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TITOLO TESI

IDENTIFICATION OF SURFACE PROTEIN COMPLEXES OF *STREPTOCOCCUS PYOGENES* THROUGH PROTEIN MICROARRAY TECHNOLOGY

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Abstract

A systematic characterization of the composition and structure of the bacterial cell-surface proteome and its complexes can provide an invaluable tool for its comprehensive understanding.

The knowledge of protein complexes composition and structure could offer new, more effective targets for a more specific and consequently effective immune response against a complex instead of a single protein.

Large-scale protein-protein interaction screens are the first step towards the identification of complexes and their attribution to specific pathways. Currently, several methods exist for identifying protein interactions and protein microarrays provide the most appealing alternative to existing techniques for a high throughput screening of protein-protein interactions in vitro under reasonably straightforward conditions.

In this study approximately 100 proteins of Group A Streptococcus (GAS) predicted to be secreted or surface exposed by genomic and proteomic approaches were purified in a His-tagged form and used to generate protein microarrays on nitrocellulose-coated slides. To identify protein-protein interactions each purified protein was then labeled with biotin, hybridized to the microarray and interactions were detected with Cy3-labelled streptavidin. Only reciprocal interactions, i. e. binding of the same two interactors irrespective of which of the two partners is in solid-phase or in solution, were taken as bona fide protein-protein interactions. Using this approach, we have identified 20 interactors of one of the potent toxins secreted by GAS and known as superantigens. Several of these interactors belong to the molecular chaperone or protein folding catalyst families and presumably are involved in the secretion and folding of the superantigen. In addition, a very interesting interaction was found between the superantigen and the substrate binding subunit of a well characterized ABC transporter. This finding opens a new perspective on the current understanding of how superantigens are modified by the bacterial cell in order to become major players in causing disease.

1. Introduction

1.1 *Streptococcus pyogenes*

Group A streptococcus (GAS) or *Streptococcus pyogenes* is a Gram-positive, non-motile, non-spore forming coccus. It divides in one plane and this it forms pairs of cells or (especially in liquid media) chains of various length (Figure 1.1). The bacterial cell is mostly round to ovoid, about 0.6 to 1.0 micrometer in diameter. *S. pyogenes* is a catalase-negative aero-tolerant anaerobe (facultative anaerobe) organism with fermentative metabolism and requires enriched medium containing blood in order to grow. Group A streptococci typically have a capsule composed of hyaluronic acid and exhibit beta (clear) hemolysis on blood agar (Wannamaker 1970).

GAS is a common pathogen of humans, present usually in the respiratory tract in 5 to 15% of normal individuals without signs of disease. As part of the normal flora, if defenses are compromised or when the organisms are able to penetrate the constitutive defenses *S. pyogenes* can generate a variety of suppurative infections.

GAS has been the main cause of puerperal fever (sepsis after childbirth) and scarlet fever in the last century, but now, due to antibiotic treatment, is mostly known for causing streptococcal pharyngitis whereas severe complications such as erysipelas (a form of cellulitis accompanied by fever and systemic toxicity) have become less common. Recently however the severity and sequelae of GAS infections have increased and severe invasive streptococcal infections lead to the definition of *S. pyogenes* as the "flesh eating bacteria" in the news media (Wannamaker 1970).

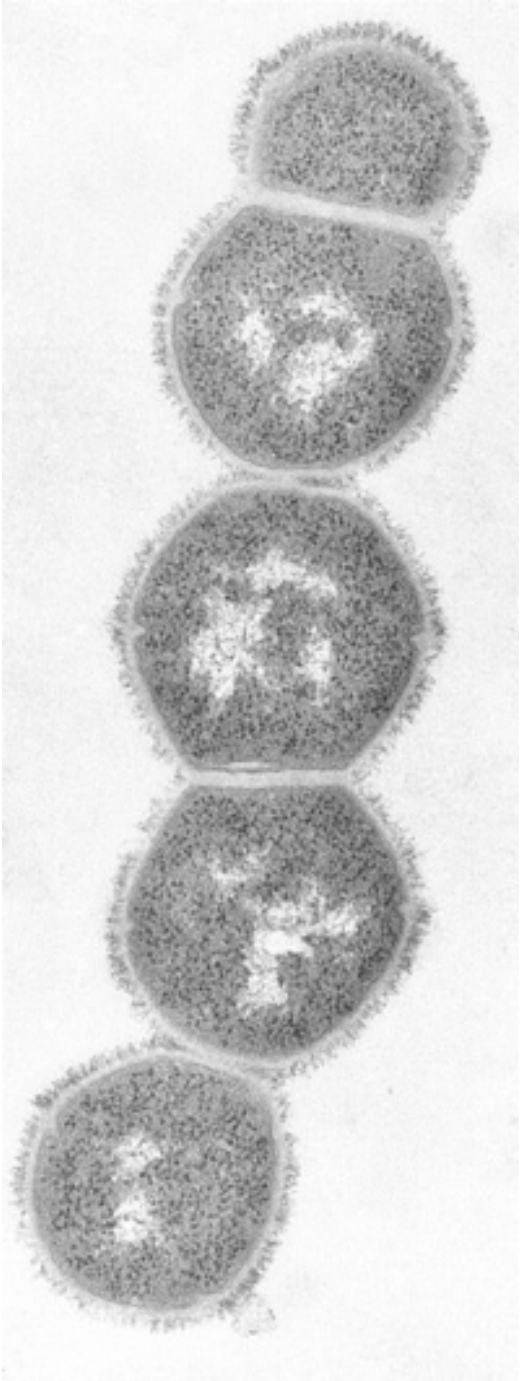


Figure 1.1. Electron micrograph of an ultra-thin section of a chain of group A streptococci (20,000X). The cell surface fibrils, consisting primarily of M protein, are clearly evident. The bacterial cell wall, to which the fibrils are attached. Incipient cell division is also indicated by the nascent septum formation near the cell equator. The streptococcal cell diameter is equal to approximately one micron.
Electron micrograph of *Streptococcus pyogenes* by Maria Fazio and Vincent A. Fischetti, Ph.D., Laboratory of Bacterial Pathogenesis and Immunology, Rockefeller University.

Today GAS remains a major worldwide health concern in cases involving rapidly progressive disease and serious sequelae of untreated infections and efforts are directed toward a better characterization of infection mechanisms and identifying rheumatogenic and nephritogenic strains of streptococci.

Today's acute GAS infections are mainly pharyngitis (strep throat), scarlet fever (rash), impetigo (superficial skin layers infection) or cellulitis (deep skin layers infection) but, when the pathogen becomes invasive, sequelae can result in necrotizing fasciitis, myositis and streptococcal toxic shock syndrome. Following acute infections also immune-mediated sequelae may occur such as acute rheumatic fever and acute glomerulonephritis.

GAS produces in a very large number of diseases and a wide array of virulence factors including: (1) M protein, fibronectin-binding protein (Protein F) and lipoteichoic acid for adherence; (2) hyaluronic acid capsule as an immunological disguise and to inhibit

phagocytosis; M-protein to inhibit phagocytosis (3) invasins such as streptokinase, streptodornase (DNAse B), hyaluronidase, and streptolysins; (4) exotoxins, such as pyrogenic (erythrogenic) toxin which causes the rash of scarlet fever and systemic toxic shock syndrome.

1.1.2 Classification of Streptococci

Streptococci have long been classified by hemolytic reaction type displayed on blood agar: alpha-hemolysis is a partial hemolysis resulting in reduction of red cell hemoglobin, whereas beta-hemolysis results in complete red cell lysis in the colony surroundings (Figure 1.2). Non hemolytic type is defined gamma-hemolytic. Group A streptococci are mostly beta-hemolytic whereas for example Group B streptococci can generate alpha, beta or gamma hemolysis. Most of the oral streptococci are non hemolytic making hemolysis not a very reliable tool for absolute identification, but it is still widely used for rapid screens for identification of *S. pyogenes*.

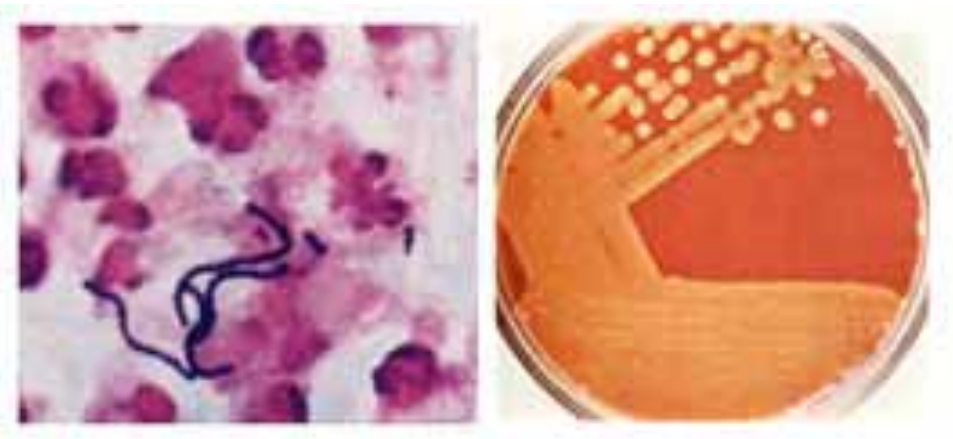


Figure 1.2. *Streptococcus pyogenes*. **Left**: Gram stain of *S. pyogenes* in a clinical specimen. **Right**: Colonies of *S. pyogenes* on blood agar exhibiting beta (clear) hemolysis (Todar 2008).

Historically, the definitive identification of streptococci has rested on the serologic reactivity of "cell wall" polysaccharide antigens as originally described by Rebecca Lancefield (Lancefield 1933). Eighteen group-specific antigens (Lancefield groups) were established. The Group A polysaccharide is a polymer of N-acetylglucosamine and rhamnose. Some group antigens are shared by more than one species. This polysaccharide is also called the C substance or group carbohydrate antigen.

1.1.3 Pathogenesis

Streptococcus pyogenes successfully colonizes and rapidly multiplies, spreading while evading phagocytosis and confusing the immune system. The respiratory tract, bloodstream, or the skin are targets for acute diseases but Streptococcal disease is most often a respiratory (pharyngitis or tonsillitis) or skin (pyoderma) infection (Bisno and Stevens 1996).

S. pyogenes remains the leading cause of bacterial pharyngitis and tonsillitis (strep throat) causing also other infections such as otitis, sinusitis, and pneumonia and skin infections: noninvasive (impetigo) or invasive (cellulitis). Deep GAS infections may reach joint or bone infections and cause also necrotizing fasciitis, myositis, meningitis and endocarditis. Rheumatic fever and glomerulonephritis, two post streptococcal sequelae, occur in 1-3% of untreated infections following streptococcal disease (Bisno 1991).

These pathologies are caused by immunological reactions to GAS antigens where circulating streptococcal toxins trigger systemic responses that result in scarlet fever and streptococcal toxic shock syndrome. Many GAS virulence factors are present on the

bacterial cell surface, and of particular interest are those involved in colonization and evasion of the host immune responses (Kaplan 1991). Group A Streptococcus has a highly complex and diverse cell surface, rich of antigenic components such as the capsule polysaccharide (C-substance), the cell wall peptidoglycan and many surface proteins including the M protein, fimbrial and fibronectin-binding proteins. A few antigens mimic molecularly human tissue of the skeleton, smooth muscle, heart and nervous system to avoid host immune response causing autoimmune sequelae.

In Group A streptococci, also the carbohydrate group (N-acetylglucosamine and rhamnose) wasn't thought to be antigenic, but emerging highly invasive strains present mucoid colonies. This suggests a role of the capsule in virulence.

1.1.4 GAS surface

M Protein

Major virulence factor involved in colonization and phagocytosis resistance is the M proteins class. This protein class is the primary cause of the high antigenic variability of GAS resulting in more than 100 serotypes of Group A Streptococcus identified based on M proteins antigenic specificity.

The M proteins are able to bind serum fibrinogen allowing the bacteria to resist phagocytosis and contain those particular epitopes that mimic human tissues some of these are the cause of rheumatic fever, leading to an autoimmune carditis.

The Capsule

Even though non antigenic, the capsule of *S. pyogenes*, because of its hyaluronic acid composition (chemically similar to host connective tissue), allows the bacterium to hide from the host immune system preventing neutrophils and macrophages phagocytosis.

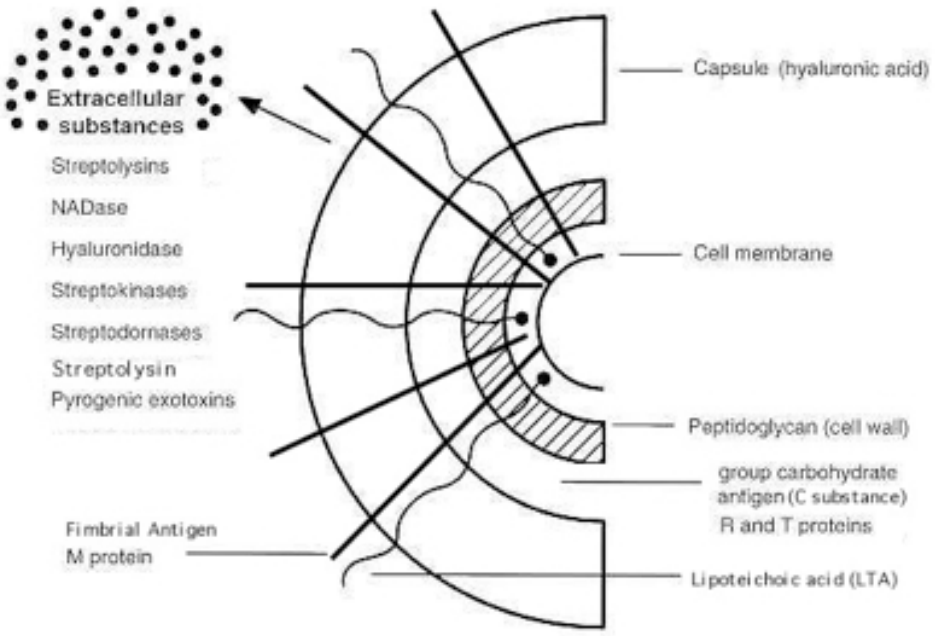


Figure 1.3. Cell surface structure of *Streptococcus pyogenes* and secreted products involved in virulence.

Adhesins

Group A Streptococcus is known (as other pathogens) to produce adhesins with different specificities. GAS adhesins identification have long been subject of conflict and debate but it is believed that they participate in bacterial adherence to host epithelial cells. M protein itself is part of this group (Caparon, Stephens et al. 1991) as well as some fibronectin-binding proteins (that mediate streptococcal adherence to the amino

terminus of fibronectin on mucosal surfaces) and lipoteichoic acids (LTA) anchored to proteins on the bacterial surface (including the M protein) (Hasty, Ofek et al. 1992).

Invasins and Exotoxins

During colonization of the upper respiratory tract and acute pharyngitis GAS may spread to other portions of the upper or lower respiratory tracts resulting in infections of the middle ear (otitis), sinuses (sinusitis) or lungs (pneumonia). If untreated, the infection may extends to the meninges (meningitis) as well as bones (osteomyelitis) or joints (arthritis). During these aspects of acute disease streptococci express a variety of secretory proteins that mediate the invasion, interacting with host blood and tissue components, killing cells and inducing a damaging inflammatory response.

Among the major proteins involved in this phase such as GAS toxins and a part of the soluble extracellular growth products, there are Streptolysin S, an oxygen-stable leukocidin; Streptolysin O, an oxygen-labile leukocidin; NADase, leukotoxic; Hyaluronidase, known as "the spreading factor", which can digest hyaluronic acid of connective tissue; Streptokinases, that participate in fibrin lysis; Streptodornases A-D, characterized by deoxyribonuclease activity and Streptodornases B and D, with a ribonuclease activity.

This large repertoire of products is important in the pathogenesis of *S. pyogenes* infections. Streptococcal invasins act in a variety of ways lysing red blood cells, phagocytes and other host macromolecules as they allow the bacteria to spread among tissues. Invasins are key proteins in GAS pathogenesis and the effects of antibodies against these proteins resulted in relatively insignificant host protection.

1.1.5 Streptococcal Superantigens: Pyrogenic Exotoxins

An interesting role in this study is played by a particular type of toxin belonging to the superantigen (SAgs) class. During the late 1980s some unexpected immunological properties were discovered and attributed to the highly mitogenic staphylococcal enterotoxins (SEs) on T lymphocytes: the triggering of lymphocyte proliferation by SEs requires selective and simultaneous binding of these molecules by both major histocompatibility complex (MHC) class II molecules at the surface of accessory cells and T cell receptors (TcR) on target lymphocytes. This confers SEs the properties of functionally bivalent molecules by cross-linking TcR and MHC class II molecules in a unique manner. Dependence on class II molecules was not due to an immunological "recognition" of SEs (as is commonly known for conventional antigens) since they stimulate T cells by binding class II MHC molecules directly and nonspecifically (Figure 1.4) so that about 20% of T cells may be stimulated (whereas 1 every 10.000 T cells are stimulated by conventional antigens) resulting in massive cytokine release.

