Multi High-Throughput Approach for Highly Selective Identification of Vaccine Candidates: the Group A Streptococcus Case*[S]

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We propose an experimental strategy for highly accurate selection of candidates for bacterial vaccines without using *in vitro* and/or *in vivo* protection assays. Starting from the observation that efficacious vaccines are constituted by conserved, surface-associated and/or secreted components, the strategy contemplates the parallel application of three high throughput technologies, *i.e.* mass spectrometry-based proteomics, protein array, and flow-cytometry analysis, to identify this category of proteins, and is based on the assumption that the antigens identified by all three technologies are the protective ones. When we tested this strategy for Group A S*treptococcus*, we selected a total of 40 proteins, of which only six identified by all three approaches. When the 40 proteins were tested in a mouse model, only six were found to be protective and five of these belonged to the group of antigens in common to the three technologies. Finally, a combination of three protective antigens conferred broad protection against a panel of four different Group A S*treptococcus* strains. This approach may find general application as an accelerated and highly accurate path to bacterial vaccine discovery. *Molecular & Cellular Proteomics 11: 10.1074/mcp.M111.015693, 1–12, 2012.*

Genome sequence data mining for bacterial secreted and surface proteins has successfully been exploited for the discovery of vaccine candidates against relevant bacterial pathogens (1, 2). A common drawback of these high throughput approaches is that a large number of candidates needs to go through *in vivo* and/or *in vitro* screening assays to identify the very few antigens conferring protection. Such assays, being laborious, expensive, and time-consuming, represent the real bottle neck of the entire vaccine discovery process. Therefore, prescreening strategies capable of reducing the number of antigens that have to undergo the biological testing would substantially shorten the time needed to identify the final vaccine formulation to enter development. Based on the observation that all bacterial vaccines inducing protective antibodies are exclusively constituted by highly expressed, surface-exposed antigens and/or secreted toxins (3), we recently demonstrated how proteomic-based methods, which selectively identify these categories of proteins, can significantly accelerate the identification of protective antigens in preclinical models (4–7). These proteomic approaches, which for Gram-positive bacteria are based on MS analysis of peptides generated after cell surface "shaving" with proteases, and for Gram-negative bacteria on MS characterization of outer membrane vesicles released in culture supernatants, significantly reduce the number of vaccine candidates to be analyzed.

To further single out the few protective antigens, here we propose a strategy that combines the MS proteomic approach with two additional technologies: high throughput analysis of immunogenic antigens by protein array and quantification of surface proteins by FACS analysis using specific polyclonal antibodies. The three technologies can be applied in parallel to a large number of samples (bacterial strains in the case of the proteome and FACS analyses and sera in the case of protein array). Therefore, when applied in a systematic manner, the combined technologies allow the identification of sufficiently conserved, well expressed, surface/secreted proteins, representing the antigens one should test first as vaccine candidates.

To test the effectiveness of our strategy, as a model system we used Group A *Streptococcus* (GAS)[1], a human pathogen

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[1] The abbreviations used are: GAS, Group A *Streptococcus*; CFU, colony forming unit; FI, fluorescence intensity; i.n., intranasal; MFI, Mean fluorescence intensity; SLO, Streptolysin O; SpyCEP, *S. pyogenes* cell envelope protease.

causing a wide range of mild and severe diseases, that still awaits an effective vaccine (8, 9). So far the most advanced GAS vaccine candidates are based on the M protein, a major surface component and virulence factor shown to induce highly protective opsonophagocytic antibodies. However, such vaccines have not reached full development for two main drawbacks. First, M protein is a highly variable antigen (more than 150 variants identified), and this results in variant-specific protection. Therefore, the development of a universal M-protein vaccine necessarily implies the combination of a large representation of the prevalent circulating serotypes (10, 11). Second, the partial homology of M protein to human components has been associated with autoimmune sequelae (12). Our tripartite approach allowed the highly selective identification of very few conserved protective antigens and the definition of a three-protein combination conferring consistent protection against multiple GAS serotypes in mice. Protection was at least partially mediated by functional antibodies, which also promote GAS killing in a classical whole blood bactericidal assay.

The proposed three-technology approach represents a general strategy to select conserved protective antigens against any pathogen for which functional antibody responses are needed. Therefore, the approach is expected to accelerate the path to vaccine development by dramatically reducing the number of proteins to be tested in preclinical models.

EXPERIMENTAL PROCEDURES

Selection, Expression, and Purification of GAS Antigens—The 126 GAS antigens reported in supplemental Table S1 were selected *in silico* by analyzing the SF370 strain using the classical reverse vaccinology and genome comparison approaches (1, 2). Presence of the antigen coding genes in at least seven of the 13 available complete genome sequences was assessed. Genes with a degree of identity equal to or higher than 75% on three-fourths of the total protein length were considered conserved in different strains. The 61 antigens selected for further studies were expressed in *E. coli*, deprived of their leader and anchor sequences, as either glutathione S-transferase (GST) or His-tagged fusions and purified from the cytoplasmic fraction as soluble forms as previously described (5). Streptolysin O (SLO, aa 32–571), SPy0269 (aa 27–849), and *S. pyogenes* cell envelope protease (SpyCEP, aa 34–1613) tagless versions were cloned in the pET24b+ *E. coli* expression vector and purified.

Bacterial Strains and Growth Conditions—GAS strains were provided by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM2071, M23 strain), by ATCC (M1-SF370, M12–2728), by the Istituto Superiore di Sanità, Rome, Italy (M1–3348), by Laboratory of Bacterial Pathogenesis and Immunology, Rockefeller University, New York, NY (M6-S43), and by University of Rostock (M28-HRO-K-06). The other GAS strains were available in-house. Bacterial cultures for "surfome" analysis as well as for *in vivo* infection experiments were grown at 37 °C in Todd-Hewitt broth (Difco Laboratories). Cultures for cytofluorymetric analysis were instead grown in Todd-Hewitt broth supplemented with yeast extract. In all cases, bacteria were grown until the optical density at 600 nm (OD_{600}) was between 0.3 and 0.4 and subsequently processed accordingly to the type of assay performed.

Flow Cytometry—To evaluate antigen exposure on the bacterial surface, GAS cultures strains were grown in Todd-Hewitt supplemented with yeast extract broth medium to OD_{600} = 0.32, washed twice with phosphate-buffered saline (PBS), suspended in newborn calf serum (Sigma), incubated for 20 min at room temperature and dispensed in 96-well plate (20 μl/well). Bacteria were then incubated for 30 min at 4 °C with preimmune or immune mice polyclonal antiserum, diluted 1:200 in dilution buffer (PBS, 20% newborn calf serum, 0.1% bovine serum albumin). After centrifugation and washing in PBS and 0.1% bovine serum albumin, samples were incubated for 30 min at 4 °C with R-phycoerythrin-conjugated F(ab)2 goat anti-mouse immunoglobulin (Jackson ImmunoResearch), diluted 1:100. Bacteria were washed and suspended in PBS. Stained samples were analyzed with a FACS Calibur cytometer (Becton Dickinson), and Mean Fluorescence Intensity (MFI) values were calculated using CellQuest software (Becton Dickinson).

Antigen FACS positivity in the different tested strains was obtained as follows: (1) for each antigen the difference between the MFIs of the immune and preimmune sera were defined (ΔMFIs); (2) the MFI mean values obtained with the preimmune sera and the different strains were used to calculate the standard deviation (SD = 50 channels), (3) antigens with a ΔMFIs higher than 100 (two times the SD) in at least 30% of the tested strains were positively prioritized.

Protein Chip Analysis—The GAS protein array was generated as previously described (13). Briefly, affinity-purified GAS recombinant antigens were spotted on nitrocellulose-coated slides (FAST slides, Schleicher and Schuell) with the Chipwriter Pro spotter (Bio-Rad, Hercules, CA). Following slide saturation with 3% Top Block (Fluka-BioChemiKa, Buchs, Switzerland) - 0,1% Tween 20 in PBS (TPBS), incubation with sera from pharyngitis patients was carried out (1:1000 dilutions in TPBS) for 1 h at room temperature. Cy3 conjugated anti-human IgG (Southern Biotech) were added for 1 h at room temperature in the dark, before proceeding with slide scanning. Fluorescence signals were detected by using a ScanArray 5000 Unit (Packard, Billerica, MA) and the 16-bit images were generated with ScanArrayTM software at 10 μm per pixel resolution and processed using ImaGene 6.0 software (Biodiscovery Inc, CA). Elaboration and analysis of image raw fluorescence Intensity (FI) data was performed using in-house developed software. Normalized FI values higher than 15,000 were chosen to consider an antigen as positively recognized by human sera (13). "Pharyngitis" sera were collected from 239 patients aged 4–14 with clinical symptoms of pharyngitis. Isolation of the GAS infective strain by a throat swab performed at diagnosis confirmed pharyngitis being GAS-associated and allowed serotypes definition. The Institutional Review Board of the Methodist Hospital Research Institute authorized the use of sera for research purposes.

