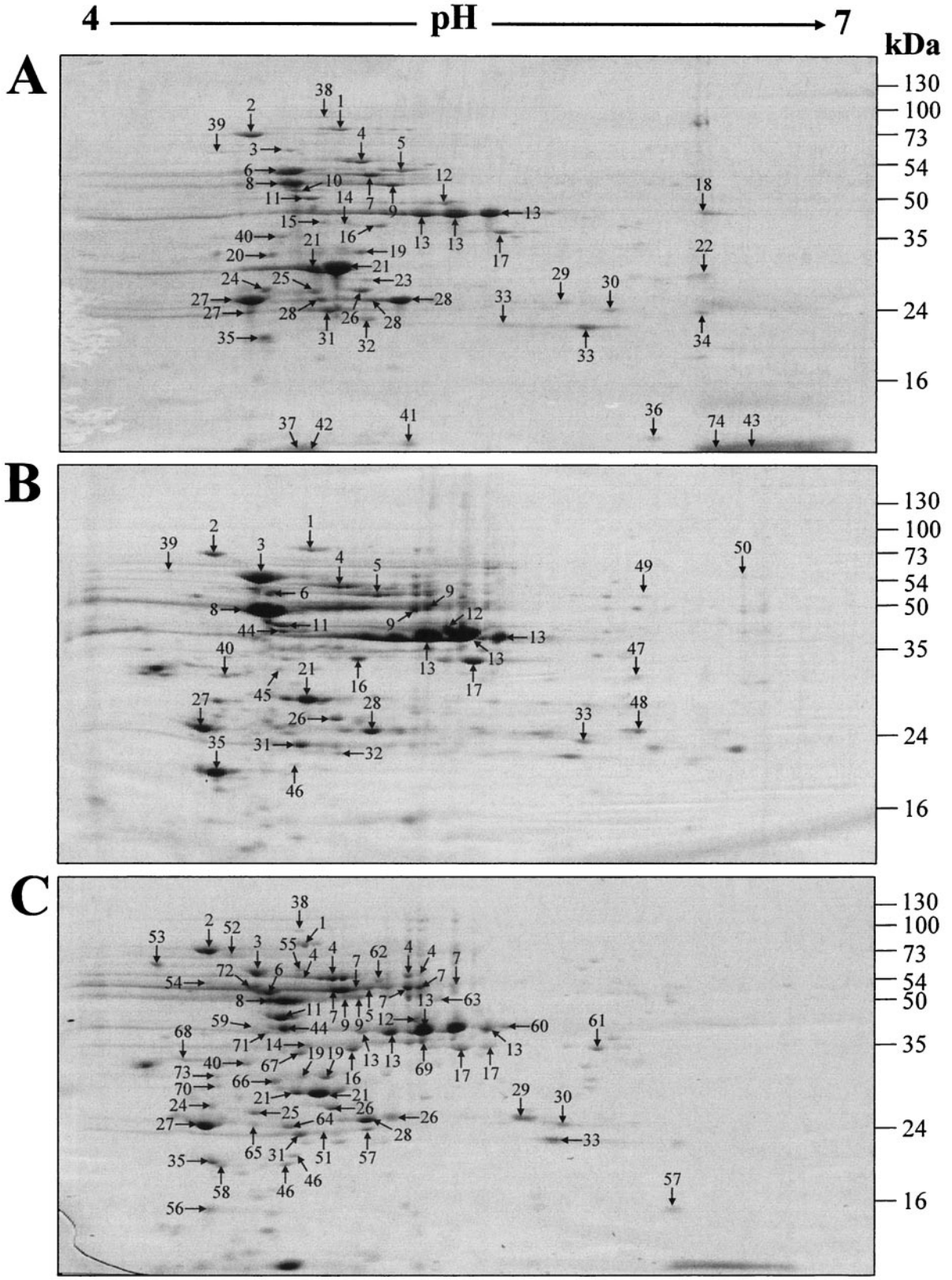