To date the constantly expanding literature describes at least 40 bacterial SAgs produced by Gram-positive bacteria like Staphylococcus aureus, *Streptococcus pyogenes* (and other species), as well as those produced by Gram-negative bacteria such as *Yersinia pseudotuberculosis* and the wall-less *Mycoplasma arthritidis*.

The repertoire of *S. pyogenes* SAgs includes 11 structurally and functionally related single-chain proteins with molecular weights ranging from about 23 to 27 kDa (Alouf and Muller-Alouf 2003; Petersson, Forsberg et al. 2004). Most of these proteins have been purified to homogeneity including the classical erythrogenic (scarlet fever) toxins A and C, also designated streptococcal pyrogenic exotoxins A and C (SPE A, SPE C) identified

in 1924 and 1960 respectively(Alouf 1980; Norgren 1997; Alouf 1999; Alouf and Muller-Alouf 2003). SPE A (25,787 Da) and SPE C (24,354 Da) are respectively encoded by bacteriophage genes *speA* and *speC*; and a series of recently discovered mitogenic exoproteins, namely streptococcal superantigen (SSA) (Mollick, Miller et al. 1993), SMEZ (streptococcal mitogenic exotoxin Z) isolated by Kamezawa and colleagues (Kamezawa, Nakahara et al. 1997), SPE G, SPE H, SPE I, SPE J (Proft, Moffatt et al. 1999; Proft, Moffatt et al. 2000; McCormick, Pragman et al. 2001), SPE L, and SPE M (Smoot, McCormick et al. 2002; Proft 2003).

The occurrence of some of these exotoxins discovered over the past twelve years was initially inferred by genome mapping of *S. pyogenes* serotypes M1, M3, and M18 (Ferretti, McShan et al. 2001; Beres, Sylva et al. 2002; Smoot, Barbian et al. 2002). The respective genes were further transcribed and the superantigenicity, as well as lethality of corresponding SAg proteins, established experimentally (Smoot, McCormick et al. 2002). Serologic studies showed the presence of antibodies against these SAgs in patients (Proft 2003). All known streptococcal SAgs with the exception of SMEZ, SPE G, and SPE J are encoded by bacteriophage genes integrated into the bacterial chromosome (Ferretti, McShan et al. 2001; Proft, Sriskandan et al. 2003). Four naturally occurring *speA* alleles have been found in strains recovered from patients with severe invasive diseases.

Determination of the nucleotide sequences from the eleven streptococcal SAgs identified to date has revealed various degrees of structural relatedness at both the amino acid sequence and three-dimensional levels. These SAgs share important structural homologies with various *S. aureus* enterotoxin SAgs. Interestingly, certain streptococcal SAgs are more similar to some staphylococcal SAgs than to other streptococcal SAgs. The genomic and structural relatedness suggests that these SAgs share a common ancestor (Arcus, Proft et

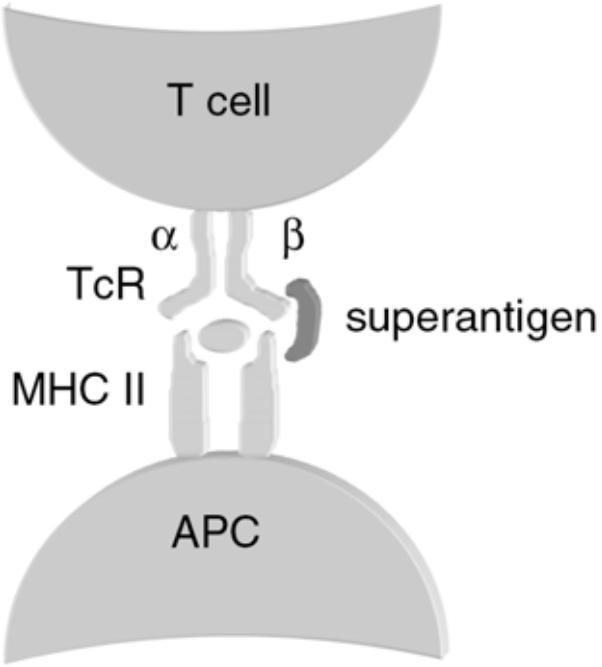


Figure 1.4. Representation of T cell activation by a conventional peptide antigen (Ag) or by a superantigenic toxin (SAg). **TcR:** T cell receptor; **APC:** antigen-presenting cell; **MHC II:** major histocompatibility class II complex molecule (Alouf 1999).

al. 2000; Sundberg, Li et al. 2002). Other streptococcal superantigens, SPE M, SSA, and SME Z have been identified from invasive strains of group C and G *Streptococcus dysgalactiae*.

The molecular structure, the genetic aspects and the interaction of these fascinating molecules with the immune system remains their most interesting aspects. The key role of SAgs in the pathogenesis of acute, chronic, and some autoimmune diseases has offered new insights into elucidating patho-physiological effects of these molecules. Many questions remain unanswered, but new achievements will certainly emerge in the coming years, such as the design and use of novel therapeutical strategies (drugs and vaccines) in the management of SAgs-induced diseases.

1.2 *S. Pyogenes* secretome: The ExPortal

Recent studies (Buist, Ridder et al. 2006) on secretions system types in Gram-positive bacteria describe how they may employ unique strategies to ensure proper folding of the prodigious quantities of proteins these organisms typically secrete. In the past two years three papers appeared that demonstrated the localization of components of the Sec pathway in the pathogen *Streptococcus pyogenes.* Immunogold electron microscopy analysis of thin sections of *S. pyogenes* showed that a microdomain with a high concentration of Sec translocons called the ExPortal is present in this coccoid bacterium (Rosch and Caparon 2004). In particular, for example, the secreted cysteine protease SpeB, its maturation protein HtrA and the heterologous alkaline phosphatase PhoZ all co-localize with SecA in this domain (Rosch and Caparon 2004; Rosch and Caparon 2005).

The ExPortal (Figure 1.5) is proposed to function as an organelle that promotes biogenesis of secreted proteins by coordinating interactions between nascent unfolded secretory proteins and membrane-associated chaperones.

The surface localization of the covalently cell-wall-bound M protein of *S. pyogenes* and the way it fits in this mechanism remains to be explained. Although a single microdomain for protein secretion was identified in this bacterium, the M protein is present on the whole cell surface. How the distribution of this protein is directed remains to be elucidated.

These findings are confirmed by a recent localization study of Sortase A in *S. pyogenes* (Raz and Fischetti 2008). Cell wall peptidoglycan-anchored surface proteins are essential virulence factors in many gram-positive bacteria. The attachment of these proteins to the

peptidoglycan is achieved through a transpeptidation reaction, whereby sortase cleaves a conserved C-terminal LPXTG motif and covalently attaches the protein to the peptidoglycan precursor lipid II.

The LPXTG sequence presenting proteins are of particular interest as potential vaccine candidates for their localization on the outer membrane and consequently they are part of this study on surface protein complexes.

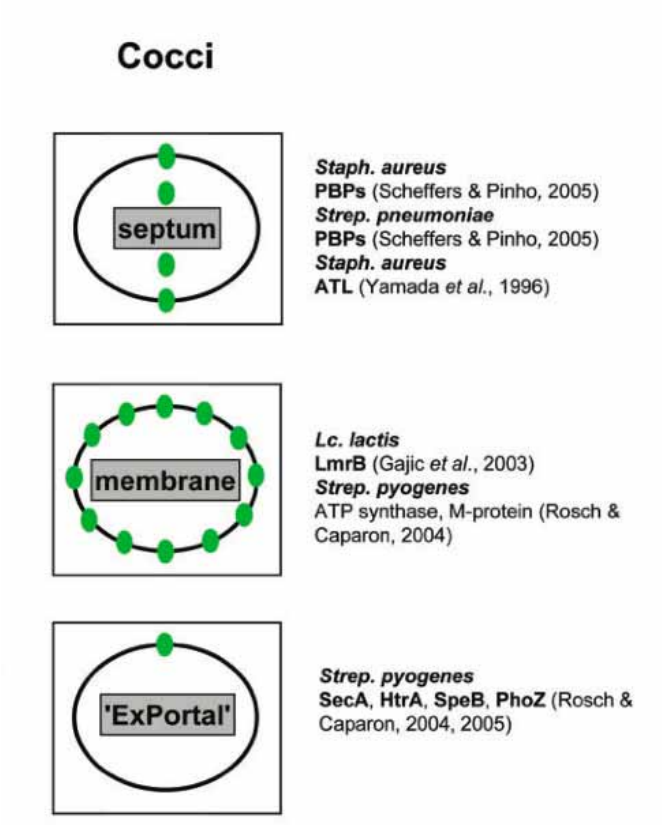


Figure 1.5. Graphical representation of the protein localization patterns in different bacteria and especially in *S. Pyogenes* (Buist, Ridder et al. 2006).

1.3 Vaccine against Group A Streptococcus

In the past decades many genomes of several microorganisms have been sequenced and sequence analysis improved considerably developing invaluable tools for pharmaceutical research. *In silico* prediction of structure and function of proteins starting from the genome sequence has become more accurate changing the approach for drug discovery and especially the development of a vaccine.

1.3.1 Reverse Vaccinology

In 2000 Rappuoli and colleagues published the Reverse Vaccinology approach for vaccine development using recombinant proteins (Rappuoli 2000). Until then the study for a new vaccine started from the microorganism and its exposed factors focusing towards identifying the DNA and its characterization. Reverse vaccinology first step is "the genome" (Masignani, Rappuoli et al. 2002). Looking into the gene sequences it's possible to identify all the pathogen proteins and among them the potential antigenic candidates starting with those whose sequence hint surface exposure or secretion (Rappuoli and Covacci 2003). This process is not dependent on single proteins properties or the pathogen characteristics (Mora, Veggi et al. 2003).

Conventional vaccines are obtained from attenuated live pathogens, inactivated ones or subunits able to confer protection. Reverse vaccinology screening for protective antigens that started *in silico* is continued both *in vitro* and *in vivo.* Once identified, genes coding for pathogen proteins are cloned in *E. coli*, expressed and purified. Purified proteins are

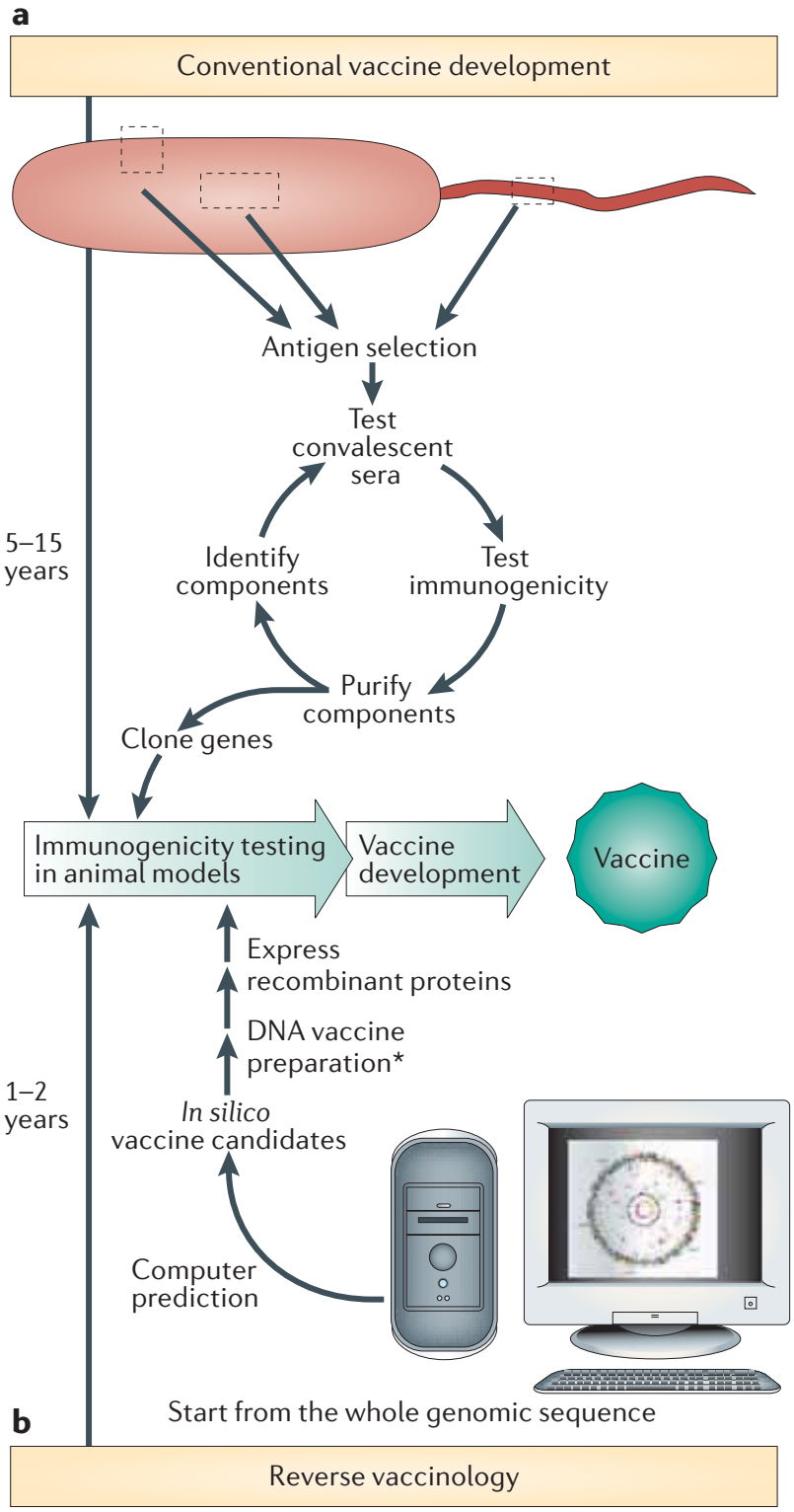


Figure 1.6. Approaches to vaccine development. Schematic demonstration of the essential steps required for vaccine development using the conventional approach (**a**), and reverse vaccinology (**b**) (Johri, Paoletti et al. 2006).

used to immunize mice and obtain sera, that once tested, are used to verify the predicted exposure and the ability to trigger an immune response. Protective antigens are analyzed again *in vitro* with opsonophagocytosis and bactericidal assays focused towards final antigen selection. Interesting candidates are also analyzed for sequence conservation among different pathogen strains.

Reverse Vaccinology, schematized in figure 1.6, is able to succeed where traditional methodology fails (e.g. non-culturable or highly infective pathogens) and also to improve existing products.

1.3.2 The Novartis Approach

Reverse Vaccinology pointed out how antigens that confers a broad range of protective antibody responses are highly expressed surface exposed components, usually secreted toxins or virulence factors well conserved among the pathogen strains. The search for protective antigen can last, however, even years: time is the main limitation of this methodology and the biological assay for candidates identification is a huge bottle-neck. For Group A Streptococcus vaccine study, Reverse Vaccinology has been integrated with proteomic analysis of GAS cell surface and both DNA and protein microarray screening of GAS-infected patient sera.

The proteomic approach was used to identify bacterial surface-exposed proteins for their use as vaccine candidates. To achieve this whole cells are treated with proteases to selectively digest protruding proteins that are subsequently identified by mass spectrometry analysis of the released peptides. Applying this approach to GAS

M1_SF370 strain, resulted in 68 PSORT-predicted surface-associated proteins identified on its surface (Figure 1.7), including most of the protective antigens described in the literature. Different capsule content influence the number of identified surface-exposed proteins which varies from strain to strain. This strategy overcomes the difficulties so far encountered in surface protein characterization and has great potential in vaccine discovery (Musser 2006).