Bacterial Surfome Analysis—Surfome analysis was performed with the following GAS strains: M1–3348, M3–40603, M6–3650, M12–2728, M23-DSM2071, M28–10266. Bacteria were plated overnight onto blood agar (TrypticaseTM, Soy Agar II with 5% sheep blood, BD Science) and colonies were grown at 37 °C in 200 ml of THB in the presence of 5% CO_2 until an OD_{600} of 0.4 was reached. Bacteria were harvested by centrifugation at 3500 × *g* for 10 min at 4 °C and washed twice with PBS. Cells were suspended in 800 μl of PBS containing 40% sucrose (pH 7.4 for trypsin digestion and pH 6.0 for proteinase K digestion). Digestions were carried out with 10 μg trypsin (Promega, Madison, WI) or 5 μg proteinase K (Sigma), for 30 min at 37 °C. Bacterial cells were then spun down at 3,500 × *g* for 10 min at 4 °C and the supernatants were filtered through 0.22-μm pore-size filters (Millex, Millipore, Bedford, MA). Protease reactions were stopped with formic acid at 0.1% final concentration. Before analysis, PBS and sucrose were removed by off-line desalting procedure using OASIS cartridges (Waters, Milford, MA) following the producer's protocol. Desalted peptides were concentrated with a Centrivap Concentrator (Labconco, Kansas City, MI), solubilized in 3% acetonitrile, 0.1% formic acid and stored at −20 °C until further analysis. Peptides

obtained were separated by nano-LC on a NanoAcquity UPLC system (Waters) connected to a Q-ToF Premier Electro Spray Ionization mass spectrometer equipped with a nanospray source (Waters). Analytical methods and the parameters applied were reported in Doro *et al.* (4). The Mascot Daemon application (Matrix Science Ltd., London, UK) was used for the automatic submission of data files to an in-house licensed Mascot, version 2.2.3, and it was running on a local server. Protein identification was achieved by searching in a locally curated database containing 56,406 sequences and 16,305,282 residues and obtained by combining protein sequence data derived from the completely sequenced GAS strains, downloaded from the J. Craig Venter Institute website (http://www.jcvi.org/) and the *Streptococcus pyogenes* section of the NCBInr database. The Mascot search parameters were set to (1) 2 as number of allowed missed cleavages (only for trypsin digestion), (2) methionine oxidation, glutamine and asparagine deamidation as variable modifications, (3) 0.3 Da as peptide tolerance, and (4) 0.3 Da as the MS/MS tolerance. Only significant hits were considered, as defined by the Mascot scoring and probability system (Mowse score $\geq$ 35).

Bacterial Secretome Analysis—Secretome analysis was performed on the following seven GAS strains: M1-SF370, M1–3348, M1–5005, M3–2721, M3–315, M3–40427, and M28-HRO-K-066. Bacteria were grown as described above. After PBS washes, the bacterial pellet was resuspended and diluted in chemically defined medium (14) to OD_{600} of 0.05 and grown until a final OD_{600} of 0.4. Bacteria were removed by centrifugation at 3500 $\times$ *g* for 10 min at 4 °C and the supernatant was filtered through a 0.22-μm pore size filter (Millipore). Complete Protease Inhibitor Mixture Tablets (Roche) were added. Filtrates were subjected to high-speed centrifugation (200,000 $\times$ *g*, 90 min.) to eliminate membrane blebs. Proteins present in the supernatant were precipitated with 10% w/v trichloroacetic acid, 0.04% w/v sodium deoxycholate. Proteins were suspended in PBS. Different aliquots were suspended on LDS buffer (Invitrogen, Carlsbad, CA), separated on 4–12% polyacrylamide SDS-PAGE and stained with R-250 Brilliant Blue Coomassie. Protein bands of interest were subsequently analyzed by in-gel trypsin digestion and matrix-assisted laser desorption ionization/time of flight Mass Spectrometry, as previously described (6).

Whole Blood Assay—Bacterial cultures were grown in Todd-Hewitt supplemented with yeast extract overnight, washed with PBS and diluted to obtain a theoretical number of 10–50 colony forming units (CFU)/25 μl for each reaction mixture verified by plating and counting the following day. The bacterial culture samples were mixed with 25 μl of heat-inactivated (30 min at 56 °C) sera from rabbits immunized either with a multiprotein Combo (SPy0416, SPy0269 and SPy0167) or adjuvant only (negative control) and incubated 20 min at room temperature, before adding 200 μl of heparinized whole blood from a single naïve donor rabbit, as source of complement and blood cells, and 10% of baby rabbit complement. The reactions were then incubated with "end over end" rotation for 5 h at 37 °C and then diluted in Minimum Essential Medium Eagle (MEM, 750 μl). A volume of 100 μl from appropriate dilutions was plated on blood agar plates that were incubated overnight at 37 °C to count the CFU number/ml and to evaluate the bactericidal effect of blood/serum samples. Bacterial reduction was calculated using the following equation:

$$((\text{mean CFU neg. control}) - (\text{mean CFU sample to be tested}))/\text{mean CFU neg. control} \times 100$$

In vitro hemolysis and IL-8 cleavage inhibition assays—Hemolysis inhibition assay was performed using a blood cell suspension prepared washing sheep blood cells three times in PBS, then suspended in five volumes of PBS (20% in PBS sheep blood cells suspension). Serial twofold dilutions of either anti-Combo or negative control sera were prepared in 96-well plates with U-shaped bottoms using 0.5% BSA in PBS plus 10 ng of SLO toxin (diluted in BSA 0.5% in PBS) were added and plates were incubated at room temperature for 20 min. Following addition of 50 μl of sheep blood cells suspension, incubation was continued for 30 min at 37 °C. Plates were finally centrifuged for 5 min at 1000 $\times$ *g* and the supernatant was carefully transferred to 96-well flat-bottomed plates. The absorbance was read at 540 nm. Inhibition titer was expressed as the lowest serum dilution factor that completely inhibited SLO induced hemolysis.

To perform IL-8 inhibition assay, SpyCEP (0.1 μg/ml) was pre-incubated with mice polyclonal anti-Combo serum at five different dilutions (1:50, 1:100, 1:400, 1:800, and 1:1600) for 5 min at 4 °C in PBS 0.5 mg/ml BSA (pre-incubation of SpyCEP with buffer only and with anti-C5a peptidase serum were used as negative controls). Then IL-8 was added (10 μg/ml) and the reaction mix was incubated at 37 °C for 2 h. The amount of IL-8 was quantified after 2 h by ELISA (human IL-8 Immunoassay kit, Invitrogen) and expressed as percentage of uncleaved IL-8 in the reaction (incubation with SpyCEP) compared with IL-8 in the control reaction (incubation with buffer only). The same samples were analyzed by SDS-PAGE and Silver staining (SilverQuest™ Silver Staining Kit, Invitrogen).

In Vivo Protection Assays—Female CD1 5-week old mice were immunized intraperitoneally (intraperitoneal) on days 0, 21, and 35 with a vaccine formulation including 20 μg of each antigen, formulated either in Freund's or in Alum hydroxide adjuvant. Negative control groups consisted of mice immunized with adjuvant alone. Positive control groups consisted of mice immunized with the M protein variant of the GAS challenge strain used for infection (homologous M). Three weeks after the third immunization, mice were infected either intranasally (i.n.) or intraperitoneally (intraperitoneal) with 50 or 200 μl of a bacterial suspension respectively. Infectious doses were dependent on the challenge strain and the infection route used, ranging from 2.5 $\times$ 10^6 to 1.2 $\times$ 10^8 CFU for the i.n. challenge and from 50 to 2.5 $\times$ 10^6 CFU for the intraperitoneal challenge. For experiments in which protection from toxin treatment was evaluated, wild-type recombinant SLO was diluted in a solution of 2 mM dithiothreitol in PBS, then 100 μl were injected into mice tail vein of animals previously immunized either with recombinant SLO formulated in Alum hydroxide adjuvant or adjuvant alone. In all experiments, mice were monitored on a daily basis for at least 1 week after treatment and euthanized when they exhibited defined humane endpoints that had been pre-established for the study in agreement with Novartis Animal Welfare Policies. For air pouch experiments, dorsolateral air pouches were inflated by subcutaneous injection of 3 ml air on day 1 and day 4. On day 6, 1 $\times$ 10^7 CFU of *S. pyogenes* in 1 ml PBS were injected into the pouch. At 24 h after infection, the animals were euthanized and an air pouch lavage was performed by repeated injection/aspiration of 2 ml PBS. Lavage samples were frozen at −80 °C to promote cell lysis, and then subjected to serial dilutions and viable counts. The multiplication factor for each mouse was calculated dividing the CFU number at 24 h post infection with that injected at time 0.