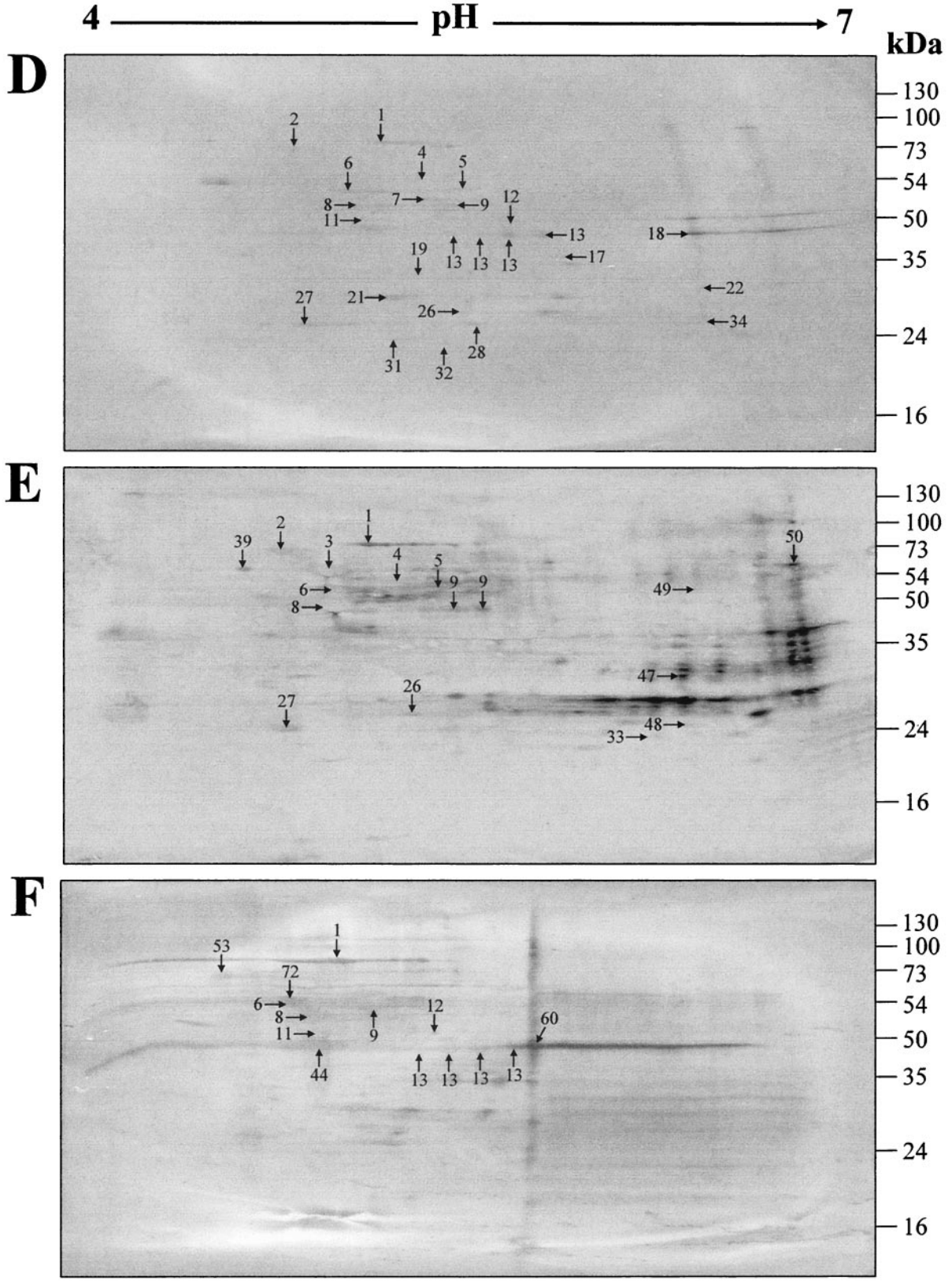