As part of the high throughput approach for vaccine studies, both DNA and protein Microarray technology have been used while screening for protective antigens. Among the other applications of this technology such as comparative genomic hybridization or detection of SNPs, DNA microarray are used for gene expression profiling, revealing the

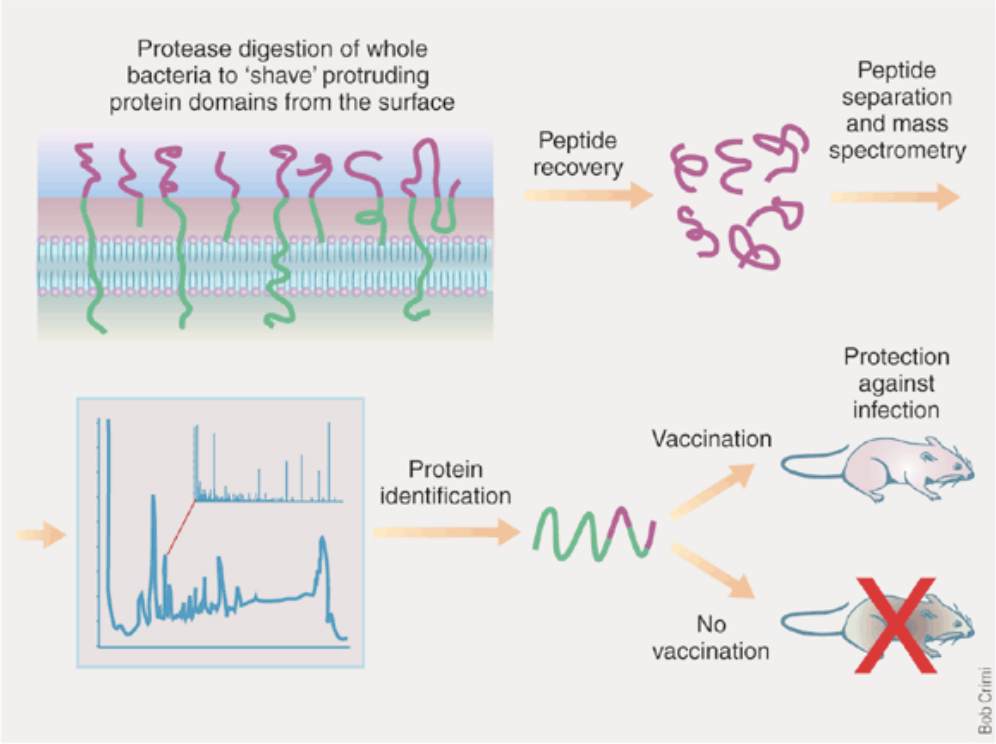


Figure 1.7. Strategy used to identify a new mouse-protective group A Streptococcus antigen. Bacteria are treated with trypsin to release surface-exposed peptides, which are subsequently identified by mass spectrometry. Genes encoding these proteins are cloned, and the overexpressed proteins are purified and used to immunize mice. The immunized animals are challenged with virulent group A Streptococcus to test for protection (Musser 2006).

most expressed genes. This technique requires the whole genome to be spotted on a microarray chip (usually glass or silicone) of DNA oligonucleotides, each containing picomoles of a specific DNA sequence such as a short section of a gene or other DNA element that are used to hybridize fluorophore-labeled mRNA probes under high-stringency conditions. Probe-target hybridization is detected and fluorescence is quantified to determine relative abundance of nucleic acid sequences.

In the study of a vaccine against GAS, microarray technology showed valuable screening potentials. Microarrays weren't only implemented for gene profiling on DNA chips, but also for identification of most immunogenic antigens, profiling of disease-specific antibody response and, what this work focuses on, for the analysis of protein-protein interactions to identify surface protein complexes. Several protein chips were spotted with pools of proteins both predicted by computational analysis to be exposed or secreted and verified by FACS and surfome analysis (Rodriguez-Ortega, Norais et al. 2006). These proteins were also screened against sera of patient infected with Group A Streptococcus. Similarly to DNA microarray but with human antibodies as probes several hybridization experiment were performed and binding detected with fluorophore labeled secondary antibodies.

These data, combined with surfome analysis and Reverse Vaccinology approach narrow the potential candidate to an even smaller array of antigens that undergo high throughput expression and purification and are used *in vitro* and *in vivo* assays to select the final vaccine candidates.

1.4 Protein Microarrays

Microarray technology allows the simultaneous analysis of many parameters within a single experiment (Figure 1.8). Molecules are immobilized in a grid of micro-spots onto a solid support and exposed to samples containing the corresponding binding molecules. Readout systems based on different techniques such as fluorescence, chemiluminescence or radioactivity can be used to detect complex formation within each spot. Recent

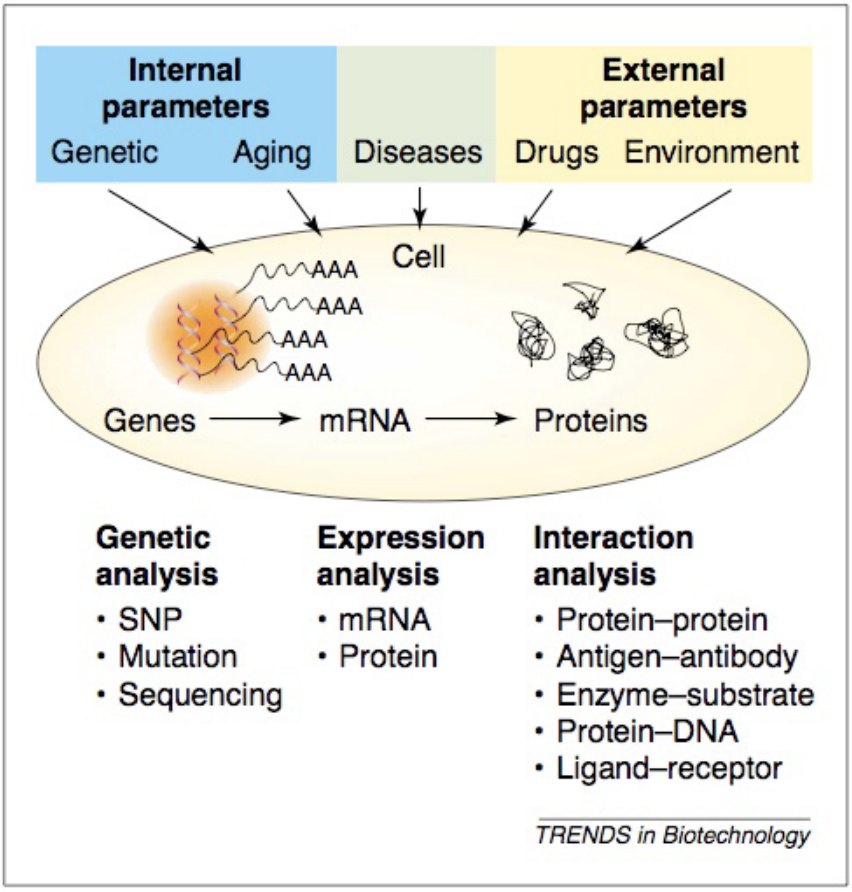


Figure 1.8. Microarrays for genomics and proteomics. The physiological state of a cell is influenced by external and internal parameters. Microarray technology can be applied to monitor intracellular gene and protein expression mechanisms. DNA microarrays are used for genetic analysis as well as expression analysis at the mRNA level. Protein microarrays are used for expression analysis at the protein level and in the expansive field of interaction analysis (Templin, Stoll et al. 2002).

developments in the field of protein microarrays show applications for enzyme–substrate, DNA–protein and different types of protein–protein interactions (Templin, Stoll et al. 2002).

The fundamental principles of miniaturized micro-spot ligand-binding assays were described more than a decade ago. In the "ambient analyte theory" Roger Ekins and coworkers (Ekins 1989; Ekins, Chu et al. 1990; Ekins and Chu 1992) explained why microspot assays are more sensitive than any other ligand-binding assay. At that time, the high sensitivity and enormous potential of micro-spot technology had already been demonstrated using miniaturized immunological assay systems. The possibility of determining thousands of different binding events in one reaction in a parallel fashion perfectly suited the needs of genomic approaches in biology. The rapid progress in whole-genome sequencing (Lander, Linton et al. 2001; Venter, Adams et al. 2001) and the increasing importance of expression studies was matched with efficient *in vitro* techniques for synthesizing specific capture molecules for ligand-binding assays. New trends in technology, mainly in microtechnology and microfluidics, newly established detection systems and improvements in computer technology and bioinformatics were rapidly integrated into the development of microarray-based assay systems. Now, microarrays, built from tens of thousands of different probes per square centimeter, are well-established high-throughput hybridization systems that generate huge sets of data within a single experiment.

In principle, any type of ligand-binding assay that relies on the product formation of an immobilized capture molecule and a target (binder or analyte) present in the surrounding solution can be miniaturized, parallelized and performed in a microarray format (Figure 1.9). Microarray-based assays using nucleic acid–nucleic acid interactions (DNA chips)

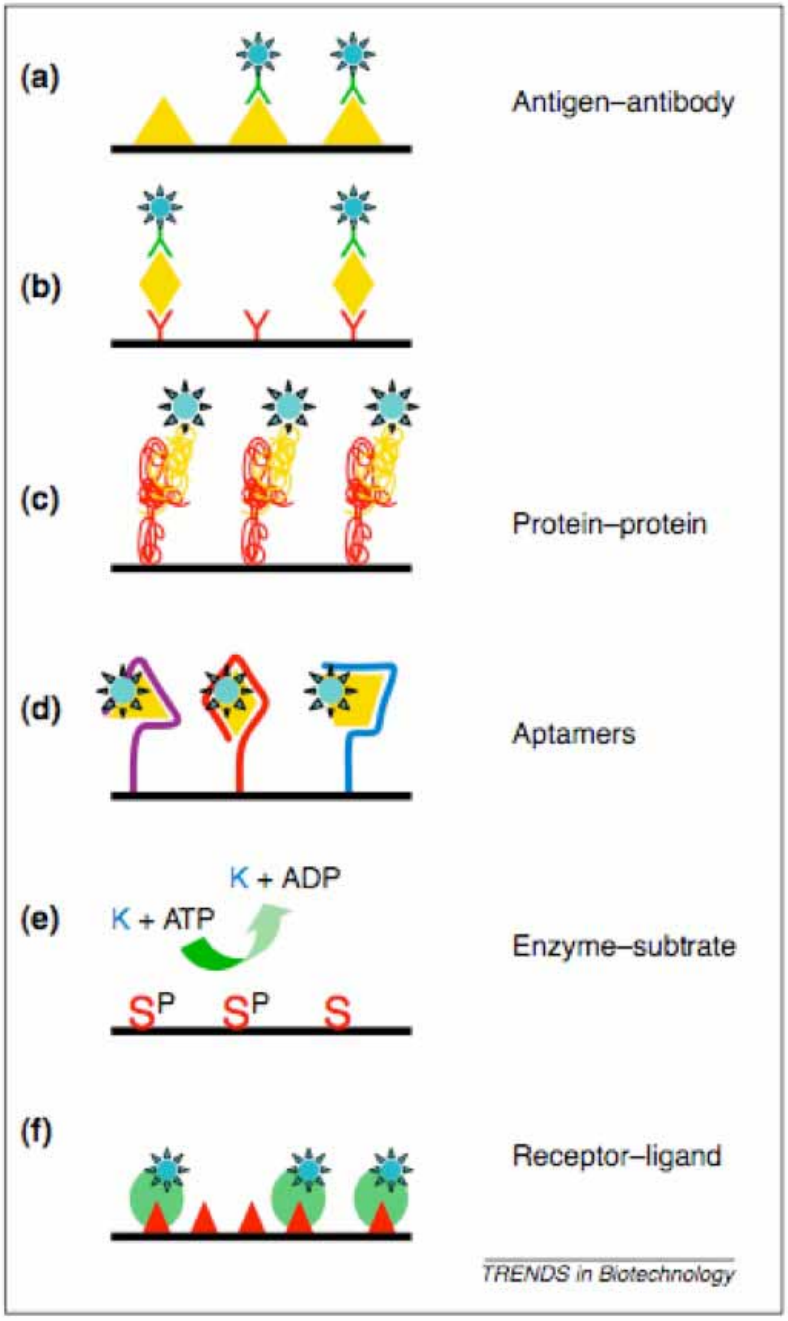


Figure 1.9. Classes of capture molecules for protein microarrays. For specific interaction analysis, different classes of molecules can be immobilized on a planar surface to act as capture molecules in a microarray assay. (**a**)Illustrates antigen–antibody interaction and (**b**) shows a scheme of a Sandwich immunoassay. In (**c**), a specific protein–protein interaction is shown. A different class of binders is shown in (**d**), where synthetic molecules referred to as aptamers act as capture molecules. They can be composed of nucleotides, ribonucleotides or peptides. Interactions of enzymes with their specific substrates are shown in (**e**), where a substrate (**S**) for kinases is immobilized and phosphorylated (**P**) by the respective kinase. A typical example for a receptor–ligand interaction is given in (**f**), where synthetic low molecular mass compounds are immobilized as capture molecules (Templin, Stoll et al. 2002).

are well established and protein microarray assays are just becoming popular. Studies on DNA–protein interactions in a microarray format were performed by Bulyk and colleagues (Bulyk, Gentalen et al. 1999) who created microarrays of double-stranded oligonucleotides. High-density microarrays of single-stranded oligonucleotides were produced using Affymetrix (Santa Clara, CA, USA) technology. In general, DNA–protein interaction assays could be useful for the characterization and identification of DNA-binding proteins, such as transcription factors. Enzyme–substrate arrays have been described for different kinds of enzymes, such as restriction enzymes, peroxidase, phosphatase and protein kinases (Bulyk, Gentalen et al. 1999; Arenkov, Kukhtin et al. 2000; MacBeath and Schreiber 2000; Zhu, Klemic et al. 2000). In a proof of concept experiment, MacBeath and Schreiber (MacBeath and Schreiber 2000) immobilized three different kinase substrates, each specific for an individual kinase, onto a planar glass surface. Using this approach, novel activities of individual kinases were identified. Sequence comparison of enzymes that could phosphorylate tyrosine residues revealed that they often share common amino acid residues around their catalytic region. For receptor–ligand assays, small organic molecules produced by combinatorial solid phase chemistry were immobilized in a microarray format. These microarrays produced by so-called small-molecule printing technology were incubated with fluorescently labeled target proteins to identify new ligands (MacBeath and Schreiber 2000). This technology enables parallel high-throughput screening for ligand–receptor interactions at very low sample consumption, which could improve screening for active substances in the pharmaceutical industry.

In the field of protein–protein interaction assays, dot-blot filter arrays were used to screen for specific interactions of immobilized proteins with other proteins (Figure 1.10).

Specific protein–protein interactions were detected between a radioactively labeled human p52 GST fusion protein and immobilized capture proteins such as nucleoline or a

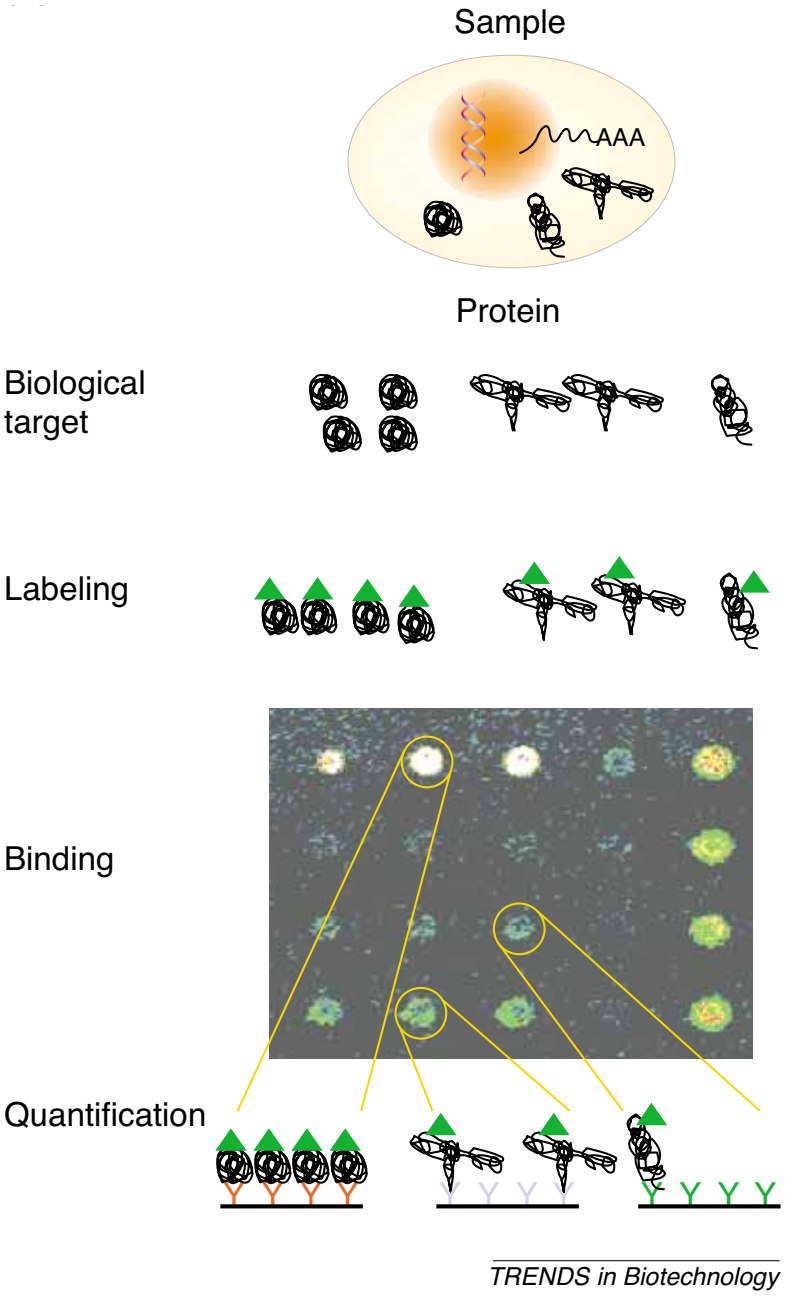


Figure 1.10. Protein Microarray. Binding of different proteins can be detected with a microarray-based assay. Specific capture proteins or antibodies immobilized in an array interact with their respective target proteins present in the solution and then labeled with fluorochromes. The resulting signal intensity correlates with the amount of captured target. Within each microarray, different kinds of control spots can be included, such as positive and negative control spots and/or internal calibration spots. This will allow accurate signal quantification (Templin, Stoll et al. 2002).

serine–arginine protein fraction isolated from HeLa cells (Ge 2000). In addition, interactions of DNA, RNA, or low molecular weight ligands with the immobilized molecules were shown. Such arrays could be further miniaturized and therefore have the potential to be performed in a microarray format.