Statistical Analysis—For the GAS intranasal and intraperitoneal infection models and for the intravenous model of Spy0167 injection, the two tailed Fisher's exact test was used. For the statistical analysis of the GAS air-pouch infection model and of the opsonophagocytosis assay, the two tailed Mann-Whitney *U* test was used. In all cases, GraphPad Prism 5 software was used to calculate statistical significance. *p* values lower than 0.05 were considered significant. Asterisks legend: *p* value $<$ 0.001 = ***; between 0.001 and 0.01 = **; between 0.01 and 0.05 = *.

RESULTS

Flowchart of Protein Selection Strategy for Vaccine Candidate Discovery—The overall strategy used to select GAS pro-

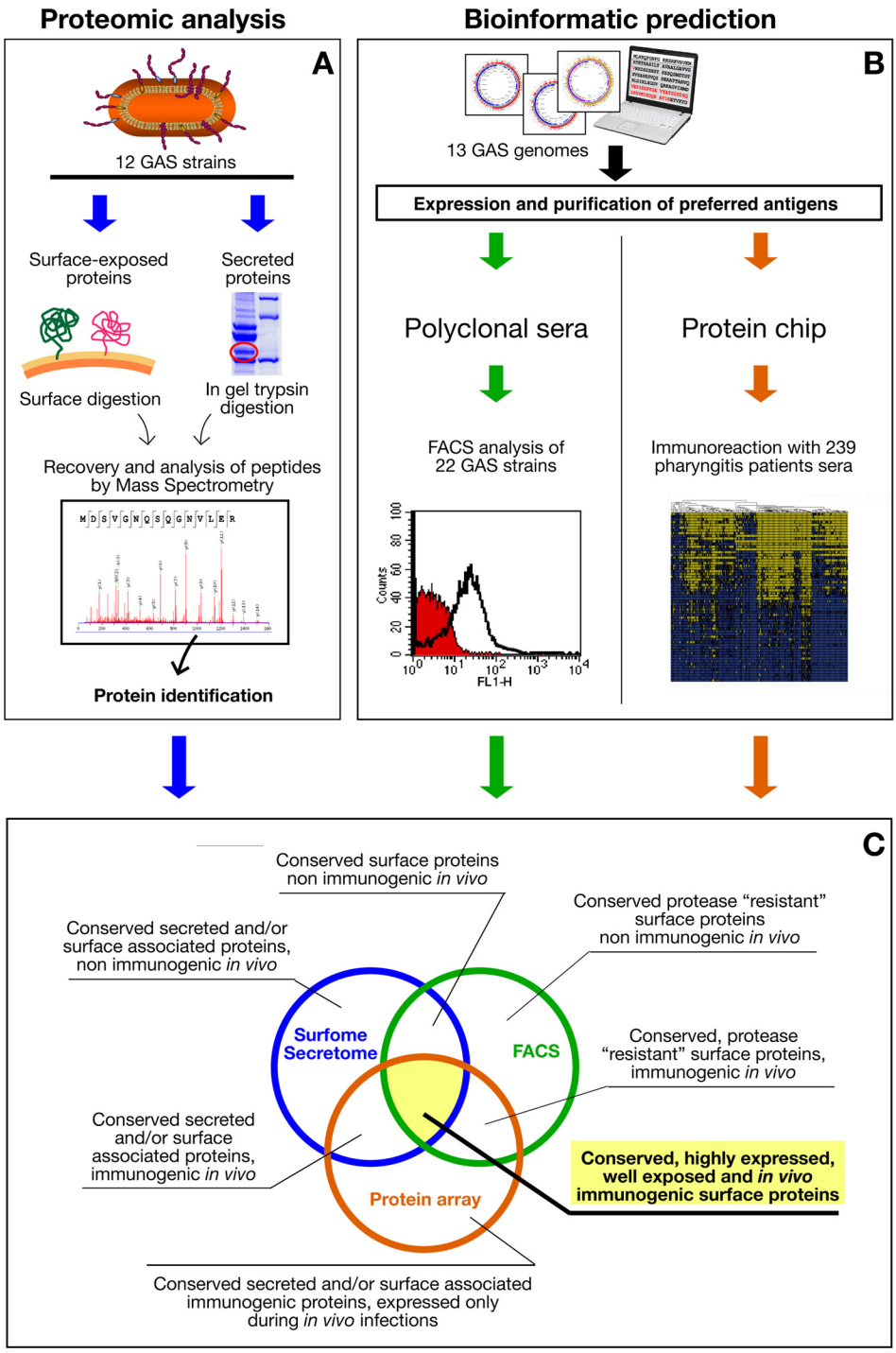


FIG. 1. **Flowchart of antigen selection strategy for vaccine candidate discovery.** The three experimental approaches used are schematically shown in panels *A* and *B*. In *C*, a Venn diagram is represented, summarizing the expected characteristics of the antigens that are positive to at least one of the approaches used.

teins to be tested in the animal model for protection can be schematized as follows (Fig. 1). First, the genome sequences of GAS isolates available in the public database were analyzed to select genes (1) potentially encoding secreted and surface associated proteins, and (2) sufficiently conserved among the different genomes. These genes were then expressed in *E. coli* and the recombinant proteins were used (1) to produce mouse polyclonal antibodies, and (2) to build protein arrays. Polyclonal antibodies were subsequently used in FACS assay to quantify the level of expression/surface exposure of the corresponding antigens in a panel of clinical isolates, leading to the selection of conserved and highly expressed surface proteins. In a parallel approach, protein arrays were exploited to screen a high number of sera from GAS-infected human patients, thus leading to the selection of antigens that were immunogenic *in vivo*. Finally, in parallel to

FACS and protein array, secreted and surface-exposed proteins were identified by MS by analyzing the supernatants (secretome) and the protease-derived peptides of "shaved" bacterial cells (surfome) from different isolates.

Once available, the lists of antigens identified by MS, FACS and protein array were merged in a Venn diagram-type representation, in which each domain of the diagram defined protein subsets with predictable features (Fig. 1). In particular, the proteins falling in the central area (proteins revealed by all three technologies) were (1) well expressed in a high number of strains, (2) immunogenic, and (3) surface exposed/secreted in multiple isolates. We hypothesized that these were the antigens that had the highest probability of inducing broadly protective antibody responses.

Bioinformatics Selection of Conserved Surface-Associated Antigens for FACS and Protein Array Analyses—Bioinformatics analysis of the publicly available complete genome sequences of 13 GAS isolates (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi) was carried out with the aim of selecting a group of conserved secreted proteins, cell wall-associated proteins, membrane-anchored proteins, and lipoproteins.

Small extracellular proteins and lipoproteins (<100 amino acids) were excluded because of naturally occurring processing *in vivo*, as they are likely not to be sufficiently long enough to cross the cell wall and the capsule and to be exposed to the bacterial surface (15). For the same reason, ~200 amino acids were considered as the minimal length for cell wall and membrane proteins. Finally, only membrane proteins with one transmembrane domain were included, because we previously observed that antigens with multiple transmembrane regions are in general poorly exposed on the bacterial surface (4, 5) and are difficult to express in *E. coli*. This selection process led us to a pool of 126 proteins conserved in multiple strains (at least seven), from which a representative subset of 61 antigens was chosen and used to establish a proof-of-concept strategy (supplemental Table S1). The protein subset included most of the lipoproteins and cell wall-associated proteins, a selection of membrane anchored and secreted proteins and few cytoplasmic proteins with the RGD motif.