FIG. 1. Two-dimensional gel electrophoresis profiles of GAS mutanolysin cell wall extracts. The extracts were harvested from GAS strains NS931 (A, D, and G), NS13 (B, E, and H), and S43 (C, F, and I) after growth to late stationary phase (37°C for 16 h) in Todd-Hewitt medium (Difco) supplemented with 1% (wt/vol) yeast extract 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 blue and destained in 1%



anti-human IgG–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 a PVDF membrane and probed with an SA-HRP conjugate prior to development with diaminobenzidine. Negative-control blots of nonbiotinylated extracts contained no labeled proteins (result not shown). Protein spots identified by peptide mass (vol/vol) acetic acid. (D-F) The proteins were transferred to a PVDF membrane and probed with a 1:100 dilution of pooled human sera from an area of endemicity. Bound antibodies were detected using a goat fingerprinting are denoted by numbered arrows, which correspond to the proteins in Table 1. Molecular mass markers are given in kilodaltons.

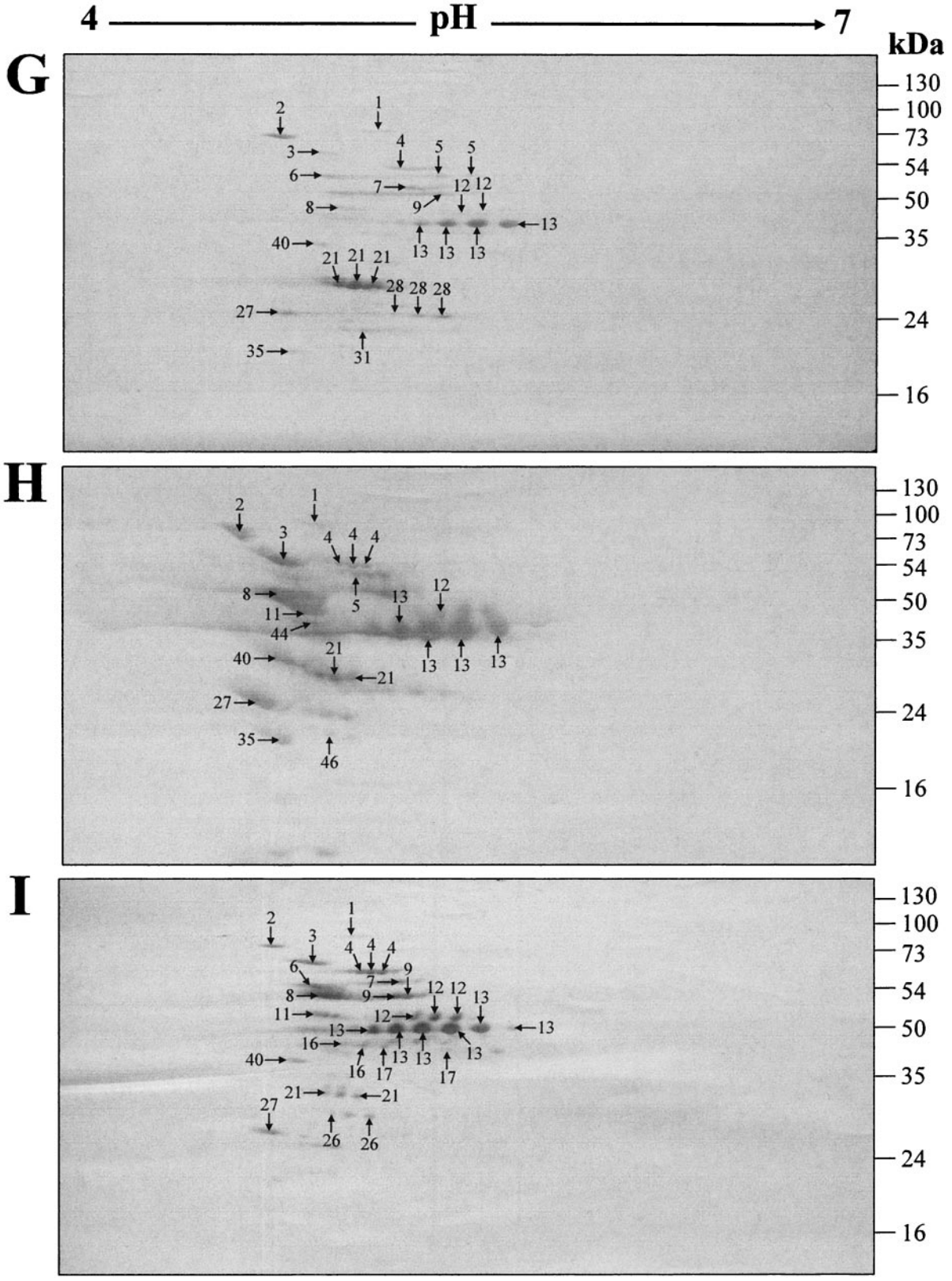


FIG. 1—*Continued*.

TABLE 1. Major cell wall-associated proteins identified in the mutanolysin extracts of GAS strains NS931, NS13, and S43 by MALDI-TOF peptide mass fingerprinting analysis[a]