Zhu et al. (Zhu, Bilgin et al. 2001) demonstrated the extraordinary power of array-based methods for proteomic approaches. After purification of 5800 different recombinant proteins from *S. cerevisiae*, the authors generated complex proteome chips that contained gene-products from >90% of the genes of the organism. These microarrays could be used to study protein–protein interactions on a genome-wide scale. Using calmodulin as a model protein to probe the arrays, many known interactions could be confirmed and a set of novel binding proteins was detected. Inspection of the sequences of these proteins revealed the presence a binding motif and therefore strongly argues for the significance of the observed binding interaction. Experiments designed to detect protein–lipid interactions showed that the identification of proteins able to bind low molecular weight compounds is possible. This opens the possibility to examine an entire proteome directly for protein–drug interactions.

Microarray immunoassays are of general interest also for all diagnostic applications where several parameters of one sample have to be analyzed in parallel (Mendoza, McQuary et al. 1999; Joos, Schrenk et al. 2000; Schweitzer, Wiltshire et al. 2000).

Accurate quantification with protein microarrays can be achieved by including positive and negative control spots and/or internal calibration spots. This will finally lead to robust and reliable diagnostic assays.

Protein microarray technology is already a useful tool to study different kinds of protein interactions. Further developments and optimization of array production and assay

performance combined with high-throughput generation of protein targets and ligands will extend the number of applications of protein microarrays dramatically. Proteomic research and diagnostic applications will be the two major fields addressed by protein microarray technologies.

In medical research, protein microarrays will accelerate immune diagnostics significantly by analyzing in parallel all relevant diagnostic parameters of interest. The reduction of sample volume is of great importance for all applications in which only minimal amounts of samples are available. One example might be the analysis of multiple tumor markers from a minimum amount of biopsy material. Furthermore, new possibilities for patient monitoring during disease treatment and therapy will be developed based on this emerging technology. Microarray-based technology beyond DNA chips will accelerate basic research in the area of protein–protein interactions and will allow protein profiling from limited numbers of proteins up to high density array-based proteomic approaches. Protein and peptide arrays will be used to analyze enzyme–substrate specificity and for measurement of enzyme activity on different kinds of substrates in a highly parallel fashion (Templin, Stoll et al. 2002).

The whole field of protein microarray technology shows a dynamic development driven by the increasing genomic information. New technologies such as automated protein expression and purification systems, used for the generation of capture molecules and the need for analysis of whole 'proteomes' will be a driving force for fast developments within the field of protein microarray technology.

1.5 Protein Microarrays in vaccine development against GAS

A systematic characterization of the composition and structure of the bacterial cell-surface proteome and its complexes can provide an invaluable tool for its comprehensive understanding.

The knowledge of protein complexes composition and structure could offer new, more effective targets for a more specific and consequently effective immune response against a complex instead of a single protein.

Large-scale protein-protein interaction screens are the first step towards the identification of complexes and their attribution to specific pathways. Currently, several methods exist for identifying protein interactions and protein microarrays provide the most appealing alternative to existing techniques for a high throughput screening of protein-protein interactions in vitro under reasonably straightforward conditions.

In this study approximately 100 proteins of Group A Streptococcus predicted to be secreted or surface exposed by genomic and proteomic approaches were purified in a His-tagged form and used to generate protein microarrays on nitrocellulose-coated slides. Spotted GAS proteins have been selected on the basis of the bioinformatic and proteomic analysis (Rodriguez-Ortega, Norais et al. 2006) that integrated the reverse vaccinology approach (Figure 1.11) and included mostly protein from M1 SF370 strain lipoproteins known to be surface exposed some of which containing the LPXTG anchor domain or know virulence factors.

To identify protein-protein interactions each purified protein was then labeled with biotin, hybridized to the microarray and interactions were detected with Cy3-labelled streptavidin. Only reciprocal interactions, i. e. binding of the same two interactors

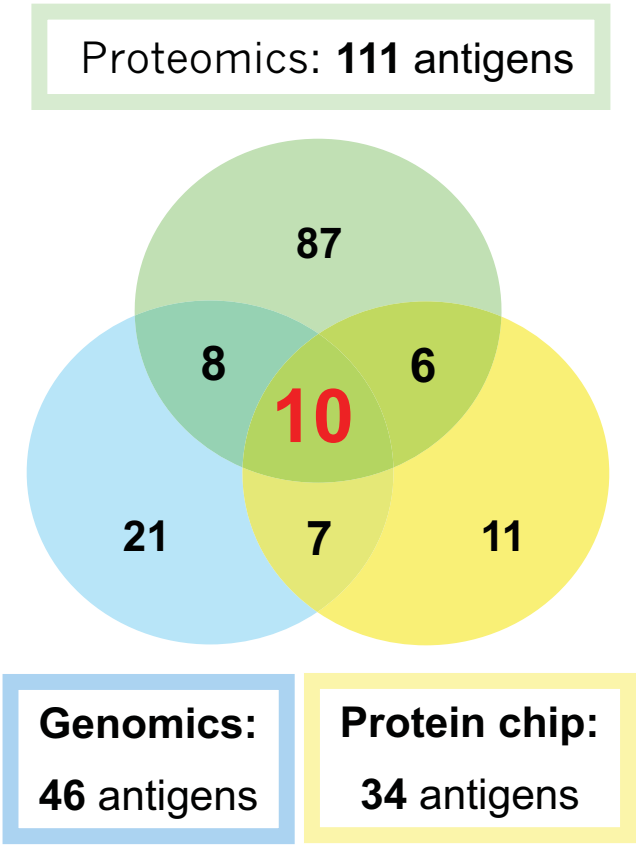


Figure 1.11 Screening for potential vaccine candidates:protein microarray results are combined with candidates obtained through genomics and proteomics approach.

irrespective of which of the two partners is in solid-phase or in solution, were taken as bona fide protein-protein interactions. Using this approach, we have identified 20 interactors of one of the potent toxins secreted by GAS and known as superantigens. Several of these interactors belong to the molecular chaperone or protein folding catalyst families and presumably are involved in the secretion and folding of the superantigen. In addition, a very interesting interaction was found between the superantigen and the substrate binding subunit of a well characterized ABC transporter. This finding opens a new perspective on the current understanding of how superantigens are modified by the bacterial cell in order to become major players in causing disease.

2. Materials and Methods

2.1 Bacterial growth medium

- LB (Luria Bertani)

Composition:

Trypton (10g/L), Yeast extract (5g/L), NaCl (10g/L) pH 7.6; ampicillin (100µg/ml)

- HTCM (High Throughput Complex Medium)

Composition:

Glycerol: 15 g/L (or Glucose); MgSO4: 0,5 g/L (2mM); YE(difco): 30 g/L; K2HPO4: 16 g/L; KH2PO4: 6 g/L; Ampicillin: 200 mg/L; pH: 7.35

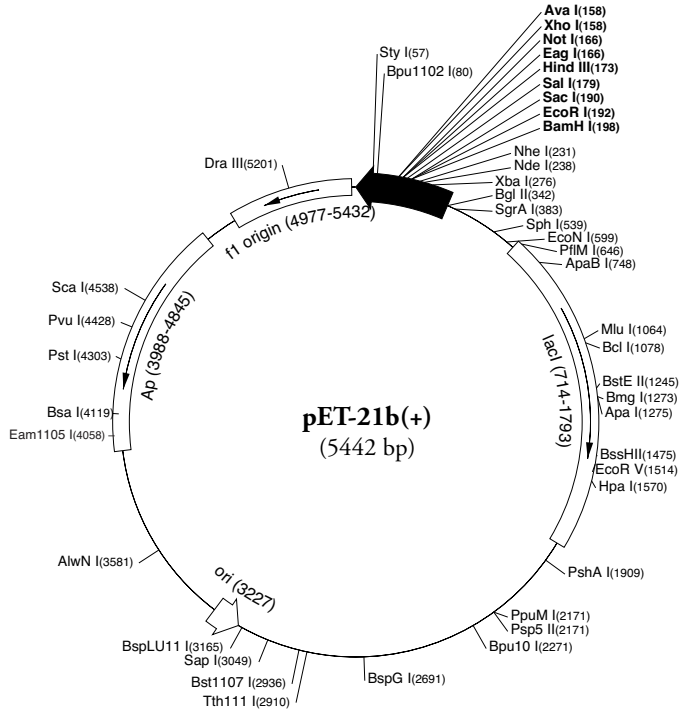
2.2 Cloning of GAS proteins

GAS genes encoding for selected protein were cloned into vectors suitable for expression in E. coli.

The vector used were pET21b(+) (plasmid for expression by T7 RNA polymerase, Novagen, Figure 2.1), which allows the expression of recombinant proteins with a 6 histidine residues tag and pGEXNNH (Amersham Bioscience, Figure 2.2) that delivers the protein fused also with Glutathione-S.transferase (GST).

The pET-21b(+) vector carry an N-terminal T7 Tag®sequence plus an optional C-terminal His Tag® sequence and carry an ampicillin selectable marker.

Unique sites are shown on the circle map. Note that the sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the circular map. The cloning and expression region of the coding strand transcribed by T7 RNA polymerase is shown below in Figure 2.1. The f1 origin is oriented so that infection with helper phage will produce virions containing single-stranded DNA that corresponds to the coding



pET-21b(+) sequence landmarks

T7 promoter	311-327
T7 transcription start	310
T7 Tag coding sequence	207-239
Multiple cloning sites (BamH I - XhoI)	158-203
His Tag coding sequence	140-157
T7 terminator	26-72
lacI coding sequence	714-1793
pBR322 origin	3227
bla coding sequence	3988-4845
f1 origin	4977-5432

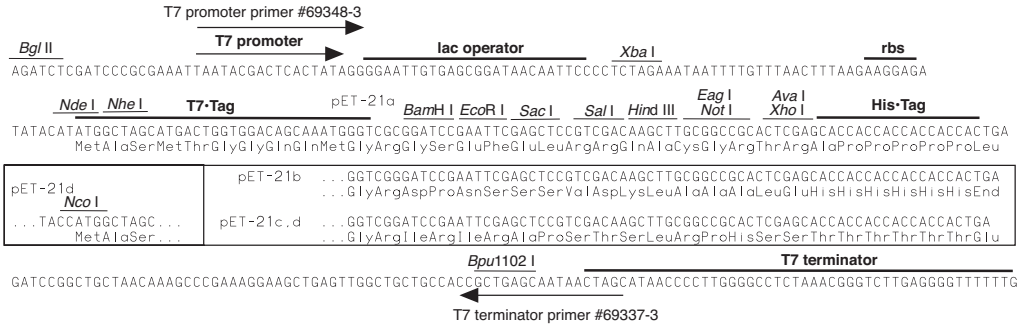


Figure 2.1. Map of pET-21b(+) expression vector and detail of the T7 cloning expression region

strand. Therefore, single-stranded sequencing should be performed using the T7 terminator primer.

Restriction sites (usually *NdeI* and *XhoI*) are determined among the ones available in the vector polylinker depending on the specific sequence of interest and primers are designed consequently, usually 20 to 30 bp long with a Melting Temperature ranging from 52 to 54° C.

The insert sequence is amplified by PCR using Pfu Ultra (Stratagene) as follows:

PCR mix composition: 100 ng GAS genomic DNA; 10 µl buffer 10x; 2 µl dNTPs 10 mM each; 2 µl taq; 20 pmol primerF (Forward); 20 pmol primerR (Reverse); H_2O to reach 100 µl final volume.

PCR cycle steps: 2 minutes at 98°C; 30 seconds at 98°C, 30 cycles; 50 seconds at 50°C; X minutes (1 minute every 1000 bp of the amplifying sequence) at 72°C and 7 minutes at 72°C. PCR products are the controlled on agarose gel and purified with QIAGEN PCR purification kit or by gel extraction (QIAGEN gel extraction kit).

Purified PCR product and vector are digested with the selected restriction enzymes (New England BioLabs) in ~60 U of each enzyme in 100 µl final volume for 3 hours or over night. Digested PCR product is purified (QIAGEN PCR purification kit) and the pET21b(+) vector is digested over night at 37°C with the same restriction enzymes (4 µg of plasmid DNA and 40 U of each enzyme in 100 µl final volume). The vector is then dephosphorylated for 1 hour at 37°C adding 2 µl (20 Units) of alkaline phosphatase (calf intestinal, CIP, New England BioLabs) in the same digestion buffer and purified by gel extraction (QIAGEN gel extraction kit).

Ligation reaction between the plasmid and the vector is performed (100 ng total between plasmid DNA and digested PCR in 10 µl final volume) for 3 hours at room temperature.

Electrocompetent *E. coli* BL21(DE3) for pET or BL21 for pGEX are transformed with 1 µl of ligation reaction. One milliliter of LB (or SOC) is added and bacteria are grown at 37°C for 45 minutes and plated on LB with 100 µg/ml ampicillin plates. Single ampicillin resistant colonies were selected and checked for the presence of recombinant plasmid by colony PCR. E.coli BL21(DE3) clones containing the recombinant plasmid are finally checked for the expression of the recombinant protein. The pGEX vector used has been engineered in-house by introducing a portion of the polylinker present in the pET vector to use the same restriction enzymes used during cloning.

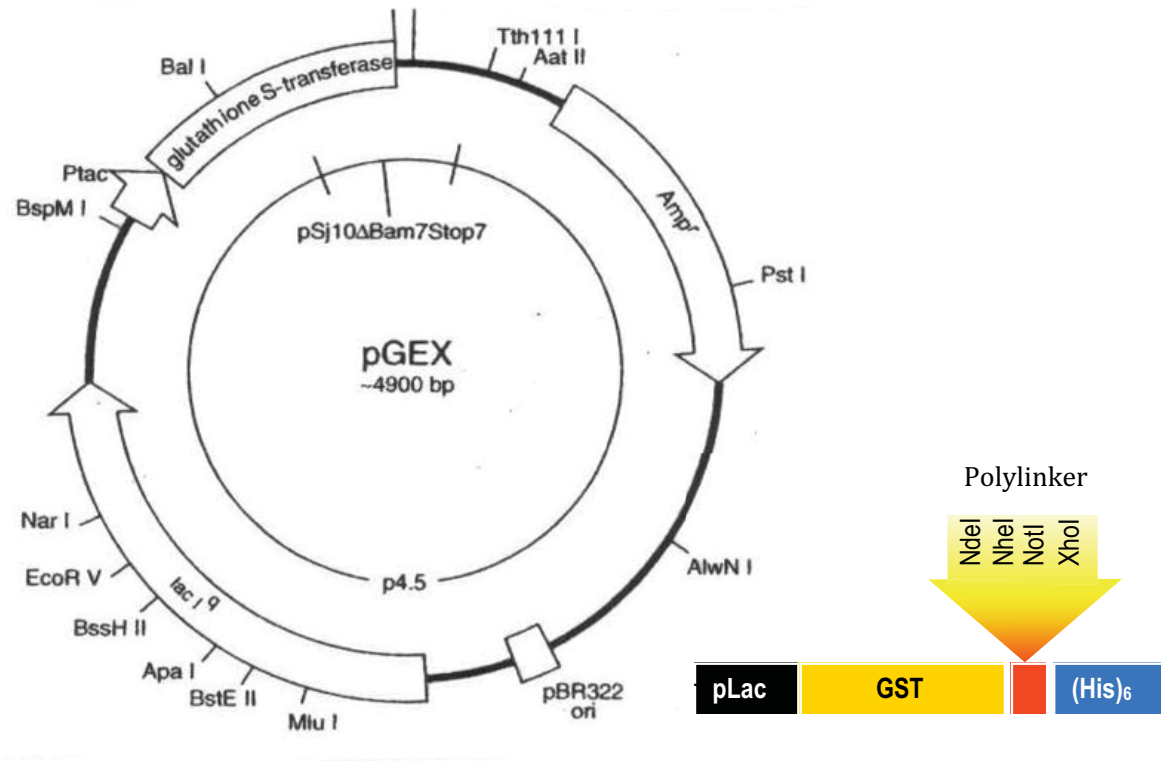


Figure 2.2. The pGEX expression vector and the in-house engineered polylinker.