Analysis of Immunogenic Antigens by Protein Array—The 61 purified proteins were spotted on nitrocellulose-coated glass slides and probed with 239 sera from children affected by GAS-associated pharyngitis. These patients were infected with strains belonging to 11 different serotypes (M1, M2, M3, M4, M6, M12, M22, M28, M75, M77, and M89), as determined by throat swabbing and *emm* gene analysis (13). The results of the microarray approach are shown in Fig. 2*A*, in which proteins listed in the *y* axis are ordered on the basis of the number of sera they reacted with. As also shown in Fig. 2*A*, 26 out of 61 antigens were positively recognized by at least 50% of the tested sera. On the *x* axis of Fig. 2*A*, sera were grouped by nonhierarchical clustering, highlighting three small groups that were poorly reactive against most of the proteins spotted on the chips, and three major clusters of sera recognizing a vast majority of the 26 prioritized proteins. Such clusters did not associate with any of the M types in the corresponding patients.

Quantification of Antigen Expression/Surface Exposure—In order to establish whether and to what extent the 61 selected proteins were exposed on the bacterial surface, we used protein-specific polyclonal antibodies to probe by FACS analysis the surface of 22 GAS strains belonging to 12 different serotypes (M1, M2, M3, M4, M5, M6, M8, M9, M11, M12, M23, an M28). In Fig. 2*B*, antigens are ordered on the basis of the number of strains found to express them to a sufficiently high level. A ΔMFI of 100 with respect to pre-immune sera, corresponding to the background intensity signal plus two standard deviations, was taken as cutoff. Twenty-four proteins met this threshold in at least 30% of the tested strains. Fig. 2*B* also shows that the top candidates detected in a highest number of isolates, which include 6 lipoproteins and four LPXTG cell wall-anchored proteins, were in general those most surface-exposed, as judged by the fluorescent values obtained with some of the strains. Interestingly, five secreted proteins including Streptokinase A (SPy1979) and SLO (SPy0167) were also detected on the surface of a significant percentage of the tested strains, indicating that these proteins are partially and/or temporarily associated to the cell surface.

MS-based Proteomic Analysis of Surface-exposed and Secreted Proteins—Twelve GAS strains representing 6 different serotypes (M1, M3, M6, M12, M23, and M28) were analyzed either by surfome or secretome proteomic approaches. In addition to the corresponding M protein variants, a total of 94 antigens were identified (supplemental Table S2). They included 38 proteins belonging to the cell wall-associated family, 11 membrane proteins, 6 lipoproteins, and 14 extracellular proteins. The remaining 25 proteins were classified as cytoplasmic. However, they included the elongation factor thermo unstable (EF-Tu) and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) proteins, reported to be associated with the cell surface and capable of conferring protection against different pathogens (16–19), as well as the L7/L12 ribosomal protein and trigger factor (TF), also shown to provide protection (20, 21). Supplemental Table S2 highlights the 38 top candidates selected by this approach, ranked on the basis of both the number of strains in which they were consistently detected (at least two out of those tested) and the number of peptides identified (at least two MS/MS fragmentations for surfome detected antigens). The list includes 12 cell wall proteins, six membrane proteins, two lipoproteins, 12 extracellular proteins, and six cytoplasmic proteins (peptide sequence details, Mascot scores and MS spectra are provided as supplemental data).

Identification of Protective Antigens—The application of the three technologies led to the generation of three antigen priority lists, constituted by 26, 24, and 38 antigens, respectively (Fig. 2*A* and 2*B*, and supplemental Table S2). The next question was whether the lists included protective antigens, and whether to elicit protection they had to fall within specific

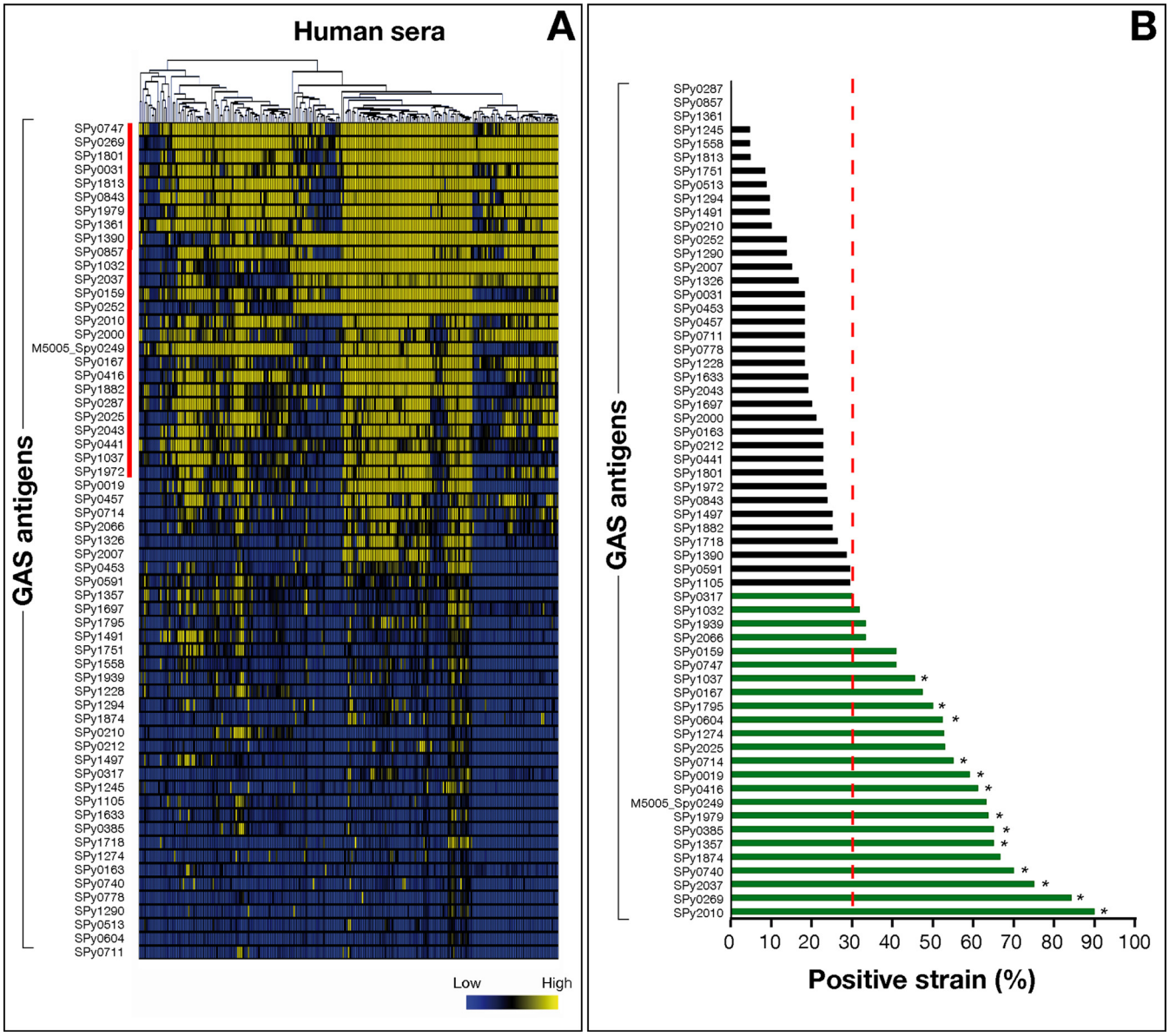


FIG. 2. **GAS antigen surface exposure and *in vivo* immunogenicity.** *A*, Unsupervised hierarchical clustering of human pharyngitis patients sera (n = 239, *x* axis), *versus* the 61 antigens considered (*y* axis). Antigen/sera interactions resulting in signals with high or low fluorescence intensity (FI) are visualized in yellow and blue respectively. Color scale of signal intensity is reported on bottom-right. Antigens and/or sera showing similar reactivity profiles are grouped in clusters. The red bar on the left of the dendrogram identifies 26 antigens that were positively recognized by at least 50% of tested sera with an FI equal to or higher than the arbitrary cut-off of 15,000. *B*, The graph reports the percentage of FACS positivity for the 61 selected antigens against a panel of 22 strains. The red dashed line defines the 30% positivity value, which was considered the cutoff to choose the 24 antigens (green bars) with highest priority. Asterisks indicate those antigens displaying surface expression levels corresponding to the mean value plus > 2 S.D. in at least 1 tested strain.

areas of the Venn diagram depicted in Fig. 1. In other words, we wanted to know whether protective antigens were selectively detected by one or more of the three technologies used. To correctly address this question, we eliminated 25 of the 38 proteins identified by the MS proteomic approach as these proteins were not included in the list of 61 antigens tested by FACS and protein array (either because they were poorly expressed in *E. coli* or not fulfilling the established bioinformatics selection criteria). By doing so, a total of 40 prioritized antigens were tested for protection. Six of them were identified by all three technologies, 11 were identified by two technologies, and 23 by only one of the three technologies (Fig. 3).