Function or pathway	Spot	Protein	Accession no.[b]	Molecular mass (kDa)[c]	pI[c]	Peptide match[d]	Coverage (%)[e]	Mutanolysin extract			Immunoreactive			Biotinylated		
								NS931	NS13	S43	NS931	NS13	S43	NS931	NS13	S43
Glycolysis	4	Putative pyruvate kinase	Q8K7A3	54.5	4.96	30	59.8	+	+	+	+	+		+	+	+
	5	Putative NADP-dependent glyceraldehyde-3-phosphate dehydrogenase	Q8K707	49.5	4.96	19	56.1	+	+	+	+	+		+	+	
	11	Phosphoglycerate kinase	Q8K5W7	42.0	4.86	23	70.0	+	+	+	+		+		+	+
	17	6-Phosphofructokinase	Q8P0S6	35.8	5.34	22	50.7	+	+	+	+					+
	21	Fructose-bisphosphate aldolase	P82486	31.1	4.87	13	50.3	+	+	+	+			+	+	+
	27	Triosephosphate isomerase	P82478	26.5	4.57	11	63.3	+	+	+	+	+		+	+	+
	28	2,3-Bisphosphoglycerate-dependent phosphoglycerate mutase	Q8P0C1	26.0	5.10	19	77.1	+	+	+	+			+		
	69	L-Lactate dehydrogenase	Q99ZN5	35.1	5.14	9	30.7			+						
Carbohydrate metabolism	1	Putative transketolase	Q8K670	77.5	4.98	21	42.1	+	+	+	+	+	+	+	+	+
	32	Putative dTDP-4-keto-6-deoxyglucose-3,5-epimerase	Q9A046	22.4	5.07	10	42.1	+	+		+					
	41	Putative lactoylglutathione lyase	Q9A121	14.4	5.09	4	30.4	+								
	52	Putative phosphoglucomutase	Q99ZH8	63.3	4.78	10	25.7			+						
	54	Putative phospho-sugar mutase	Q878L0	48.4	4.57	8	17.7			+						
	60	Putative dTDP-glucose-4,6-dehydratase	Q8P199	38.8	5.41	4	12.4			+			+			
	61	Glycerol-3-phosphate dehydrogenase [NAD(P)$^+$]	P58143	36.7	5.65	12	45.9			+						
	67	Tagatose 1,6-diphosphate aldolase 2	Q8K5U9	36.5	4.93	15	50.5			+						
Arginine degradation	12	Ornithine carbamoyltransferase, catabolic	Q8P052	37.8	5.19	19	62.8	+	+	+	+		+	+	+	+
	40[f]	Putative carbamate kinase	Q8K6Q9	33.2	4.71	12	44.3	+	+	+				+	+	+
Amino acid biosynthesis	14	Putative branched-chain-amino-acid aminotransferase	Q8K7U5	37.2	4.90	6	21.4	+		+						
	72	Putative glutamine synthetase	Q8NZG4	50.5	5.21	8	19.6			+			+			
Fatty acid and phospholipid biosynthesis	47	Putative malonyl coenzyme A-acyl carrier protein transacylase	Q879J3	34.5	6.02	5	19.1		+			+				
Pantothenate (vitamin B_5) biosynthesis	19	Putative 2-dehydropantoate 2-reductase (ketopantoate reductase)	Q8P1F1	33.8	4.93	6	24.8	+		+	+					
Pyridoxine (vitamin B_6) biosynthesis	36	Putative pyridoxamine-phosphate oxidase	Q8K7X7	14.9	5.87	10	68.4	+								

Continued on following page

TABLE 1—*Continued*

Function or pathway	Spot	Protein	Accession no.[b]	Molecular mass (kDa)[c]	pI[c]	Peptide match[d]	Coverage (%)[e]	Mutanolysin extract			Immunoreactive			Biotinylated		
								NS931	NS13	S43	NS931	NS13	S43	NS931	NS13	S43
Nucleoside metabolism	26	Putative purine nucleoside phosphorylase	Q878J4	28.5	4.98	12	44.4	+	+	+	+	+				+
	34	Uracil phosphoribosyltransferase (UMP pyrophosphorylase)	Q9A194	22.8	6.30	13	57.4	+			+					
	55	GMP synthase (glutamine hydrolyzing)	Q8K7E6	57.5	4.91	7	14.8			+						
	63	Adenylosuccinate synthetase	Q8P2U1	47.4	5.29	10	30.7			+						
Metabolic enzyme	16	Putative phosphotransacetylase	Q878S0	35.9	5.08	13	43.8	+	+	+						+
	66	Putative acetoin reductase	Q8P1U1	26.8	4.79	9	44.5			+						
	68	Probable manganese-dependent inorganic pyrophosphatase	Q9A1A2	33.6	4.47	7	23.8			+						
Virulence factor	8	Enolase (2-phosphoglycerate dehydratase)	P82479	47.2	4.74	28	65.4	+	+	+	+	+	+	+	+	+
	9	Arginine deiminase[i]	Q8K5F0	46.1	4.99	31	70.5	+	+	+	+	+	+	+		+
	13	GAPDH	P50467	35.8	5.34	18	61.8	+	+	+	+		+	+	+	+
	18[f]	Cysteine protease SpeB precursor	Q93LQ2	37.3	7.21	7	22.4	+			+					
	23	Putative C3-degrading proteinase	Q99Y63	28.6	4.89	10	43.2	+								
	31	Superoxide dismutase (Mn)	Q8P0D4	22.5	4.87	13	81.5	+	+	+	+			+		
	43[f]	Pyrogenic exotoxin M (SpeM) (fragment)	Q7WYA3	24.1	7.87	6	25.0	+								
	48[f,g]	FcrA protein precursor	Q54859	45.4	6.47	6	15.4		+			+				
	50[f,g]	M protein	Q54840	61.7	6.24	4	6.5		+			+				
	73	M protein (fragment)	Q93LJ0	27.2	5.22	6	25.0			+						
	74	M protein (fragment)	O86065	21.0	5.41	5	21.7	+								
Protein biosynthesis	7	Elongation factor Tu	Q8K872	43.8	4.91	24	58.3	+		+	+			+		+
	29	Peptide deformylase	Q8NZB7	22.9	5.51	11	72.5	+		+						
	33	Ribosome recycling factor	Q8P274	20.5	5.68	11	64.3	+	+	+		+				
	38	Elongation factor G	P82477	76.4	4.83	13	22.9	+		+						
	44	Elongation factor Ts	Q8K5L1	37.3	4.86	13	45.7		+	+			+		+	
	57	Probable sigma^{54} modulation protein (fragments)	P82482	18.4	4.45	4	30.6			+						
	62	Seryl-tRNA synthetase	Q8K635	48.1	5.17	13	38.8			+						
	64	Elongation factor P	P82459	20.5	4.85	4	29.2			+						
Protein transport	22[f]	Putative ABC transporter, substrate-binding protein	Q8P2K8	30.6	7.69	7	28.6	+			+					
	39	Trigger factor	Q879L7	47.1	4.39	11	27.6	+	+			+				
	53	Putative ABC transporter, ATP-binding protein	Q99XH2	60.7	4.77	9	21.2			+			+			
	65	Putative copper homeostasis protein (hypothetical protein)	Q8K8H0	22.6	4.79	4	24.4			+						
Proteolysis and peptidolysis	6	Putative dipeptidase	Q8K7L6	51.4	4.81	12	33.0	+	+	+	+	+	+	+		+
	10	Putative X-His dipeptidase	Q99YT8	49.1	4.74	21	46.5	+								
	30	Pyrrolidone-carboxylate peptidase	Q8K8C4	23.2	5.64	7	38.6	+		+						