2.3 Protein expression

Expression of His-tagged proteins

Selected clone is first grown in a 25 ml LB with 0.1 µg/ml ampicillin starter colture over night at 37°C in agitation at 180 rpm.

The starter colture is then diluted in 500 ml of the above growth medium in a 2 liter flask and grown at 30°C in agitation at 180 rpm. The colture $O.D._{600}$ is periodically measured. When colture $O.D._{600}$ reaches a value between 0.4 and 0.7, IPTG 0.5M (1mM final concentration) is added for induction.

The colture is grown for 3.5 hours at 25°C in agitation 180 rpm.

Expression of Gst-Tagged proteins

Selected clone is first grown in a 25 ml LB with 0.1 µg/ml ampicillin starter colture over night at 37°C in agitation at 180 rpm.

The starter colture is then diluted in 500 ml of the above growth medium in a 2 liter flask and grown at 25°C in agitation at 180 rpm. The colture $O.D._{600}$ is periodically measured. When colture $O.D._{600}$ reaches a value between 0.7 and 0.8, IPTG 0.5M (1mM final concentration) is added for induction.

The colture is grown for 3.5 hours at 25°C in agitation 180 rpm.

Protein expression using HTFS (High Throughput Fermentation System)

This system is based on in-house adapted 50 ml Falcon vials, prepared by boring three round holes in the cap: one in the center for air intake and two lateral for air out. The air intake will host a 2 ml Falcon pipette connected to a fluximeter and the air outtake will be filtered by two shortened ART1000 filtered tips (Figure 2.3).

Selected clone is then grown in 4 ml Glucose-HTCM at 37°C in agitation until bacteria reach exponential phase. Thirty five microliters of this colture are inoculated into a prepared Falcon vial containing 35 ml of Glycerol-HTCM and 50 µl PPC (poly propylene carbonate anti-foam solution, 1/10 diluted).

Falcon vials are placed on a heater set to 26.1°C (to keep the temperature from dropping under 26°C) and air flux (which also provides agitation) is set to 3 VVM (volume gas / [volume liquid / minute]).

The bacteria are grown for 36 hours, then the falcon vial caps are replaced with new ones and vials are centrifuged at 4000 xg at 4°C for 40 minutes. Supernatant is discarded and pellets are stored at -20°C.

Figure 2.3. HTFS machinery has been developed *in house* in the Novartis Vaccines Fermentation Lab, by Erwin Swennen and colleagues. The system is composed by 2 block heaters holding a maximum number of 24 standard 50 ml Falcon vials with 3 holes manually drilled in the cap. Filtered ART1000 tips are used as air-out and the central hole is attached to a fluximeter set to 3 VVM.

2.4 Protein purification

Poly-Prep Column His-Tagged protein purification

Pellets are thaw at room temperature and resuspended in 10 ml B-PER | (Bacterial-Protein Extraction Reagent, Pierce); 20 µl $MgCl_2$ 50 mM (0.1mM); 100µl DNAse I (100 Kunits Sigma D-4263) in PBS; 100 µl lysozyme (Sigma L-7651) 100 mg/ml in PBS (1 mg/ml final concentration).

Lysis solution is transferred in 50 ml centrifuge vials and is kept at room temperature fore 40 minutes and vortexed a few times and then spun at 40000 g for 25 minutes.

Poly-Prep column are prepared and equilibrated with 1 ml Ni-Activated Chelating Sepharose Fast Flow in 50mM phosphate buffer and 300mM NaCl at pH 8 and supernatant is loaded. Column flow through is discarded.

Ten milliliters of 20 mM imidazole, 50 mM phosphate, 300 mM NaCl buffer at pH 8 is added to discard impurities.

Proteins bound to the column are eluted with 4.5 ml of 250 mM imidazole, 50mM phosphate, 300 mM NaCl buffer at pH 8 and collected in three 1.5 ml fractions. 15µl DTT (Dithiothreitol) 200 mM (2mM final concentration) is added to each fraction.

Protein concentration of each fraction is estimated by Bradford assay and 10 µg of protein are loaded on SDS-PAGE gel.

Proteins are then stored at 4°C

Poly-Prep Column GST-Tagged protein purification

Pellets are thaw at room temperature and resuspended in 10 ml B-PER | (Bacterial-Protein Extraction Reagent, Pierce); 20µl $MgCl_2$ 50 mM (0.1mM); 100µl DNAse I (100 Kunits Sigma D-4263) in PBS; 100µl lysozyme (Sigma L-7651) 100 mg/ml in PBS (1 mg/ml final concentration).

Lysis solution is transferred in 50 ml centrifuge vials and is kept at room temperature for 40 minutes and vortexed a few times and then spun at 40000 xg for 25 minutes.

Poly-Prep column are prepared and equilibrated with 0,5 ml Glutathione-Sepharose 4B, washed with 2 ml H2O and 10 ml PBS, pH 7.4 and supernatant is loaded. Column flow through is discarded. 10 ml of PBS, pH 7.4 is added to discard impurities. Proteins bound to the column are eluted with 50 mM TRIS and 10mM reduced glutathione at pH 8 and collected in three 1.5 ml fractions. 15µl DTT 200 mM (2mM final concentration) is added to each fraction. Protein concentration of each fraction is estimated by Bradford assay and 10µg of protein are loaded on SDS-PAGE gel. Proteins are then stored at 4°C

AKTA System

Every "System" module can perform three purifications. The biomass is resuspended in 10 ml B-PER | (Bacterial-Protein Extraction Reagent, Pierce cat. 78266); 10 µl of 100 mM $MgCl_2$ solution (0,1 mM final concentration); 50 µl DNAse I equivalent to 100 K-Units (Sigma D-4263) in PBS and 100 µl of 100 mg/ml lysozyme solution (Sigma L-7651) in PBS equivalent to 10 mg (1 mg/ml final concentration).

Resuspended bacteria are transferred in 50 ml Falcon tubes, kept there for 30 to 40 minutes and vortexed a few times in order for the lysis reagents to act and then centrifuged at 35000 xg. The pellet is discarded and the clear supernatant is collected for chromatographical analysis with AKTAxpress. The absorbance at 280 nm is constantly read by the system to monitor the following purification.

1°) AFFINITY CHROMATOGRAPHY: the sample is loaded on a HisTrap FF column (Nickel activated) conditioned in 300 mM NaCl, 50 mM Na-Phosphate pH 8.00. The column is then washed in 20 mM Imidazole in equilibrium buffer and the protein is eluted with 500 mM Imidazole (in the same buffer). All flux rates are set to 1 ml/minute.

2°) DESALTING: the goal of this step is to prepare the sample for the ion exchange column. The HisTrap FF column eluate is loaded on the 5 ml Hi-Trap Desalting, serially attached, conditioned in 50 mM Tris-Cl pH 8.00 and the sample is eluted with equilibrium buffer with a 5 ml/minute flux rate.

3°) ION EXCHANGE: the sample eluted from desalting is loaded at 1 ml/minute flux rate on a 1 ml Hi-Trap Q HP column conditioned in 50 mM Tris-Cl pH 8.00, previously washed with the same buffer. The flow through is automatically collected by default (2 ml for each fraction). Proteins bound to the column are eluted with a NaCl gradient from 0 to 500mM in ten column volumes (CV), collecting 1 ml fractions followed by a 10CV step in 1.0 M NaCl in equilibrium buffer.

Every step is performed automatically by AKTAxpress.

COLUMN POSITION

POSITION N°	COLUMN	Column Volume
1	HisTrap FF	1 ml
2	HisTrap FF	1 ml
3	HisTrap FF	1 ml
4	HiTrap Q HP	1 ml
5	HiTrap Desalting	3 x 5 ml

INLET → BUFFER:

INLET	BUFFER
A1	300 mM NaCl, 50 mM Na-phosphate pH 8.00.
B1	500 mM Imidazole, 300 mM NaCl, 50 mM Na-Phosphate pH 8.00.
A2	50 mM Tris-Cl pH 8.00.
B2	1 M NaCl 50 mM Tris-Cl pH 8.00.
A3	20 mM Imidazole, 300 mM NaCl, 50 mM Na- Phosphate pH 8.00.
A5	H2O.
A6	0,5 M NaOH.

2.5 Tagless Proteins

To obtain purified proteins without a Histidine tag, a TEV (Tobacco Etch Virus) cleavage site was inserted at the N-terminal of the protein before the 6 Histidines.

The "PIPE" method (Polymerase Incomplete Primer Extension) was used for cloning these specific proteins (Klock, Koesema et al. 2008; Klock and Lesley 2009). The vector pSpeedET (Figure 2.5) was used that provided the cells with kanamycin resistance and therefore expression protocol was adapted to use kanamycin (30 µg/ml) instead of ampicillin (Figure 2.4).

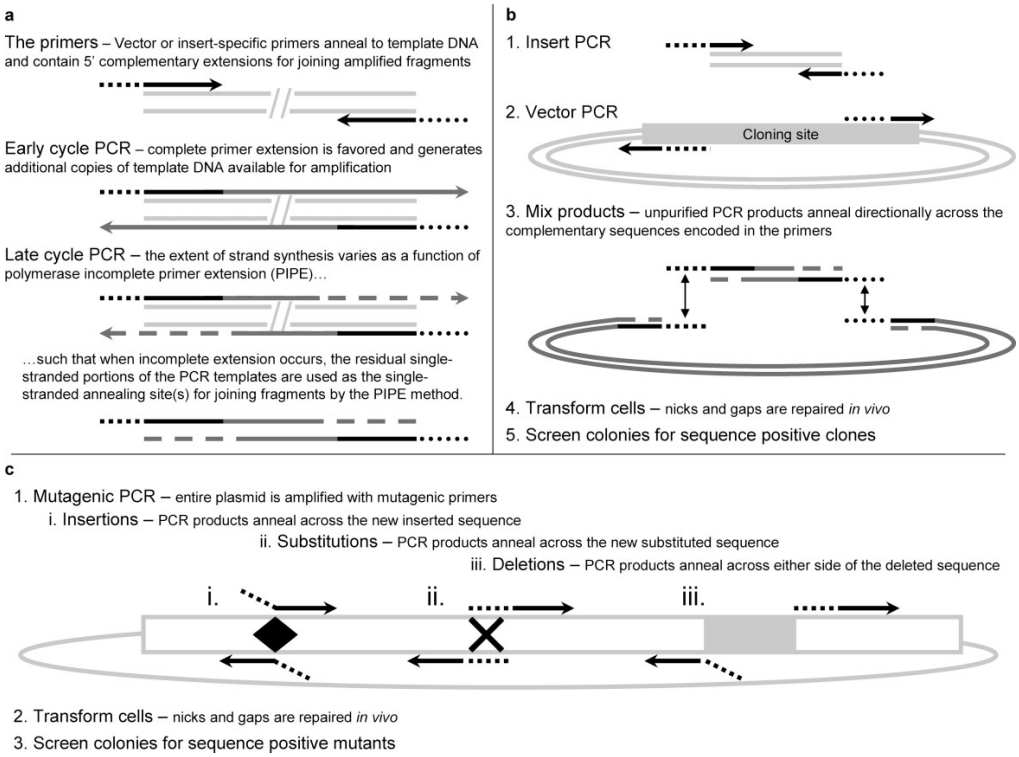


Figure 2.4. Schematics of proposed PIPE mechanism and methods. The light gray lines represent template DNA. The black lines with dashed or dotted ends represent the primers with 50 complementary extensions. The black square dashes represent sequences complementary to each other as do the black circles. The straight dark gray lines represent complete strand synthesis. The dashed dark gray lines represent heterogeneous primer extension resulting from PIPE. (**a**) Progression of PIPE during normal PCR amplification. (**b**) The PIPE Entry Cloning method. (**c**) Primer design guidelines and method for PIPE Mutagenic Cloning for creating insertion, substitution and deletion mutants (Klock, Koesema et al. 2008).

Normal PCR reactions generate mixtures of incomplete extension products. Using classical primer design rules and PCR, short, overlapping sequences are introduced at the ends of these incomplete extension mixtures which allow complementary strands to anneal and produce hybrid vector/insert combinations. These hybrids are directly transformed into recipient cells without any post-PCR enzymatic manipulations.

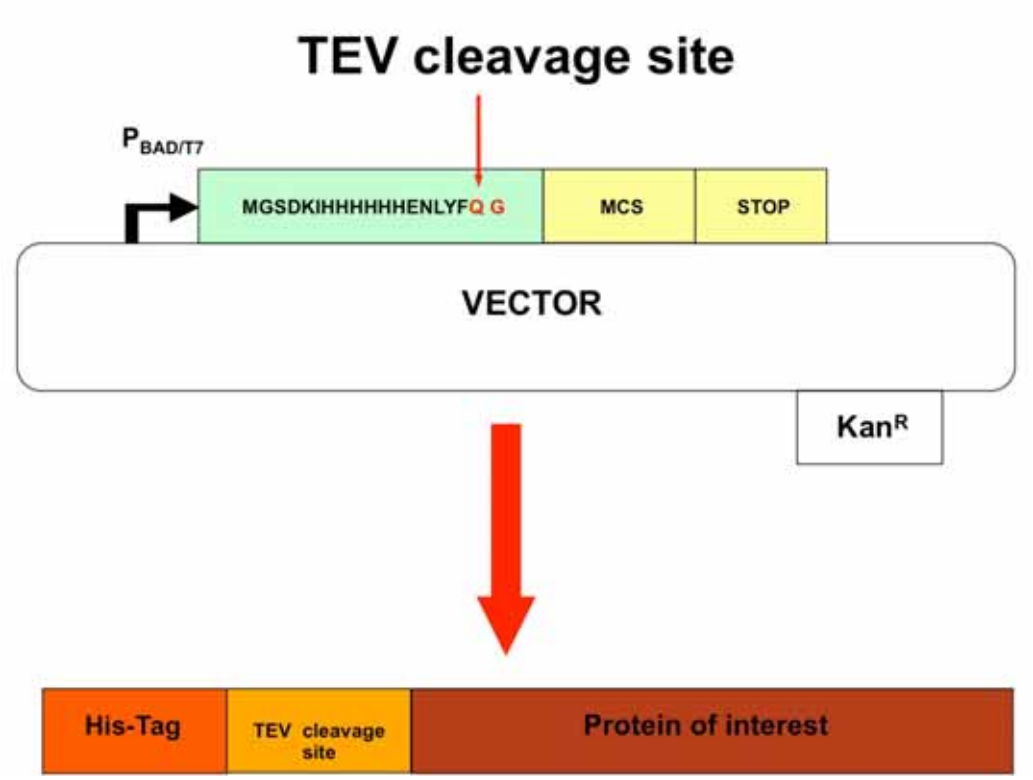


Figure 2.5. Detail of the vector used for obtaining tagless proteins exploiting the TEV cleavage site cloned between the protein and the Histidine tag.

2.6 Other Protein assays

Bradford Assay

The Bradford assay is the commonly used technique to determine concentrations of purified proteins in solution. The method of Bradford is the basis of the Bio-Rad Protein Assay and it involves the addition of an acidic dye to protein solution, and subsequent measurement at 595 nm with a spectrophotometer (an ULTROSPEC® 3000 Pharmacia Biotech was used). A differential color change of a dye occurs in response to various concentrations of the protein (Bradford 1976). The absorbance maximum for an acidic solution of Coomassie®Brilliant Blue G-250 dye shifts from 465 nm to 595 nm when binding to protein occurs (Reisner, Nemes et al. 1975; Sedmak and Grossberg 1977). The Coomassie blue dye binds to primarily basic and aromatic amino acid residues, especially arginine (Compton and Jones 1985). Spector and colleagues (Spector 1978) found that the extinction coefficient of a dye-albumin complex solution was constant over a 10-fold concentration range. Thus, Beer's law may be applied for accurate quantification of a protein by selecting an appropriate ratio of dye volume to sample concentration.

Samples were prepared using 1 part Dye Reagent Concentrate with 4 parts PBS and at least two different dilutions of the protein so that both absorbance values (A_{595}) are within the optimal reading range of the instrument (A_{595} between 0.2 and 0.8).

The determined protein concentration is:

[(A_{595} Sample) - (A_{595} Reference Sample)] * dilution factor

Considering the obtained protein concentration 5 to 10 µg are loaded on gel to verify the result of the Bradford assay.

A_{280} reading to determine protein concentration

When the Bradford assay failed to provide accurate results or when it was not recommended to use any protein amount in concentration determination assays the A_{280} reading provided an appealing alternative approach for protein concentration determination without wasting the protein.