Each of the 40 proteins was used to immunize mice that were subsequently challenged with two different strains, M1–3348 and M23–2071. This exercise resulted in the identification of six antigens that induced protection in mice against one or both strains (Table I). These antigens were the SpyCEP protease (SPy0416), the secreted protein SLO (SPy0167),

SPy0269 that in the SF370 genome was annotated as "putative surface exclusion protein," SPy0019 annotated as a putative secreted protein, SPy1361 belonging to internalin A protein family, and C5a peptidase (SPy2010).

When the six protective antigens were mapped in the Venn diagram depicted in Fig. 3, four of them, SPy2010, SPy0167, SPy0416, and SPy0269, fell in the subset resulting from the superimposition of the three technologies (Table II). Of the two remaining proteins, SPy0019 just missed the central domain of the diagram in that it was recognized by 49% of the sera by protein array, only 1% below the arbitrary threshold (50%) used to include antigens in the protein array priority list. Spy1361 was instead highly immunogenic *in vivo* but was not detected by FACS analysis and identified in only one of the strains subjected to proteomics analysis (Table II).

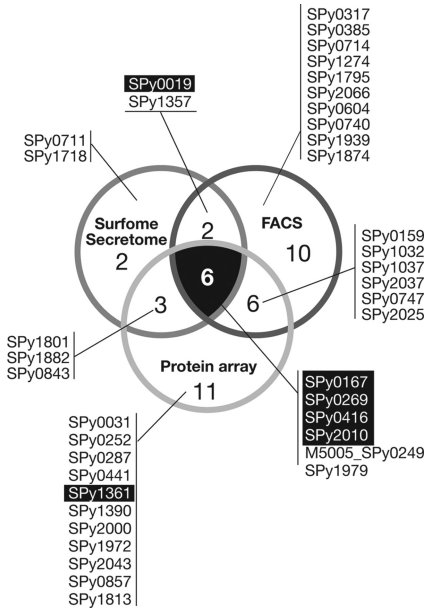


FIG. 3. **The three-approach strategy identifies six top priority antigens.** The Venn diagram reports the antigen distribution obtained on the basis of antigen positivity with the three technologies. Antigens highlighted in black are those conferring protection in the animal models against infection with M1 and M23 GAS isolates (Table I).

In conclusion, the protection data reported above indicate that (1) the large majority of protective antigens fall in the central domain of the Venn diagram and 2) the same domain mostly include antigens that are protective (4 out of 6).

Selection of a Broad Coverage Protective Antigen Combination—We next asked the question whether the proteins that conferred protection in mice when challenged with M1–3348 and/or M23–2071 strains could also protect mice against additional GAS isolates. Mice were immunized with each of the five antigens (C5 peptidase was excluded from our analysis because it has been well characterized by others) (22, 23) and subsequently challenged with bacteria belonging to serotypes M6 and M12. As shown in Table I, SPy0416, SPy0269, and SPy0167 elicited protection against at least one of these additional strains.

We next combined SPy0416, SPy0269, and SPy0167 and tested the "Combo" for cross-protective activity against M1, M6, M12, and M23 strains. For each challenge strain the level of protection achieved with the Combo was compared with that obtained with the corresponding M protein variant, known to induce strong but variant-specific protection. For this analysis the proteins were formulated with Alum adjuvant to be compatible with future human studies. As highlighted in Fig. 4*A* and 4*B*, the three-antigen Combo was protective against all four tested strains in two mouse models differing for the challenge route. Protection ranged from 80% to 50%, and was close to the levels achieved when mice were immunized with the homologous M protein used as control.

We also tested the protective efficacy of the three-antigen Combo in an air pouch subcutaneous mouse model, which provides data on the immediate/early response to infection in immunized mice by following the rate of bacterial growth *in situ*. As shown in Fig. 4*C*, Combo-immunized mice had a bacterial load that was one order of magnitude lower than mock immunized animals. This degree of growth inhibition was comparable to what was observed in mice immunized with the homologous M protein.

To establish the possible mechanisms of protection induced by the Combo vaccine, sera from Combo-immunized

TABLE I
Results of antigen in vivo protection analysis against multiple GAS strains

	GAS strains							
	M1–3348		M23–2071		M12–2728		M6-S43	
Antigen	Alive/tested	Survival (%)	Alive/tested	Survival (%)	Alive/tested	Survival (%)	Alive/tested	Survival (%)
Adjuvant[a]	20/72	28	11/44	25	20/96	21	44/144	31
SPy0269	41/73	**56****	23/35	**65****	37/83	**44****	27/48[c]	**56***
SPy0416[b]	22/35	**63****	21/40	**52***	45/69	**65****	49/112[c]	**44***
SPy0167	35/38	**92****	7/20	35	31/64	**48****	30/48[c]	**63****
SPy0019	28/40	**70****	5/25	20	24/40	**53***	Not tested	
SPy1361	19/40	**47***	2/20	10	Not tested		5/32	16

[a] Freund, unless indicated otherwise.
[b] Protein fragment including aminoacids 34–898.
[c] Tested with aluminum hydroxide adjuvant.
* p value < 0.05, ** p value < 0.001 (Fisher's exact test against control mice immunized with adjuvant only).

TABLE II
Expression and immunogenicity of the identified protective GAS antigens

SPy	PSORTb	Product	Proteome[a]	Protein array[b]	FACS[c]
SPy0167	extracellular	streptolysin O precursor	3/6	63	47
SPy0269	cell wall	putative surface exclusion protein	4/6	89	84
SPy0416	cell wall	putative cell envelope proteinase	4/6	62	61
SPy0019	extracellular	putative secreted protein	2/6	49	59
SPy1361	lipoprotein	putative internalin A precursor	1/6	80	0
SPy2010	cell wall	C5a peptidase precursor	6/6	67	90

[a] Number of positive strains on the total number of strains analyzed.
[b] Percent of sera which positively recognize the antigen.
[c] Percent of positive strains.

mice were analyzed for functional activities. In particular, sera were tested for their capacity to neutralize the hemolytic and IL-8 proteolytic activity of SPy0167, and SPy0416 respectively, and for bactericidal activity using the classical whole blood killing assay. As shown in Fig. 5*A*, a 1:8,000 dilution of the Combo immune serum was sufficient to block 100% of the hemolytic activity of 10 ng of toxin. Furthermore, SPy0167 immunized mice survived intravenous administration of a lethal dose of toxin (Fig. 5*B*). Additionally, sera from Combo immunized mice could neutralize SPy0416 proteolytic activity on IL-8 in a dose dependent manner (Fig. 5*C*), indicating that antibodies against this protein prevent the inhibition of neutrophil recruitment *in vivo*, thus favoring bacterial phagocytosis and killing (24, 25). Finally, Combo immunization also induced antibodies mediating GAS opsonophagocytic killing, as revealed by the capacity of rabbit immune serum to facilitate killing of 70% of M1–3348 bacterial cells in the presence of blood from a naïve donor animal, as source of complement and immune cells (Fig. 5*D*). Comparable results were obtained when blood from Combo immunized rabbits was directly used to set up the bactericidal assay (data not shown).

DISCUSSION

The ultimate goal of Vaccinology is to learn how to select protective antigens for a given bacterial pathogen by "simply" reading its genome sequence. When this "Holy Grail" is reached, vaccine research will mostly be performed *in silico*, animal use will be restricted to toxicity studies only, and vaccine efficacy will be directly demonstrated in humans.

Although we are still a few years away from this ambitious goal, rational approaches have been developed that allow to successfully reduce the list of candidates entering the animal screening assays down to few tens. They include bioinformatics analysis to select membrane and surface-associated proteins (2, 26), high throughput strategies to identify antigens that are immunogenic during infection (13, 27), and proteomic analyses aimed at the identification of proteins protruding out of the bacterial cell (3).

The present work describes a further, substantial improvement of the strategies so far employed in that it singles out the relevant protective antigens without using functional assays in animals. Starting from the observation that all vaccines inducing broadly protective antibody responses are constituted by bacterial components that are sufficiently conserved and are either secreted or abundantly expressed on the surface, we reasoned that if such category of proteins are searched by using a battery of experimental protocols, those proteins that are consistently identified by all the experimental approaches employed would be the protective ones. We have tested this strategy on Group A *Streptococcus*, a human pathogen for which an effective vaccine is still missing despite decades of intensive research (9). Three different approaches were used for antigen identification. The first was based on bacterial protease digestion coupled to mass spectrometry methodology (3) with which we scanned the surface of several clinical isolates for protruding proteins by "piling" off and analyzing their exposed moieties. With the second approach, the bacterial surface of several clinical isolates was probed with antibodies produced against a group of recombinant proteins selected by multiple genome analysis on the basis of their predicted surface location and sequence conservation. The third experimental strategy indirectly established which of the recombinant proteins selected for antibody production was well expressed during infection by determining their immunogenicity in sera from pharyngitis patients. Once the three lists of antigens derived from the application of the three technologies were available, all proteins were tested in the mouse model to determine which technology was capable of pinning down the protective ones.