Continued on facing page

TABLE 1—*Continued*

Function or pathway	Spot	Protein	Accession no.[b]	Molecular mass (kDa)[c]	pI[c]	Peptide match[d]	Coverage (%)[e]	Mutanolysin extract			Immunoreactive			Biotinylated		
								NS931	NS13	S43	NS931	NS13	S43	NS931	NS13	S43
	45	Putative methionine aminopeptidase	Q8K718	31.6	4.84	5	31.5		+							
Chaperone	2	Chaperone protein DnaK	P95831	64.8	4.62	24	47.6	+	+	+	+	+		+	+	+
	3	60-kDa chaperonin GroEL	Q8K5M5	56.9	4.75	35	69.6	+	+	+		+		+	+	+
Stress protein	35	Putative alkyl hydroperoxidase	Q99XR7	20.5	4.65	9	58.6	+	+	+				+	+	
	49	Putative glutathione reductase	Q8P1H3	48.9	5.66	5	11.1		+			+				
Transcription factor	58	Transcription elongation factor GreA	Q9A1C4	17.7	4.67	7	61.3			+						
Adhesin	59[f,g]	Collagen-like protein B (fragment)	Q9AGC4	46.7	4.60	4	10.8			+						
Housekeeping	15	Putative alcohol dehydrogenase I	Q9A1X7	35.4	4.89	6	20.7	+								
	24	Putative phosphoprotein phosphatase	Q99YM9	27.0	4.60	19	80.9	+		+						
	25	Adenylate kinase	Q8K8X1	23.7	4.83	13	79.7	+		+						
Unknown function	20	Hypothetical UPF0082 protein SPy0316/ SpyM3_0231/SPs16 28/ spyM18_0311	Q9A1E6	25.9	4.49	6	29.0	+								
	37	Hypothetical phage protein spyM18_0356	Q8P2H6	25.1	5.37	4	13.6	+								
	42	Hypothetical phage protein spyM18_1764	Q8NZS3	22.7	4.63	3	20.2	+								
	46	Hypothetical protein SPy1262	Q99ZE5	19.9	4.93	9	42.5		+	+					+	
	51	Hypothetical protein SpyM3_0548	Q8K7Z2	15.4	6.05	4	21.2			+						
	56	Conserved hypothetical protein SPs1095	Q878P1	17.5	4.65	6	54.6			+						
	70	Conserved protein SpyM18_1567	Q8P050	26.6	4.67	5	28.6			+						
	71	Hypothetical protein (phage associated) SPs0647	Q879B2	26.8	5.10	5	21.0			+						

[a] Identified proteins are indicated by a plus sign.
[b] Swiss-Prot or TrEMBL accession number.
[c] Theoretical values obtained from Swiss-Prot or TrEMBL database.
[d] Number of tryptic peptides detected by MALDI-TOF MS that could be matched to the protein.
[e] Percentage of the protein sequence covered by the matched peptides.
[f] Contains a putative secretion signal sequence identified by SignalP3.0 signal peptide prediction server (http://www.cbs.dtu.dk/services/SignalP/).
[g] Contains a C-terminal LPXTG membrane anchor motif identified by Pfam motif search (http://pfam.wustl.edu/hmmsearch.shtml).