To use this method the protein sequence must be known and provided to computer software (such as VectorNTI or free online tools at ExPASy Proteomics Server) that returns the protein concentration at which A_{280} is 1. This concentration value is used to determine the concentration of the sample based on its A_{280} reading.

2.7 Protein chip

Design

A pool of 93 proteins has been selected among those predicted and found to be on the Group A Streptococcus cell surface or secreted. Designing the chip layout is a key step in microarray based research to overcome some of the technique limitations. For this particular experiment, because of the number of samples and controls we chose a 12-pin array setup with 384 wells plates. The pins were arranged in two rows of six and each one printing a grid.

Due to the morphology of the BioRad ChipWriter™ Pro used to print the microarray and capable also of printing nucleic acids, the carry-over of protein from one spot to the next one is the major limitation. Therefore half of the spots in the microarray layout are considered empty, filled with only the H_2O spotted, to control and avoid carry-over. These spots will be analyzed after every experiment looking for fluorescence signals that will determine the background value.

The design also included various types of controls, mostly serial dilutions of proteins or antibodies and they have all been arranged to be spotted at the side of the grid for the most user friendly analysis possible that fitted the sample number and the 12 pin array. Double water spots were included after the first four spots of the controls serial dilutions to avoid carry over of certain types of controls (e.g. antibodies) and for additional background control (Figure 2.6).

This particular design is able to fit protein-protein interaction experiments using various detection techniques: biotinylated protein and fluorophore conjugated streptavidin as

well as mouse or rabbit protein specific antibody and conjugated secondary antibody.

Both Cy3 and Cy5 fluorophores can be used.

Figure 2.6. Microarray layout. Surface GAS proteins are represented by orange boxes. H20 spotter between proteins to avoid carry over is highlighter in blue. Experimental and spotting controls in other colors.

Preparation

Purified GAS proteins are dialyzed in PBS and eventually concentrated to obtain a concentration of 0.5 mg/ml or higher. Each protein is then loaded in four wells (6 µl per well) of a 384 well polypropylene micro plate.

Each plate contains three standard curves: Mouse IgG, Cy3 and Cy5-labelled BSA (Amersham Biosciences) and biotin-labeled BSA. All samples are spotted on nitrocellulose slides by using the VersArray ChipWriterTM Pro System (BIO-RAD) equipped with TeleChem quill pins (TeleChem International Sunnyvale, CA, USA). The pins are previously controlled with optical microscope to check for dirt or damaged heads.

Spotting

Following the first printing of each sample, the pins are washed 7 times (6 seconds each), subjected to sonication (1 second) and dried under vacuum (2 seconds). After each printing process, each slide is scanned to check the signals of the Cy3 and Cy5-labeled BSA curves.

Hybridization

Slides are washed with PBS at 20°C for 5 minutes and then with TPBS (0.05% Tween 20 in PBS) at 20°C for 1 minute followed by 1 hour incubation in the dark for blocking with shaking in 5% Top Block (Fluka-BioChemiKa, Cat. n° 37766) in TPBS. Slides are incubated with protein probe (in 3% Top Block in PBS) for 1 h at 20°C in the dark and then washed 3 times (3 minutes each time) in TPBS.

Staining

Biotinylated protein/streptavidin:

Streptavidin-Cy3 (1:100 dilution in 3% Top Block in PBS) is added and incubation is prolonged for 1 hour at 20°C in the dark. Slides are then washed twice with TPBS (1 minute each time), twice with PBS (1 minute) and once with sterile milliQ H_2O (30 seconds).

Protein/specific antibody:

Slides are incubated with probe-specific primary antibody (1:20000 final dilution in 3% Top Block in PBS) for 1 hour at 20°C in the dark and then washed 3 times (1 minute each time) in TPBS then Alexa 546 anti-Rabbit IgGs (1:1000 dilution in 3% Top Block in PBS) are added and incubation is prolonged for 1 hour at 20°C in the dark.

Slides are then removed from the incubation chamber, washed once with sterile milliQ H_2O and air-dried.

Scan of hybridized slides

The fluorescence signal was detected using a high resolution (10 mpixel size) scanner ScanArray 5000 Unit (Packard, Billerica, MA, USA) and using lasers to detect the specific fluorophore used. The signal was then quantified with the program Imagene 7.5 (Biodiscovery Inc, CA, USA). The data collected were analyzed using the in-house developed program "Protein Chip".

The fluorescence signal of each protein was calculated as an average intensity of fluorescence of the four replicates minus the background fluorescence intensity.

To determine binding on a protein-probe to another protein cutoff fluorescence intensity value of 5000 was established as equal to the average values of intensity of the negative controls (protein contamination of *E. coli*) plus three times the value of standard deviation. Average intensities of fluorescence below this value were discarded.

2.8 Surface plasmon resonance analysis

Proteins immobilization

Experiments were performed at 25°C with a BIACORE T100 instrument (Biacore AB, Uppsala, Sweden). All the reagents were purchased from GE Healthcare, whereas not specified. Proteins SpeI, AdcA and Lmb were immobilized on a carboxymethilated dextran coated (CM5) sensor chip by amine coupling.

Briefly, a mixture of 0.2 M 1-ethyl-3-diaminopropyl-carbodiimide (EDC) and 0.05 M N-hydroxysuccinimide (NHS) was used for sensor chip surface activation. Proteins pre-concentrated in 0.01M sodium acetate pH 4.5 were injected at 50µg/ml for 7 minutes and eventually 1M Ethanolamine pH 8.5 was used to block remaining activated groups. Approximately 2000 Rus of immobilized material were achieved for the three proteins.

As for monomeric biotinylated SpeI, immobilization on a streptavidin coated (SA) sensor chip was obtained by 5 minutes injection of 50 µg/ml of the above mentioned protein in HBS-EP^{+} buffer (10 mM Hepes, NaCl 150 mM, EDTA 3.4 mM, p20 0.05%, pH 7.4) at a flow rate of 5 µl/min.

In all the following described experiments an empty flow cell was used as blank reference and subtracted sensorgrams were used for evaluation.

Zn^{++} influence on binding

Binding on immobilized proteins was investigated either in the absence or in the presence of zinc ions. A solution containing 10 mM HEPES, 150 mM NaCl, 0.05% p20, pH 7.4

(HBS-N) with increasing $ZnCl^2$ (Sigma-Aldrich) concentrations ranging from 100 nM to 50 µM was used as running buffer. Proteins diluted in the same buffer at 50 and 25 µg/ml were injected for 3 minutes at a flow rate of 20 µl/ml and regeneration of sensor chip surface was achieved with a 30 seconds pulse of 500 nM NaCl and 10 mM EDTA. HBS-EP^+ was used as running and dilution buffer for the same experiments without Zn^{2+}.

k_{off} ranking

Selected proteins were diluted at 25µg/ml in HBS-N/5µM Zn^{++} and eventually injected over the three proteins simultaneously for 3 minutes at a flow rate of 20 µl/min. Dissociation was followed for 400 seconds and regeneration was performed as already described. Whenever possible dissociation rate constants were calculated with "BiaEvaluation 4.1 software".

Kinetics characterization

Proteins SpeI and AdcA were further characterized for their association rate and affinity constants at equilibrium versus immobilized proteins.

Kinetics experiments were performed by injecting increasing concentration of the proteins in HBS-N/5 µM Zn^{2+} over the sensor chip surface for 3 minutes at a flow rate of 20 µl/minute. Complexes were left to dissociate for 500 seconds and regeneration was obtained with a 30 seconds pulse of 500 nM NaCl and 10 mM EDTA.

Dilutions of each protein were prepared shortly before injection in order to minimize potential aggregation. k_{on}, k_{off} and K_D were calculated with 1:1 Langmuir model with "BiaEvaluation 4.1".

3. Results

3.1 Obtaining the proteins for protein-protein interactions

For Microarray analysis of *S. pyogenes* protein-protein interactions a high-throughput system for expression and purification was implemented involving a High Throughput Fermentation System (HTFS) for protein expression and AKTAxpress chromatography system to obtain a highly purified proteins in high-throughput manner (Figure 3.1).

From a collection of 93 selected proteins (Table 3.1), carrying a 6-Histidine tag at the carboxyl terminus, 82 were successfully expressed in *E. Coli* using HTFS with only 35 ml of HTCM growth medium and auto-induction, reaching a final OD_{590} value between 7 and 10, corresponding to 0.5 to 0.75 grams of total biomass and 1 to 20 mg of total recombinant protein. The system can deliver 48 cultures in 2 days and a total of 192 proteins per month per technician.

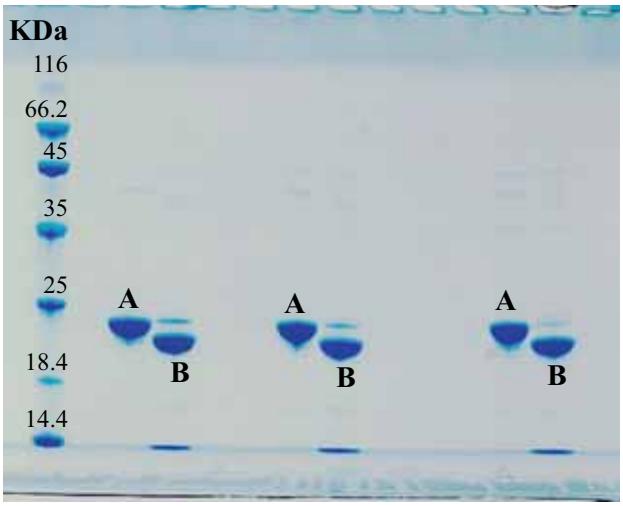


Figure 3.1. Purified SpeI on AKTAxpress before (**A**) and after (**B**) the cleavage of the His-tag by TEV protease.

Protein predicted localization	N°	Annotation (SF370 NCBI)	Locus
Lipoprotein	23	Energy metabolism	*lmb; mtsA; tlpA;* SPy0163; SPy1228
		Transport and binding proteins	*fhuD; oppA; pstS;* SPy0317*;* SPy1274*;* SPy1795
		Cell envelope	*dppA;* SPy0604*;* SPy1290
		Unknown function	*inlA; malX*; *prsA*; SPy0210; SPy0252; SPy0457; SPy0778; SPy1294; SPy1390
Membrane	45	Amino acid biosynthesis	*cysM*
		Cell envelope	*isp*; *prtS*; SPy0793; SPy0843; SPy1326; M6_Spy0160
		Cell wall/membrane biogenesis	SpyM3_0104
		Cellular processes	*hlyA1*; *emm3*; M6_Spy0159
		Central intermediary metabolism	*glmS*; SPy0380
		Energy metabolism	*guaA*; *pulA*
		Fatty acid and phospholipid metabolism	*accA*
		Protein fate	*scpA*
		Transport and binding proteins	SPy2009; SPy2033; M6_Spy0157; MGAS2096_Spy0110; MGAS2096_Spy0115; MGAS2096_Spy0119
		Unknown function	*cbp*; *cpa*; *emm1*; *fabK*; *ftsZ*; *grab*; *mf*; *pepQ*; *prgA*; SPy0128; SPy0130; SPy0838; SPy0872; SPy1054; SPy1686; SPy1874; SPy1939; M5005_Spy0107; SpyM3_0100; SpyM3_0102; M28_Spy0109; SpyM50106
Outside	20	Cell envelope	*isp2*; *mur1.2*
		Cellular processes	*hylA; ska*; *speC*; *speG*; *speI*; *speJ*
		Central intermediary metabolism	SPy1718
		Unknown function	*adcA*; *mf3*; *sic*; *slo*; SPy0019; SPy0925; SPy1037; SPy1491; SPy1733; SPy1813; SPy2066
Cytoplasm	2	Cell envelope	*fbp*
		Energy metabolism	*eno*
Unknown	3		*gid*; SPy0652; SPy1959

Table 3.1. Annotation and predicted localization of proteins used for microarrays.

The remaining 11 proteins are characterized by low expression levels and required a greater culture volume and IPTG for induction of expression.

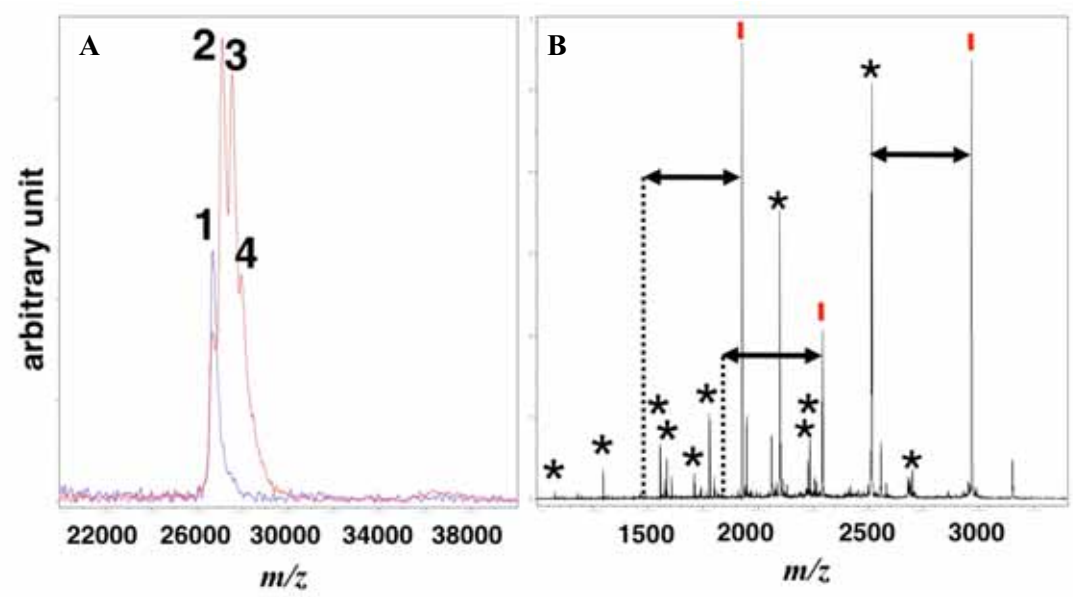
Particularly when studying protein-protein interactions it is very important to work with proteins as pure as possible and obtained in a high throughput manner. The AKTAxpress 3-step purification system is a good compromise between quality and time. The system delivers up to 48 His-tagged proteins per month per technician with purity levels of 75% to 90%. All proteins used in this study were purified using AKTAxpress and because of its versatility it could be used for both His- and GST-tagged proteins.

3.2 Biotinylation of GAS proteins

Even though the microarray design allows detection of binding between the protein probe and the array by using probe-specific mouse or rabbit sera, the detection of interactions in our experimental approach was obtained using biotinylated protein probes and Cy3-labelled streptavidin, since this is the most commonly used approach in microarray publications in the literature.

Each purified GAS protein from the 93 selected proteins was biotinylated using an amine-reactive biotinylation reagent. This reagent is able to attach a biotin molecule to the amine groups of all the exposed lysine residues by using a biotin-protein molar ratio of 20:1. To avoid biotinylation of all lysines, should a lysine be part of an active binding site, the molar ratio has been lowered to 3:1. Ten of the biotinylated proteins were analyzed by mass spectrometry and their biotinylated lysines were mapped.

A representative example of such an analysis is given in Fig. 3.1, which shows that in our experimental conditions Spy1007 (SpeI), that contains 21 lysine residues in its sequence, was biotinylated only at one, two or three lysine residues per protein molecule and biotin was linked only to Lys102 and/or Lys209 and/or Lys227. Analogous results were obtained for the other proteins analyzed by mass spectrometry. In all cases, between one and three lysine residues were biotinylated and the modification was observed to occur at a restricted number of sites.



MSSVGVINLR NLYSTYDPTE VKGKINEGPP FSGSLFYKNI PYGNSSIELK
VELNSVEKAN FFSGKRVDIF TLEYSPPCNS NIKKNSYGGI TLSDGNRIDK
KNIPVNIFID GVQQKYSYTD ISTGSTDKKE VTIQELDVKS RYYLQKHFNI
YGFGDVKDFG RSSRFQSGFE EGNIIFHLNS GERISYNLFD TGHGDRESML
KKYSDNKTAY SDQLHIDIYL VKFNKLEHHH HHH

C

Figure 3.2. Mass spectrometry analysis of SpeI biotinylation. Three forms of the biotinylated protein plus the non biotinylated form are present in the solution (**A**). After tryptic digestion only three lysins appear to have acquired a biotin molecule (**B**, **C**). The peptides containing the biotinylated lysins appear to have a mass shift correspondent to the biotin molecule mass (**B**). In the sequence of SpeI shown above in **bold** are the peptides found in the MS analysis and in **bold red** the biotinylated lysins (**C**).