The most striking result from our study is that of the 40 unique proteins identified by one or more of the three technologies only six were protective and most of them belonged to the group of proteins identified by all three approaches (Fig. 3). These included C5a peptidase (9, 11, 23), SpyCEP (5, 9), and Spy0269 (28, 29), already reported to be protective, and Streptolysin O. As far as the protective antigen SPy0019 is concerned, it was also identified by the three approaches but it does not graphically fall in the central part of the Venn diagram because it was just 1% below the 50% threshold we arbitrarily imposed to include a protein in the protein array priority list. Therefore, the only protective protein showing a different identification pattern was SPy1361, which was almost negative by both proteomic and FACS analyses but

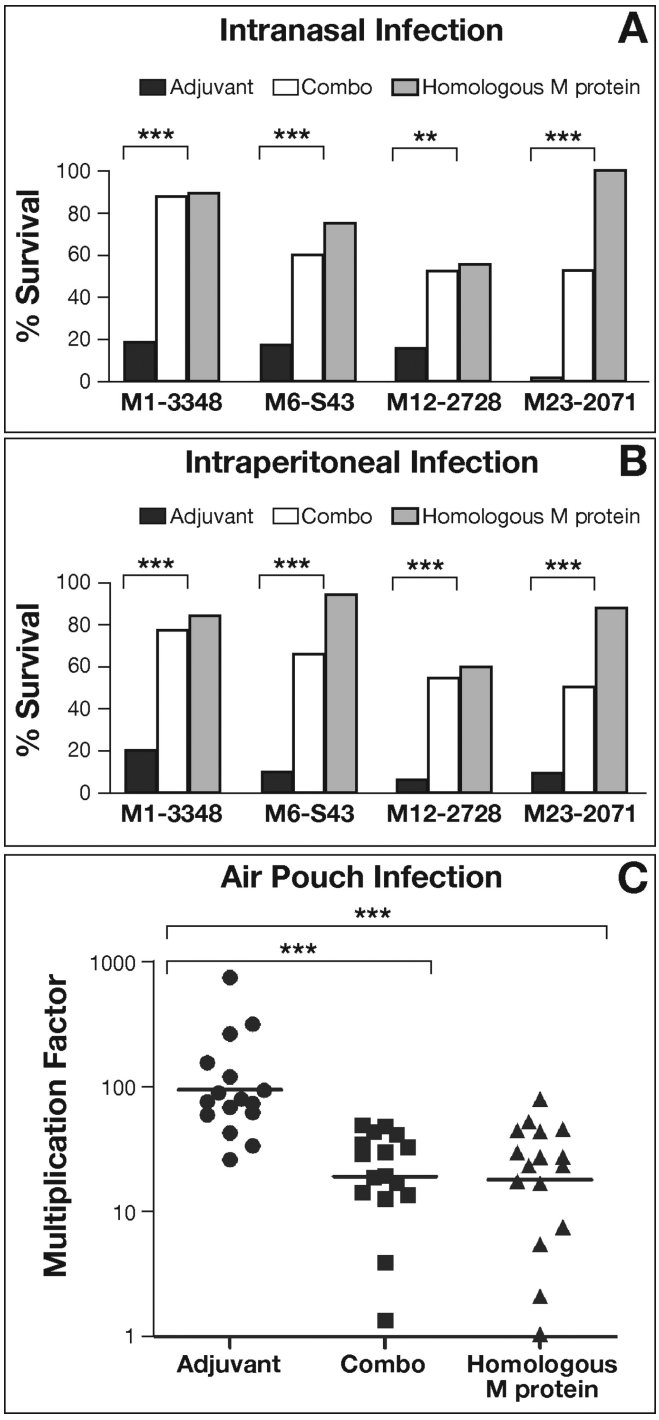
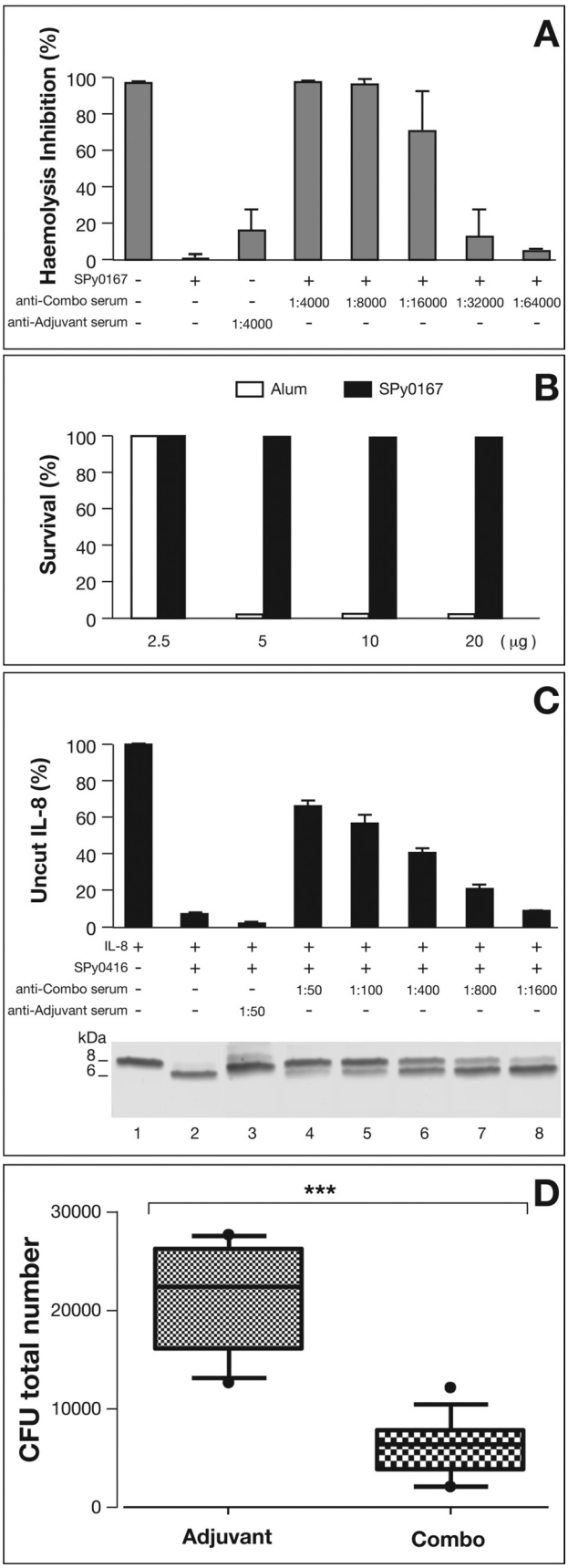


FIG. 4. **Immunization with the protein Combo confers consistent protection against infection with multiple GAS serotypes.** Panels *A* and *B* show the survival rates of Combo immunized mice infected with four strains of different serotypes intranasally and intraperitoneally respectively. Survival of mice immunized either with adjuvant only (negative control groups) or with each of the four homologous M proteins (positive control groups) are also reported. Data for each group result from a minimum of three independent experiments with a total number of at least 32 mice. Asterisks indicate statistically significant difference between groups immunized either with adjuvant or with Combo (** = p value < 0.01; *** = p value <0.001, Fisher's exact test). The diagram in *C* reports the rate of bacterial growth (multiplication factor, *y* axis) in the air pouches of individual mice immunized with the protein Combo (black squares), adjuvant only (black circles) or M1 positive control protein (black triangles) and then infected in the pouch with the M1–3348 strain. A multiplication factor was obtained for each mouse as the ratio between the CFU number 24 h post-infection and time 0. Mann-Whitney *U* test was used for statistical analysis (*** = p value <0.001).

remarkably immunogenic, suggesting that it is primarily expressed during infection.