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 surfaces of GAS strains NS931, NS13, and S43 were biotinylated (1) prior to mutanolysin extraction and subsequent 2D Western blot analysis. Biotin-labeled cell surface proteins were detected using a streptavidin-HRP (SA-HRP) conjugate (Sigma). Two-dimensional blots containing nonbiotinylated 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 blue-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. 1G to 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

TABLE 2. Cell-wall associated proteins previously characterized as cellular or secreted in GAS

Functional category	Protein	Positivity for indicated characteristic		
		Cellular[a]	Secreted[b]	Cell wall[c]
Chaperonin	DnaK	+	+	+
	GroEL	+	+	+
Plasminogen binding	Enolase	+	+	+
	Glyceraldehyde-3-phosphate dehydrogenase	+	+	+
Glycolytic pathway	6-Phosphofructokinase	+	+	+
	Phosphoglycerate kinase	+	+	+
	Fructose-bisphosphate aldolase	+	+	+
	Triosephosphate isomerase	+	+	+
	Phosphoglycerate mutase	+	+	+
	Pyruvate kinase	+	+	+
Virulence factor	M protein	+	+	+
	SpeB		+	+
Protein synthesis	Ribosome recycling factor	+	+	+
	Elongation factor Tu	+	+	+
	Elongation factor Ts	+	+	+
	Elongation factor G	+	+	+
	Elongation factor P	+		+
	Peptide deformylase	+		+
Urea cycle pathway	Arginine deaminase		+	+
	Ornithine carbamoyltransferase		+	+
	Carbamate kinase		+	+
Cell wall synthesis	dTDP-4-keto-6-deoxyglucose-3,5-epimerase	+		+
Stress protein	Alkyl hydroperoxidase	+		+
	Superoxide dismutase (Mn)	+		+
Nucleotide synthesis	GMP synthase	+		+
Housekeeping	L-Lactate dehydrogenase	+	+	+
	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase	+	+	+
	2-Dehydropantoate 2-reductase	+	+	+
	Transketolase	+	+	+
	Manganese-dependent inorganic pyrophosphatase	+	+	+
	Dipeptidase		+	+
	Adenylate kinase	+		+
	ABC transporter (ATP-binding protein)	+		+
	Branched-chain-amino-acid aminotransferase	+		+
	Phosphotransacetylase	+		+

[a] Data obtained from the work of Thongboonkerd et al. (37).
[b] Data obtained from the work of Aziz et al. (2), Chaussee et al. (4), Lei et al. (22), Nakamura et al. (27), and Thongboonkerd et al. (37).
[c] This study.

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, posttranslational 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. We 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-five 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). The identification of these proteins in the cell walls of SpeB-positive strains suggests that while these proteins are associated with the GAS cell wall during late stationary phase, they are efficiently protected from SpeB-mediated degrada-

tion. 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 GAPDH, also referred to as the plasmin receptor protein (Plr), is a well-characterized GAS cell surface protein with plasminogen binding (40) and ADP-ribosylating (29) activities. This multifunctional protein binds fibronectin, lysozyme, myosin, and actin (30) and elicits signal transduction events in human pharyngeal cells (32). Streptococcal enolase is a glycolytic and major plasminogen-binding protein located on the cell surfaces of most GAS strains (12). Streptococcal enolase 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 poststreptococcal 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 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, CO_2, 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, 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 either are 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, spot 50) 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 an area of endemicity from whom the 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 23 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.

We thank Tove' Bolken (SIGA Research Laboratories, Oregon) for providing *S. pyogenes* strain S43 and Jody Wilton for assisting with the 2D gel electrophoresis.

J. N. Cole is the recipient of an Australian postgraduate award. This work was supported by the National Health and Medical Research Council (NHMRC) of Australia.