3.3 Chip validation

Mouse and rabbit sera raised against the spotted recombinant proteins were used to verify the presence of the protein on the microarray and carry over of the protein in other spots. Serial dilution curves of Biotinylated Bovine Serum Albumine (BSA), Cy3/Cy5-labeled BSA, Mouse and Rabbit IgG are spotted in triplicates on the chip as controls for fluorophore conjugated secondary antibodies and streptavidin and for the behavior of every pin on each printed slide during the spotting session.

Among the 93 proteins selected for this screening 4 are well know Fibronectin Binding Proteins. These were used as positive controls for validating both the printed microarrays and the experimental conditions used in the screening for protein-protein interactions.

Human fibronectin was biotinylated under non-saturating conditions and it was incubated with the GAS proteins printed on the chip following the screening protocols. Binding was detected only for the spots of the fibronectin binding proteins (Figure 3.3). The binding detection technique did not generate any false positive signal due to the conjugated streptavidin or the chip saturation.

Figure 3.3. Fibronectin binding proteins spots fluorescence signal after hybridization with human fibronectin. The for proteins present on the chip are: gi-19224141 (**1**), M6_Spy0157 (**2**), SpyM3_0104 (**3**) and gi-19224134 (**4**). It is also possible to notice the absence of any protein carry other to other spots and any other nonspecific binding caused by the detection system.

3.4 Protein-protein interactions

Considering the chip validation results criteria were determined for positive interaction results determination. First the fluorescence value for positive binding was set, in a scale from 0 to 65000, to 5000 corresponding to the mean fluorescence value of the negative control spots plus 3 times the standard deviation value. In addition, only reciprocal interactions, i.e. binding of the same two interactors irrespective of which of the two partners was immobilized on the chip or in solution, were taken as bona fide protein-protein interactions as schematized in Figure 3.4.

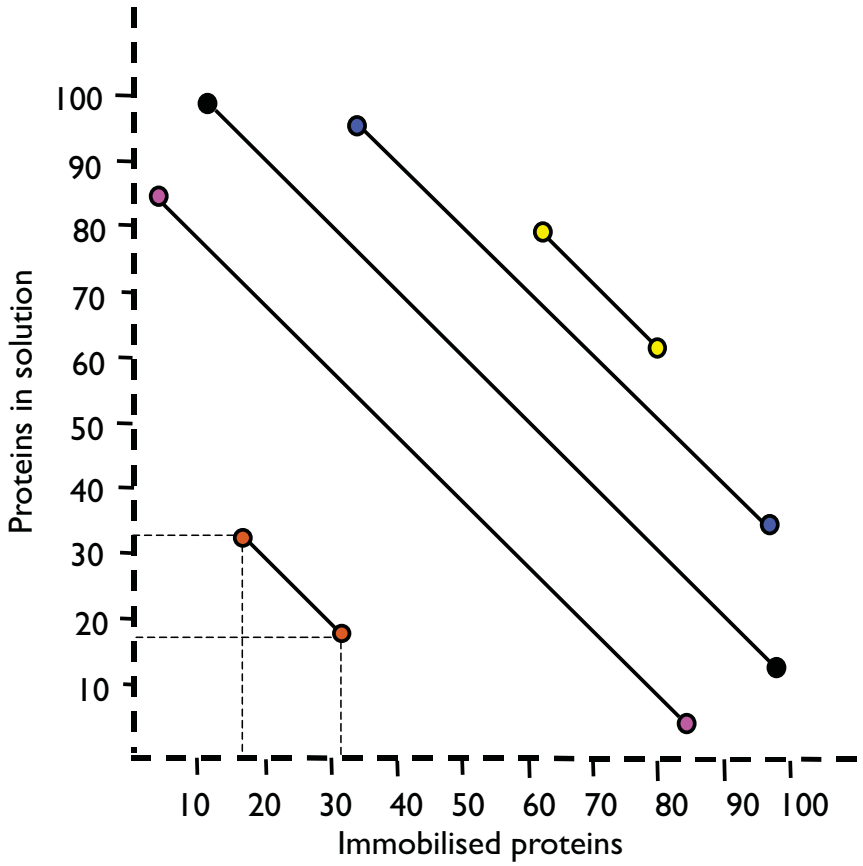


Figure 3.4. Explanatory reciprocal interaction diagram. The proteins found to be reciprocally interacting are highlighted through parallel lines.

Using these criteria, 25 reciprocal interaction have been identified, 20 of them regarding Streptococcal Pyrogenic Exotoxin I (SpeI), that belongs to the superantigen protein family secreted by GAS (Figure 3.5).

Among the SpeI interactors, 8 proteins are annotated as “hypothetical”. This shows the potential of this type of approach for characterization of proteins of unknown functions. The other SpeI interactors mostly pertain to the molecular chaperone proteins family

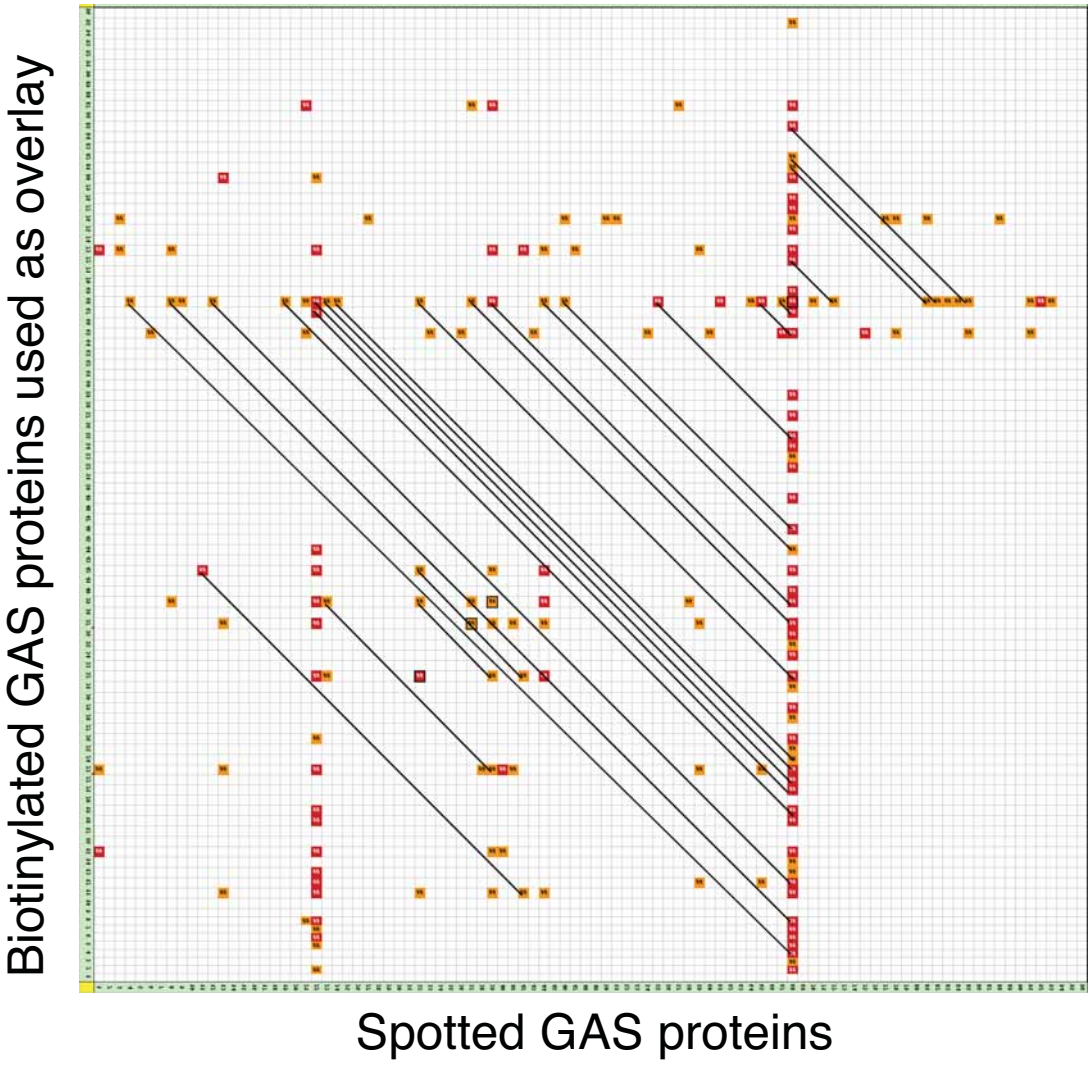


Figure 3.5. Results of the protein-protein interaction screening. All the binding detections are shown in this figure. Yellow squares correspond to a fluorescence intensity signal from 5000 to 10000. **Red** squares stand for a signal of 10000 and above. Reciprocal interactions are highlighted by parallels lines.

dedicated to folding and thus probably involved in maturation and secretion of the superantigen.

SpeI is know to dimerize forming a disulfide bond between two of the monomeric form of the protein that carry one single cysteine in its sequence. It has also been shown to have a monomer-dimer concentration dependant equilibrium (Brouillard, Gunther et al. 2007). This observation was confirmed by this approach and SpeI was found to interact with itself. When coming to "self" interaction detection, it was not possible for obvious reasons to apply the reciprocal interaction rule, but SpeI was also found interacting with SPyl558 (TlpA) a protein disulfide reductase and SPy0925, a putative oxidoreductase as another confirmation for this sort of behavior.

SpeG was another protein found to be reciprocally interacting with SpeI. It's a group IV SAg as SpeC and SpeJ (whereas SpeI belongs to group V), also present in the microarray, and it is described in the literature as capable of forming concentration-dependent homodimers (Proft, Moffatt et al. 1999; Sachse, Seidel et al. 2002). In this case the interaction between the two SAgs may suggest the formation of heterodimers.

SpeI has been shown to bind MHC-II molecules in a zinc-dependent manner (Proft, Arcus et al. 2001) and in this screening was found interacting with AdcA and Lmb, which belong to the transition metals transporter family. In particular AdcA is an orthologue of ZnuA: the substrate binding protein of the high affinity zinc uptake system and Lmb is part of another transition metal transporter but with a general affinity for Mn^{2+}, Fe and, interestingly, Zn2+ (Desrosiers, Sun et al. 2007).

Figure 3.6 and Table 3.2 show the network of reciprocal interactions and annotation of the proteins.

SPy number	Protein name	Annotation
M5005_ SPy0249	OppA	oligopeptidepermease
SPy0130		hypothetical protein
SPy0212	SpeG	exotoxin G precursor
SPy0317		hypothetical protein
SPy0604		hypothetical protein
SPy0714	AdcA	Zinc-binding protein adcA/ putative adhesion protein
SPy0793		hypothetical protein
SPy0838		hypothetical protein
SPy0857	Mur1.2	putative peptidoglycan hydrolase
SPy0925		putative oxidoreductase
SPy1007	SpeI	streptococcal exotoxin I
SPy1032	HylA	Extracellular hyaluronate lyase
SPy1037		hypothetical protein
SPy1054		putative collagen-like protein
SPy1228		putative lipoprotein
SPy1326		hypothetical protein
SPy1520	FtsZ	cell division protein
SPy1558	TlpA[a]	Thioredoxin[b]
SPy1743	AccA	acetyl-CoA carboxylase alpha subunit
SPy2000	DppA	Dipeptide-binding protein
SPy2007	Lmb	putative laminin adhesion
SPy2037	PrsA	peptidylprolyl isomerase Foldase protein prsA
SPy2066		putative dipeptidase

[a] (Koski, Saarilahti et al. 1992)

[b] (Lei, Liu et al. 2004)

Table 3.2. Annotation of proteins reciprocally interacting proteins.

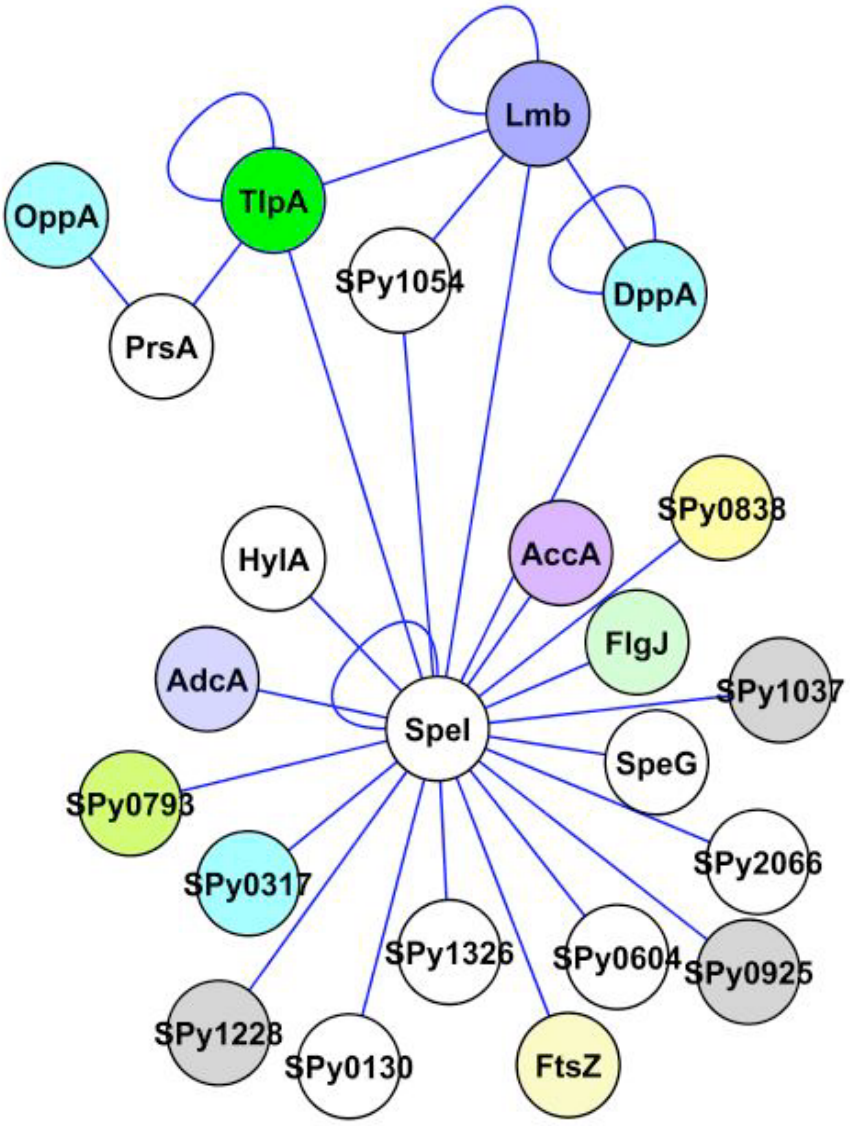


Figure 3.6. Reciprocal interaction network

3.5 Characterizing interactions by SPR

Surface Plasmon Resonance: the Biacore Technology

Biacore® T100 is a system for comprehensive protein interaction analysis (Figure 3.7). It allows to measure kinetic rate constants over the broadest range, from the fastest on-rates to the slowest off-rates, to compare buffer effects in a single run, to work with low molecular weight compounds and, particularly, to study interactions at physiological temperatures and above.

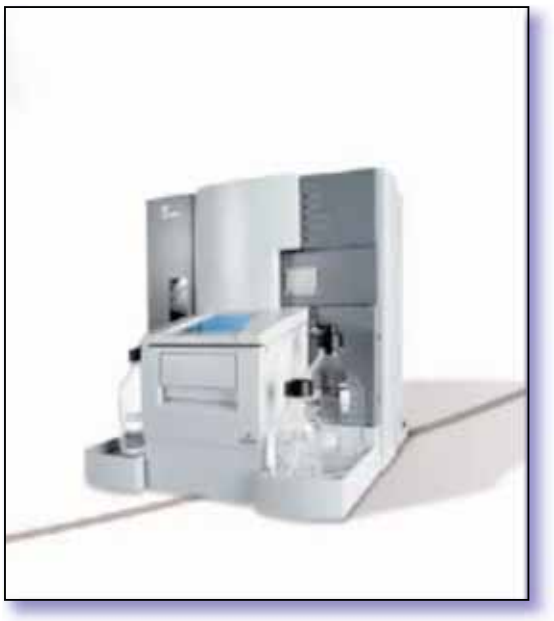


Figure 3.7. The Biacore T100.

It is based on Surface Plasmon Resonance (SPR). The SPR is a physical phenomenon occurring when monochromatic polarized light hits a metal film under total internal reflection conditions. Incident photons are adsorbed and converted in plasmons thus creating a gap in the intensity of reflected light at a particular angle (resonance angle). These dips of light related to change in resonance angle are detected by the instrument.