A key step of the strategy here proposed is the upfront bioinformatics analysis of several bacterial genomes to select among the proteins that have to be expressed for antibody production and protein array preparation only those that are sufficiently conserved. In the case of GAS, our analysis of the 13 publicly available complete genome sequences led us to select 126 conserved genes encoding predicted surface-associated and secreted proteins. Based on our previous experience with other pathogens indicating that most of the exposed proteins in Gram-positive bacteria are the cell-anchored proteins, the lipoproteins and some secreted proteins, we restricted the FACS and protein array analyses to a subgroup of 61 proteins. A legitimate question is whether protective antigens exist among the excluded 65 proteins. Even though we do not have yet a definite answer to this question, only six of these 65 proteins were found by mass spectrometry (supplemental Table S2), and thus the large majority of these antigens could not fall in the central area of the diagram. Furthermore, we have recently completed the analysis of the protective activity of 39 of those 65 proteins on strain M23–2071, and partially on M1–3348 strain and none of them turned out to be protective (unpublished results). Hence, the concept that most protective antigens fall in the central sector of the Venn diagram and that this sector includes almost exclusively protective antigens appears to be further strengthened.

The three platforms here proposed are robust, reliable, and sufficiently fast to be carried out on a large number of samples. This is particularly important because, given the genetic variability of bacterial species, several isolates need to be analyzed to select antigens that are widely expressed in order to demonstrate broad coverage. Proteome analysis of both supernatant and cell surface can be completed in a few days and several strains can be analyzed in parallel. High-throughput expression and purification allows obtaining hundreds of recombinant proteins in few weeks. Finally, protein array allows the rapid analysis of several hundreds of sera and FACS analysis can be performed on several strains simultaneously once polyclonal antibodies to the recombinant proteins are available. In conclusion, in a sufficiently organized laboratory, the entire process, starting with the selection of antigens by genomes analysis and ending with the generation of the Venn diagram derived from the three approaches and the analysis



of protective activity, can be completed in a few months, a time substantially shorter than what required for any other genome-based vaccine discovery process.

As already pointed out, the number of proteins detected by all three technologies was limited to six, in line with the notion that broadly protective protein antigens are a minute fraction of whole bacterial proteomes that have in common the property of being sufficiently well expressed and exposed on the bacterial surface. Even secreted proteins, such as SLO, can be recognized on live bacteria by specific antibodies, most likely because they are transiently retained on the bacterial cell surface during their way out of the cell.

The attractiveness of the tripartite-platform technology here proposed is that, although it allows the immediate selection of the most probably protective antigens, particularly promising outliers that do not fall in the central domain of the Venn diagram, like SPy1361, can be easily picked up and included in the list of antigens to be tested in the animal model.

As a value-added result of the tripartite approach, we were able to show that a combination of three conserved protective antigens conferred consistent cross-protection against a wide range of GAS strains in different mouse models of infection. This three-antigen Combo is the first proposed multiprotein formulation, which may represent a valid alternative to M-protein-based vaccines, the only formulations that have been tested in humans so far (30). A great advantage of the proposed combination is that, being constituted by highly conserved proteins, it could become a truly universal solution to GAS infections. On the contrary, because of the high varia-

FIG. 5. **Immunization with the GAS protein Combo induces functional antibodies.** *A*, Combo immune sera inhibit SPy0167-dependent hemolysis of sheep erythrocytes. Sheep blood cells were incubated with 10 ng of recombinant SPy0167 in the presence of increasing dilutions of sera from mice immunized with the alum-formulated Combo vaccine. Negative control reactions were incubated either without any serum or in the presence of serum from mice immunized with adjuvant only. *B*, Mice immunized with SPy0167 are protected by treatment with the toxin. The diagram shows the survival of mice immunized either with adjuvant alone or with SPy0167 following intravenous injection of increasing doses of recombinant SPy0167 (4–8 mice/group). *C*, Combo immune sera inhibit SPy0416-mediated processing of IL-8. The diagram in the upper panel shows the percent of uncleaved IL-8 in the presence of 100 ng of recombinant SPy0416 and different dilutions of Combo immune sera, as determined by ELISA assay. The results obtained with negative control samples without immune serum or with serum of mice immunized with adjuvant only are also shown. A silver-stained polyacrylamide gel showing the two IL-8 forms generated by cleavage is shown in the lower panel. *D*, Whole blood bactericidal assay with immune sera from Combo- and adjuvant-immunized rabbits. The data shown derive from 16 independent reactions set up using blood from three different naive rabbits incubated with rabbit immune sera. Statistically significant differences (*** = p value <0.0001) were obtained by Mann-Whitney analysis. Data are represented by box-and-whiskers plot analysis, showing the median, the 10 and 90 percentiles and extreme values (black dots).

bility of M-protein, M-protein based vaccines inevitably protect against a restricted number of isolates that may vary in different geographic areas and as a consequence of vaccination practice.

Not only the Combo induces bactericidal antibodies, a functional activity that is known to correlate with protection against infection (31–33), but it also elicits antibodies capable of neutralizing two important virulence factors expressed by a large fraction of GAS isolates. In particular, our data clearly demonstrated that the vaccine can neutralize the activity of SpyCEP, a serine protease that degrades IL-8 and other chemokines, preventing neutrophils recruitment at the infection site (24, 34). In addition, antibodies induced by our Combo vaccine efficiently prevent the activity of SLO, a secreted toxin that kills eukaryotic cells through the formation of membrane pores (35). Finally, preliminary data from our laboratory suggest that Spy0269 is involved in bacterial cell division (data not shown) and therefore specific antibodies might act by interfering with GAS septation and proliferation. Therefore, our vaccine is expected to operate through different mechanisms and this aspect could be particularly relevant to prevent the infection of a pathogen that causes such a wide range of disease.

There are several pathogens causing severe human and animal diseases for which vaccines do not yet exist. Among them, important bacteria such as *Staphylococcus aureus*, different *Streptococcal* species, *Pseudomonas spp*, pathogenic *E. coli*, and parasites such as the *Plasmodium* species. The application of genomic strategies for vaccine development against these pathogens has been hampered by the extremely high number of animals that would be required for antigen screening and, particularly important, by the absence of reliable animal models to be applicable in high throughput modalities. For instance, several of these pathogens require models based on transgenic animals and/or non human primates. The approach here proposed has the great advantage that the very few antigens eliciting protective antibodies are selected with a high degree of resolution without the need of animal screening. Therefore, we believe that our approach could become a valid strategy for an accelerated development of vaccines against many other clinically important microorganisms.

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REFERENCES

1. Maione, D., Margarit, I., Rinaudo, C. D., Masignani, V., Mora, M., Scarselli, M., Tettelin, H., Brettoni, C., Iacobini, E. T., Rosini, R., D'Agostino, N., Miorin, L., Buccato, S., Mariani, M., Galli, G., Nogarotto, R., Dei, V. N., Vegni, F., Fraser, C., Mancuso, G., Teti, G., Madoff, L. C., Paoletti, L. C., Rappuoli, R., Kasper, D. L., Telford, J. L., and Grandi, G. (2005) Identification of a universal group B streptococcus vaccine by multiple genome screen. *Science* **309,** 148–150
2. Pizza, M., Scarlato, V., Masignani, V., Giuliani, M. M., Arico, B., Comanducci, M., Jennings, G. T., Baldi, L., Bartolini, E., Capecchi, B., Galeotti, C. L., Luzzi, E., Manetti, R., Marchetti, E., Mora, M., Nuti, S., Ratti, G., Santini, L., Savino, S., Scarselli, M., Storni, E., Zuo, P., Broeker, M., Hundt, E., Knapp, B., Blair, E., Mason, T., Tettelin, H., eacute, Hood, D. W., Jeffries, A. C., Saunders, N. J., Granoff, D. M., Venter, J. C., Moxon, E. R., Grandi, G., and Rappuoli, R. (2000) Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing. *Science* **287,** 1816–1820
3. Grandi, G. (2010) Bacterial surface proteins and vaccines. *Biol. Reports* **2,** 36–39
4. Doro, F., Liberatori, S., Rodriguez-Ortega, M. J., Rinaudo, C. D., Rosini, R., Mora, M., Scarselli, M., Altindis, E., D'Aurizio, R., Stella, M., Margarit, I., Maione, D., Telford, J. L., Norais, N., and Grandi, G. (2009) Surfome analysis as a fast track to vaccine discovery: identification of a novel protective antigen for group B streptococcus hypervirulent strain COH1. *Mol. Cell. Proteomics* **8,** 1728–1737
5. Rodriguez-Ortega, M. J., Norais, N., Bensi, G., Liberatori, S., Capo, S., Mora, M., Scarselli, M., Doro, F., Ferrari, G., Garaguso, I., Maggi, T., Neumann, A., Covre, A., Telford, J. L., and Grandi, G. (2006) Characterization and identification of vaccine candidate proteins through analysis of the group A streptococcus surface proteome. *Nat. Biotechnol.* **24,** 191–197
6. Berlanda Scorza, F., Doro, F., Rodriguez-Ortega, M. J., Stella, M., Liberatori, S., Taddei, A. R., Serino, L., Gomes Moriel, D., Nesta, B., Fontana, M. R., Spagnuolo, A., Pizza, M., Norais, N., and Grandi, G. (2008) Proteomics characterization of outer membrane vesicles from the extraintestinal pathogenic Escherichia coli delta *tol*R IHE3034 mutant. *Mol. Cell. Proteomics* **7,** 473–485
7. Ferrari, G., Garaguso, I., Adu-Bobie, J., Doro, F., Taddei, A. R., Biolchi, A., Brunelli, B., Giuliani, M. M., Pizza, M., Norais, N., and Grandi, G. (2006) Outer membrane vesicles from group B Neisseria meningitidis delta gna33 mutant: proteomic and immunological comparison with deter-