REFERENCES

1. **Altin, J. G., and E. B. Pagler.** 1995. A one-step procedure for biotinylation and chemical cross-linking of lymphocyte surface and intracellular membrane-associated molecules. Anal. Biochem. **224:**382–389.
2. **Aziz, R. K., M. J. Pabst, A. Jeng, R. Kansal, D. E. Low, V. Nizet, and M. Kotb.** 2004. Invasive M1T1 group A streptococcus undergoes a phase-shift *in vivo* to prevent proteolytic degradation of multiple virulence factors by SpeB. Mol. Microbiol. **51:**123–134.
3. **Burnette, W. N.** 1981. Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112:**195–203.
4. **Chaussee, M. S., R. O. Watson, J. C. Smoot, and J. M. Musser.** 2001. Identification of Rgg-regulated exoproteins of *Streptococcus pyogenes.* Infect. Immun. **69:**822–831.
5. **Chhatwal, G. S.** 2002. Anchorless adhesins and invasins of Gram-positive bacteria: a new class of virulence factors. Trends Microbiol. **10:**205–208.
6. **Cordwell, S. J., D. J. Basseal, B. Bjellqvist, D. C. Shaw, and I. Humphery-Smith.** 1997. Characterisation of basic proteins from *Spiroplasma melliferum* using novel immobilised pH gradients. Electrophoresis **18:**1393–1398.
7. **Cunin, R., N. Glansdorff, A. Pierard, and V. Stalon.** 1986. Biosynthesis and metabolism of arginine in bacteria. Microbiol. Rev. **50:**314–352.
8. **Cunningham, M. W.** 2000. Pathogenesis of group A streptococcal infections. Clin. Microbiol. Rev. **13:**470–511.
9. **Currie, B. J., and J. R. Carapetis.** 2000. Skin infections and infestations in Aboriginal communities in northern Australia. Australas. J. Dermatol. **41:** 139–143.
10. **Degnan, B. A., M. C. Fontaine, A. H. Doebereiner, J. J. Lee, P. Mastroeni, G. Dougan, J. A. Goodacre, and M. A. Kehoe.** 2000. Characterization of an isogenic mutant of *Streptococcus pyogenes* Manfredo lacking the ability to make streptococcal acid glycoprotein. Infect. Immun. **68:**2441–2448.
11. **Delvecchio, A., B. J. Currie, J. D. McArthur, M. J. Walker, and K. S. Sriprakash.** 2002. *Streptococcus pyogenes prtFII*, but not *sfbI*, *sfbII* or *fbp54*, is represented more frequently among invasive-disease isolates of tropical Australia. Epidemiol. Infect. **128:**391–396.
12. **Derbise, A., Y. P. Song, S. Parikh, V. A. Fischetti, and V. Pancholi.** 2004. Role of the C-terminal lysine residues of streptococcal surface enolase in Glu- and Lys-plasminogen-binding activities of group A streptococci. Infect. Immun. **72:**94–105.
13. **Fischetti, V. A.** 1989. Streptococcal M protein: molecular design and biological behavior. Clin. Microbiol. Rev. **2:**285–314.
14. **Floderus, E., L. E. Linder, and M. L. Sund.** 1990. Arginine catabolism by strains of oral streptococci. APMIS **98:**1045–1052.
15. **Fontan, P. A., V. Pancholi, M. M. Nociari, and V. A. Fischetti.** 2000. Antibodies to streptococcal surface enolase react with human alpha-enolase: implications in poststreptococcal sequelae. J. Infect. Dis. **182:**1712–1721.
16. **Hollingshead, S. K., J. Arnold, T. L. Readdy, and D. E. Bessen.** 1994. Molecular evolution of a multigene family in group A streptococci. Mol. Biol. Evol. **11:**208–219.
17. **Hughes, M. J., J. C. Moore, J. D. Lane, R. Wilson, P. K. Pribul, Z. N. Younes, R. J. Dobson, P. Everest, A. J. Reason, J. M. Redfern, F. M. Greer, T. Paxton,**