gent-derived outer membrane vesicles. *Proteomics* **6,** 1856–1866
8. Cunningham, M. W. (2000) Pathogenesis of group A streptococcal infections. *Clin. Microbiol. Rev.* **13,** 470–511
9. Steer, A. C., Batzloff, M. R., Mulholland, K., and Carapetis, J. R. (2009) Group A streptococcal vaccines: facts versus fantasy. *Current Opinion Infectious Dis.* **22,** 544–552
10. Dale, J. B., Penfound, T., Chiang, E. Y., Long, V., Shulman, S. T., and Beall, B. (2005) Multivalent group A streptococcal vaccine elicits bactericidal antibodies against variant M subtypes. *Clin. Diagn. Lab. Immunol.* **12,** 833–836
11. Dale, J. B. (2008) Current status of group A streptococcal vaccine development. *Hot Topics Infection Immunity in Children IV*, pp. 53–63
12. Cunningham, M. W. (2008) Pathogenesis of group A streptococcal infections and their sequelae. *Hot Topics in Infection and Immunity in Children IV*, pp. 29–42
13. Bombaci, M., Grifantini, R., Mora, M., Reguzzi, V., Petracca, R., Meoni, E., Balloni, S., Zingaretti, C., Falugi, F., Manetti, A. G. O., Margarit, I., Musser, J. M., Cardona, F., Orefici, G., Grandi, G., and Bensi, G. (2009) Protein array profiling of tic patient sera reveals a broad range and enhanced immune response against group A *Streptococcus* antigens. *PLoS ONE* **4,** p.e6332
14. Mickelson, M. N. (1964) Chemically defined medium for growth of Streptococcus pyogenes. *J. Bacteriol.* **88,** 158–164
15. Barinov, A., Loux, V., Hammani, A., Nicolas, P., Langella, P., Ehrlich, D., Maguin, E., and van de Guchte, M. (2009) Prediction of surface exposed proteins in Streptococcus pyogenes, with a potential application to other Gram-positive bacteria. *Proteomics* **9,** 61–73
16. Dallo, S. F., Kannan, T. R., Blaylock, M. W., and Baseman, J. B. (2002) Elongation factor Tu and E1 β subunit of pyruvate dehydrogenase complex act as fibronectin binding proteins in Mycoplasma pneumoniae. *Mol. Microbiol.* **46,** 1041–1051
17. Jin, H., Agarwal, S., and Pancholi, V. (2011) Surface export of GAPDH/SDH, a glycolytic enzyme, is essential for Streptococcus pyogenes virulence. *MBio* **2,** e00068–00011
18. Ling, E., Feldman, G., Portnoi, M., Dagan, R., Overweg, K., Mulholland, F., Chalifa-Caspi, V., Wells, J., and Mizrachi-Nebenzahl, Y. (2004) Glycolytic enzymes associated with the cell surface of Streptococcus pneumoniae are antigenic in humans and elicit protective immune responses in the mouse. *Clin. Exp. Immunol.* **138,** 290–298
19. Niewes, W., Heang, J., Asakrah, S., Honer zu Bentrup, K., Roy, C. J., and Morici, L. A. (2010) Immunospecific responses to bacterial elongation factor Tu during Burkholderia infection and immunization. *PLos One* **5,** p.e14361
20. Oliveira, S. C., and Splitter, G. A. (1996) Immunization of mice with recombinant L7/L12 ribosomal protein confers protection against Brucella abortus infection. *Vaccine* **14,** 959–962
21. Yang, X., Walters, N., Robison, A., Trunkle, T., and Pascual, D. W. (2007) Nasal immunization with recombinant Brucella melitensis bp26 and trigger factor with cholera toxin reduces B. melitensis colonization. *Vaccine* **25,** 2261–2268
22. Ji, Y., Carlson, B., Kondagunta, A., and Cleary, P. P. (1997) Intranasal immunization with C5a peptidase prevents nasopharyngeal colonization of mice by the group A Streptococcus. *Infect. Immun.* **65,** 2080–2087
23. Shet, A., K. E., Johnson, D., and Cleary, P. P. (2004) Human immunogenicity studies on group A streptococcal C5a peptidase (SCPA) as a potential vaccine against group A streptococcal infections. *Indian J. Med. Res.* **119,**
24. Edwards, R. J., Taylor, Graham W., Ferguson, M., Murray, S., Rendell, N., Wrigley, A., Bai, Z., Boyle, J., Finney, Simon J., Jones, A., Russell, Hugh H., Turner, C., Cohen, J., Faulkner, L., and Sriskandan, S. (2005) Specific C-terminal cleavage and inactivation of interleukin-8 by invasive disease isolates of Streptococcus pyogenes. *J. Inf. Dis.* **192,** 783–790
25. Zinkernagel, A. S., Timmer, A. M., Pence, M. A., Locke, J. B., Buchanan, J. T., Turner, C. E., Mishalian, I., Sriskandan, S., Hanski, E., and Nizet, V. (2008) The IL-8 protease SpyCEP/ScpC of group A streptococcus promotes resistance to neutrophil killing. *Cell Host Microbe* **4,** 170–178
26. Mora, M., Veggi, D., Santini, L., Pizza, M., and Rappuoli, R. (2003) Reverse vaccinology. *Drug Discovery Today* **8,** 459–464
27. Meinke, A., Henics, T., Hanner, M., Minh, D. B., and Nagy, E. (2005) Antigenome technology: a novel approach for the selection of bacterial vaccine candidate antigens. *Vaccine* **23,** 2035–2041
28. Fritzer, A., Senn, B. M., Minh, D. B., Hanner, M., Gelbmann, D., Noiges, B., Henics, T., Schulze, K., Guzman, C. A., Goodacre, J., von Gabain, A., Nagy, E., and Meinke, A. L. (2010) Novel conserved group A streptococcal proteins identified by the antigenome technology as vaccine candidates for a non-M protein-based vaccine. *Infect. Immun.* **78,** 4051–4067
29. Grandi, G. (2005) Genomics and proteomics in reverse vaccines. *Microbial Proteomics*, pp. 379–393, John Wiley & Sons, Inc.
30. McNeil, S. A., Halperin, S. A., Langley, J. M., Smith, B., Warren, A., Sharratt, G. P., Baxendale, D. M., Reddish, M. A., Hu, M. C., Stroop, S. D., Linden, J., Fries, L. F., Vink, P. E., and Dale, J. B. (2005) Safety and immunogenicity of 26-valent group A streptococcus vaccine in healthy adult volunteers. *Clin. Inf. Dis.* **41,** 1114–1122
31. Lancefield, R. C. (1957) Differentiation of group A streptococci with a common R antigen into three serological types, with special reference to the bactericidal test. *J. Exp. Med.* **106,** 525–544
32. Lancefield, R. C. (1959) Persistence of type-specific antibodies in man following infection with group A streptococci. *J. Exp. Med.* **110,** 271–292
33. Lancefield, R. C. (1962) Current knowledge of type-specific M antigens of group A streptococci. *J. Immunol.* **89,** 307–313
34. Sumby, P., Zhang, S., Whitney, A. R., Falugi, F., Grandi, G., Graviss, E. A., DeLeo, F. R., and Musser, J. M. (2008) A chemokine-degrading extracellular protease made by group A streptococcus alters pathogenesis by enhancing evasion of the innate immune response. *Infect. Immun.* **76,** 978–985
35. Alouf, J. E. (1980) Streptococcal toxins (streptolysin O, streptolysin S, erythrogenic toxin). *Pharmac. Ther.* **11,** 661–717