M. Panico, H. R. Morris, R. G. Feldman, and J. D. Santangelo. 2002. Identification of major outer surface proteins of *Streptococcus agalactiae.* Infect. Immun. **70:**1254–1259.
18. **Hytonen, J., S. Haataja, D. Gerlach, A. Podbielski, and J. Finne.** 2001. The SpeB virulence factor of *Streptococcus pyogenes*, a multifunctional secreted and cell surface molecule with strepadhesin, laminin-binding and cysteine protease activity. Mol. Microbiol. **39:**512–519.
19. **Ji, Y., N. Schnitzler, E. DeMaster, and P. Cleary.** 1998. Impact of M49, Mrp, Enn, and C5a peptidase proteins on colonization of the mouse oral mucosa by *Streptococcus pyogenes.* Infect. Immun. **66:**5399–5405.
20. **Kansal, R. G., A. McGeer, D. E. Low, A. Norrby-Teglund, and M. Kotb.** 2000. Inverse relation between disease severity and expression of the streptococcal cysteine protease, SpeB, among clonal M1T1 isolates recovered from invasive group A streptococcal infection cases. Infect. Immun. **68:**6362–6369.
21. **Lancefield, R. C., and E. W. Todd.** 1928. Antigenic differences between matt hemolytic streptococci and their glossy variants. J. Exp. Med. **48:**769–790.
22. **Lei, B., S. Mackie, S. Lukomski, and J. M. Musser.** 2000. Identification and immunogenicity of group A streptococcus culture supernatant proteins. Infect. Immun. **68:**6807–6818.
23. **Lemos, J. A., R. A. Burne, and A. C. Castro.** 2000. Molecular cloning, purification and immunological responses of recombinants GroEL and DnaK from *Streptococcus pyogenes.* FEMS Immunol. Med. Microbiol. **28:** 121–128.
24. **Lemos, J. A., M. Giambiagi-Demarval, and A. C. Castro.** 1998. Expression of heat-shock proteins in *Streptococcus pyogenes* and their immunoreactivity with sera from patients with streptococcal diseases. J. Med. Microbiol. **47:** 711–715.
25. **Marouni, M. J., E. Ziomek, and S. Sela.** 2003. Influence of group A streptococcal acid glycoprotein on expression of major virulence factors and internalization by epithelial cells. Microb. Pathog. **35:**63–72.
26. **McMillan, D. J., M. R. Davies, M. F. Good, and K. S. Sriprakash.** 2004. Immune response to superoxide dismutase in group A streptococcal infection. FEMS Immunol. Med. Microbiol. **40:**249–256.
27. **Nakamura, T., T. Hasegawa, K. Torii, Y. Hasegawa, K. Shimokata, and M. Ohta.** 2004. Two-dimensional gel electrophoresis analysis of the abundance of virulent exoproteins of group A streptococcus caused by environmental changes. Arch. Microbiol. **181:**74–81.
28. **Novick, R. P.** 2000. Sortase: the surface protein anchoring transpeptidase and the LPXTG motif. Trends Microbiol. **8:**148–151.
29. **Pancholi, V., and V. Fischetti.** 1993. Glyceraldehyde-3-phosphate dehydrogenase on the surface of group A streptococci is also an ADP-ribosylating enzyme. Proc. Natl. Acad. Sci. USA **90:**8154–8158.
30. **Pancholi, V., and V. Fischetti.** 1992. A major surface protein on group A streptococci is a glyceraldehyde-3-phosphate-dehydrogenase with multiple binding activity. J. Exp. Med. **176:**415–426.
31. **Pancholi, V., and V. A. Fischetti.** 1998. α-Enolase, a novel strong plasmin-(ogen) binding protein on the surface of pathogenic streptococci. J. Biol. Chem. **273:**14503–14515.
32. **Pancholi, V., and V. A. Fischetti.** 1997. Regulation of the phosphorylation of human pharyngeal cell proteins by group A streptococcal surface dehydrogenase: signal transduction between streptococci and pharyngeal cells. J. Exp. Med. **186:**1633–1643.
33. **Pancholi, V., P. Fontan, and H. Jin.** 2003. Plasminogen-mediated group A streptococcal adherence to and pericellular invasion of human pharyngeal cells. Microb. Pathog. **35:**293–303.
34. **Raeder, R., M. Woischnik, A. Podbielski, and M. D. Boyle.** 1998. A secreted streptococcal cysteine protease can cleave a surface-expressed M1 protein and alter the immunoglobulin binding properties. Res. Microbiol. **149:**539–548.
35. **Rosch, J., and M. Caparon.** 2004. A microdomain for protein secretion in Gram-positive bacteria. Science **304:**1513–1515.
36. **Smoot, L. M., J. K. McCormick, J. C. Smoot, N. P. Hoe, I. Strickland, R. L. Cole, K. D. Barbian, C. A. Earhart, D. H. Ohlendorf, L. G. Veasy, H. R. Hill, D. Y. Leung, P. M. Schlievert, and J. M. Musser.** 2002. Characterization of two novel pyrogenic toxin superantigens made by an acute rheumatic fever clone of *Streptococcus pyogenes* associated with multiple disease outbreaks. Infect. Immun. **70:**7095–7104.
37. **Thongboonkerd, V., J. Luengpailin, J. Cao, W. M. Pierce, J. Cai, J. B. Klein, and R. J. Doyle.** 2002. Fluoride exposure attenuates expression of *Streptococcus pyogenes* virulence factors. J. Biol. Chem. **277:**16599–16605.
38. **Whatmore, A. M.** 2001. *Streptococcus pyogenes sclB* encodes a putative hypervariable surface protein with a collagen-like repetitive structure. Microbiology **147:**419–429.
39. **Wilkins, J. C., D. Beighton, and K. A. Homer.** 2003. Effect of acidic pH on expression of surface-associated proteins of *Streptococcus oralis.* Appl. Environ. Microbiol. **69:**5290–5296.
40. **Winram, S. B., and R. Lottenberg.** 1996. The plasmin-binding protein Plr of group A streptococci is identified as glyceraldehyde-3-phosphate dehydrogenase. Microbiology **142:**2311–2320.
41. **Winterhoff, N., R. Goethe, P. Gruening, M. Rohde, H. Kalisz, H. E. Smith, and P. Valentin-Weigand.** 2002. Identification and characterization of two temperature-induced surface-associated proteins of *Streptococcus suis* with high homologies to members of the arginine deiminase system of *Streptococcus pyogenes.* J. Bacteriol. **184:**6768–6776.

Editor: V. J. DiRita