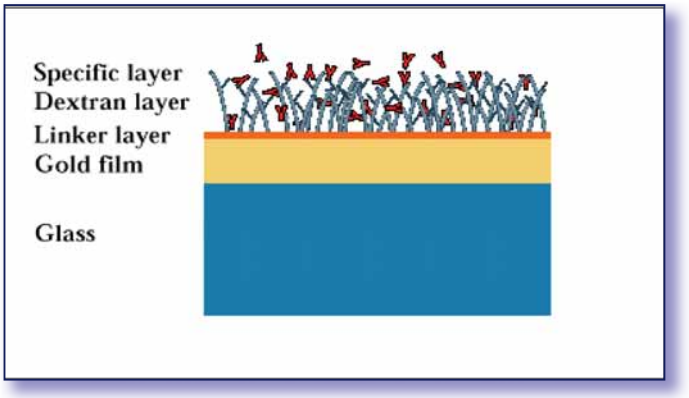


Figure 3.8. The Sensor Chip.

The core of Biacore T100 is the sensor chip (Figure 3.8). A dextran matrix covering a golden layer on a glass chip. It enables molecules to be immobilized to a sensor surface and provides a hydrophilic environment for interactions.

When screening for interactions the analyte is injected over the immobilized ligand and, whenever an interaction occurs, the change in mass on the sensor chip surface causes a change in resonance angle proportional to the amount of bound analyte. The resulting sensorgram (Figure 3.9) is the real time analysis of the biomolecular interactions and provides kinetics values for association and dissociation.

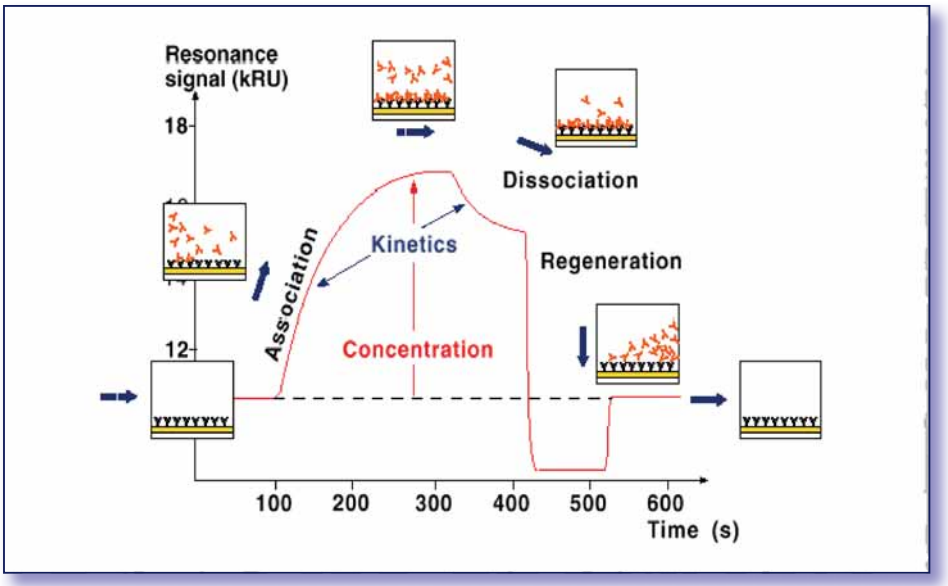


Figure 3.9. Surface Plasmon Resonance analysis of interactions.

Analysis of interactions

Biacore technology was chosen as the methodology for verifying interactions found in the protein microarray screening as a solid technique for protein-protein interaction analysis (Onell and Andersson 2005). Among the most significant interactions, 14 were selected to be analyzed on Biacore T100.

For this analysis the tagless form of the proteins were used in place of the His-tagged ones spotted on the microarray. This was done to verify also an eventual role of the Histidine tag in the interaction, especially when dealing with interaction involving metal binding proteins. Moreover the carboxymethilated dextran matrix of the sensor chip used was much more flexible than the nitrocellulose coating of the microarray slides.

Ligand protein were immobilized by amine coupling and interactions were tested in the presence or absence of Zn^{2+}.

All the selected interactions obtained using the microarray approach were confirmed and verified with the biacore analysis. Dissociation kinetics were calculated by the instrument and k_{off} values (dissociation rates) were obtained in a range typical of stable binders (from 1.4×10^{-4} to 3.9×10^{-3} s^{-1}) and are reported in Table 3.3.

The interaction between SpeI and the two transition metal binding proteins was of particular interest and a more in-depth analysis was performed focusing on the interaction of the SAg in a zinc-dependent manner (Figure 3.10).

Biacore sensorgrams of these interactions demonstrate a stable binding between the proteins in presence of zinc ions whereas the interactions were not observed in the presence of EDTA. Affinity constants (K_D) were obtained for these interactions (Table 3.4) showing AdcA interacting with SpeI with the highest affinity (K_D = 3.3 nM) when in

	In solution	*Immobilized*	k_{off} (s^{-1}) (10^{-4})	k_{off} (s^{-1}) (10^{-4})
Reciprocal	SpeG	SpeI	8.2	
	AdcA	SpeI	18.0	8.1
	SPy_1054	SpeI	2.8	
	SPy_1054	Lmb	2.0	
	DppA	SpeI	4.5	
	DppA	Lmb	1.4	
	Lmb	SpeI	35.0	7.4
	SPy_2066	SpeI	3.4	
	SPy_1326	SpeI	2.7	
	SPy_0925	SpeI	2.0	
Unilateral	SpeG	Lmb	8.7	
	SPy_2066	Lmb	3.2	
	SpeI	SpeI	10.0	
	Lmb	Lmb	38.7	

Table 3.3. k_{off} obtained by Surface Plasmon Resonance analysis of selected interactions. Depending on the proteins selected to be immobilized on the sensor chip in two cases it was possible to calculate the K_{OFF} of the reciprocal interaction as it was done on the microarray.

solution, greater than that observed for SpeI with itself (K_D = 18 nM). Moreover, when in solution, SpeI interacted with AdcA and Lmb with similar affinity (K_D = 8 and 7 nM respectively).

Sub-nanomolar dissociation constants as a result of non-covalent binding interactions between two molecules are rare. Nevertheless, there are some important exceptions.

Biotin and avidin bind with a dissociation constant of roughly 10^{-15} M (0.000001 nM) (Livnah, Bayer et al. 1993) and Ribonuclease inhibitor proteins may also bind to ribonuclease with a similar 10^{-15} M affinity (Johnson, McCoy et al. 2007). It has to be kept in mind that the dissociation constant for a particular ligand-protein interaction, and in this case for protein-protein interaction, can change significantly with experimental conditions (e.g. temperature, pH and salt concentration). The effect of different solution conditions is to effectively modify the strength of any intermolecular interactions holding a particular ligand-protein complex together.

IN SOLUTION	IMMOBILISED	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (nM)
SpeI	**SpeI**	$5.7 \pm 0.30\ 10^{4}$	$1.0 \pm 0.1\ 10^{-3}$	**18 ± 1.2**
SpeI	**Lmb**	$1.0 \pm 0.03\ 10^{5}$	$7.4 \pm 0.7\ 10^{-4}$	**7 ± 0.5**
SpeI	**AdcA**	$1.0 \pm 0.01\ 10^{5}$	$8.1 \pm 0.6\ 10^{-4}$	**8 ± 0.4**
AdcA	**SpeI**	$5.5 \pm 3.23\ 10^{5}$	$1.8 \pm 0.2\ 10^{-3}$	**3.3 ± 0.1**

Table 3.4. For the most interesting interactions a more in-depth analysis was performed on biacore T100 and KD values obtained are in the same range of those of Ras-GTP and RafRBD interaction (Nassar, Horn et al. 1996).

In pharmaceutical research aimed at drug production it is known that harmful side effects can occur through drug molecules interactions with proteins for which they were not meant to or designed to interact with. Therefore research is aimed at designing drugs that bind to only their target proteins with high affinity, typically from 0.1 to 10 nM which is mostly within the range observed for the analyzed interactions. In addition, the SpeI dimer formation affinity value observed in the Biacore T100 analysis is the same as the

one of Ras-GTP interacting with RafRBD: a reference value for protein-protein interactions (Nassar, Horn et al. 1996).

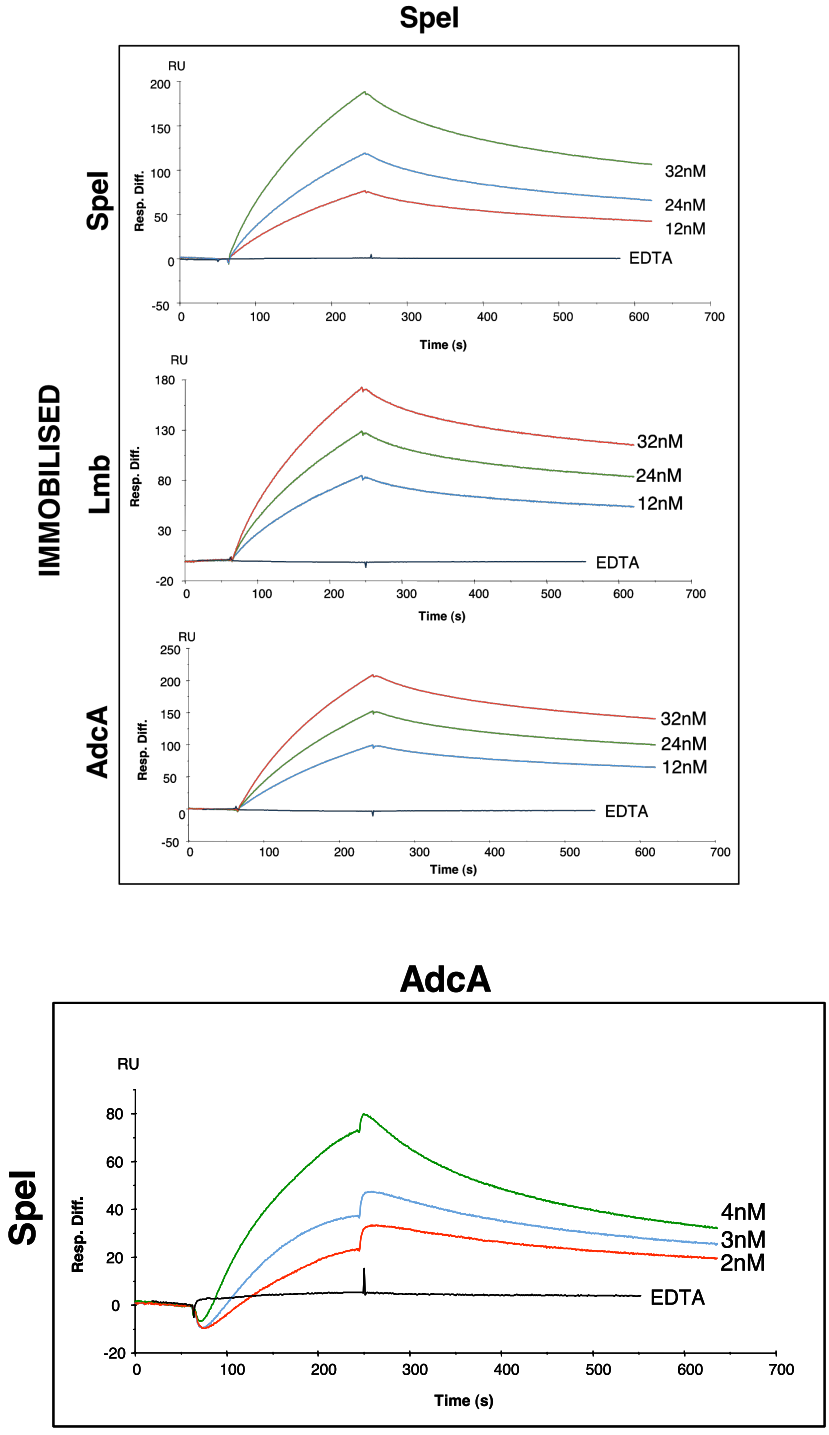


Figure 3.10. Sensorgrams of the zinc dependent interaction of SpeI with the two transition metal transporter AdcA and Lmb with respectively high and low affinity for zinc. The interaction is dependent on the concentration of the injected protein and it is not verified in presence of EDTA.

3.6 Sequence analysis of SpeI interactors

As part of a more accurate analysis of SpeI interactors the aminoacid sequence was compared between SpeI interacting and non-interacting proteins.

The analysis was done on the basis of the single aminoacids as well as the amino acid groups frequency to verify if a particular aminoacid or a specific aminoacid group was playing a role in the interaction with the superantigen.

As Figure 3.11 shows, the pattern of aminoacid and aminoacid group frequencies of the interacting proteins is similar to the frequencies found for the microarray proteins that do not interact with SpeI.

The relative frequency of each aminoacid or aminoacid group was also analyzed leading to the same conclusions.

Whereas a different aminoacid variability does not suggest any particular conclusion regarding the analyzed proteins, a very similar variability suggests that they carry the same properties as all the other proteins and actually the same as the average GAS surface protein.

As shown in Figure 3.12, there is not a significant difference in aminoacid composition among SpeI interactors when compared to the other spotted proteins.

This suggests a more specific interaction with the superantigen not dependent simply on sequence composition, but, perhaps, on a defined binding site pertaining to a specific function.

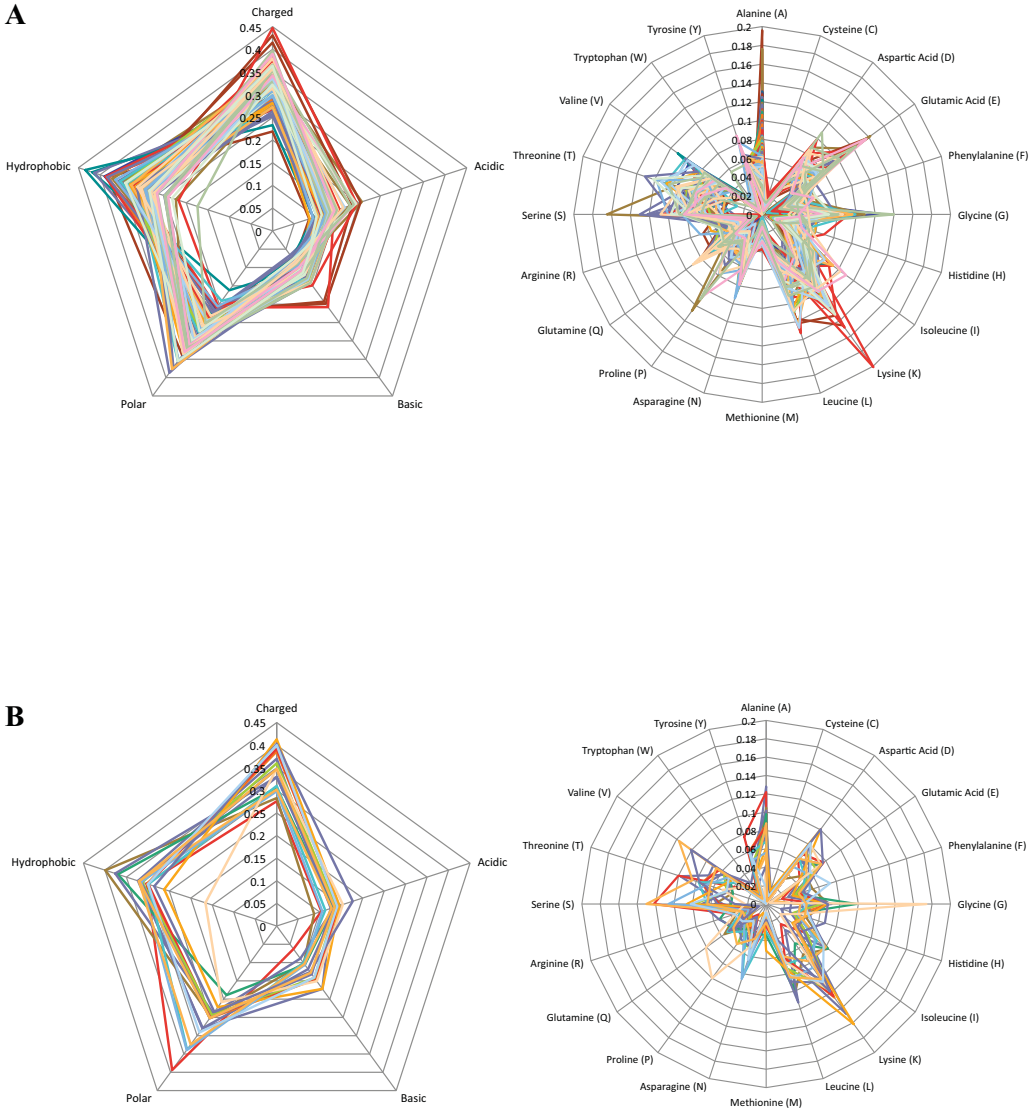


Figure 3.11. Sequence analysis of the SpeI interactors (**B**) compared to the other GAS proteins (**A**) present on the microarray. The sequence percentage of the single aminoacids or the percentage of a specific aminoacid group is comparable among the two groups.

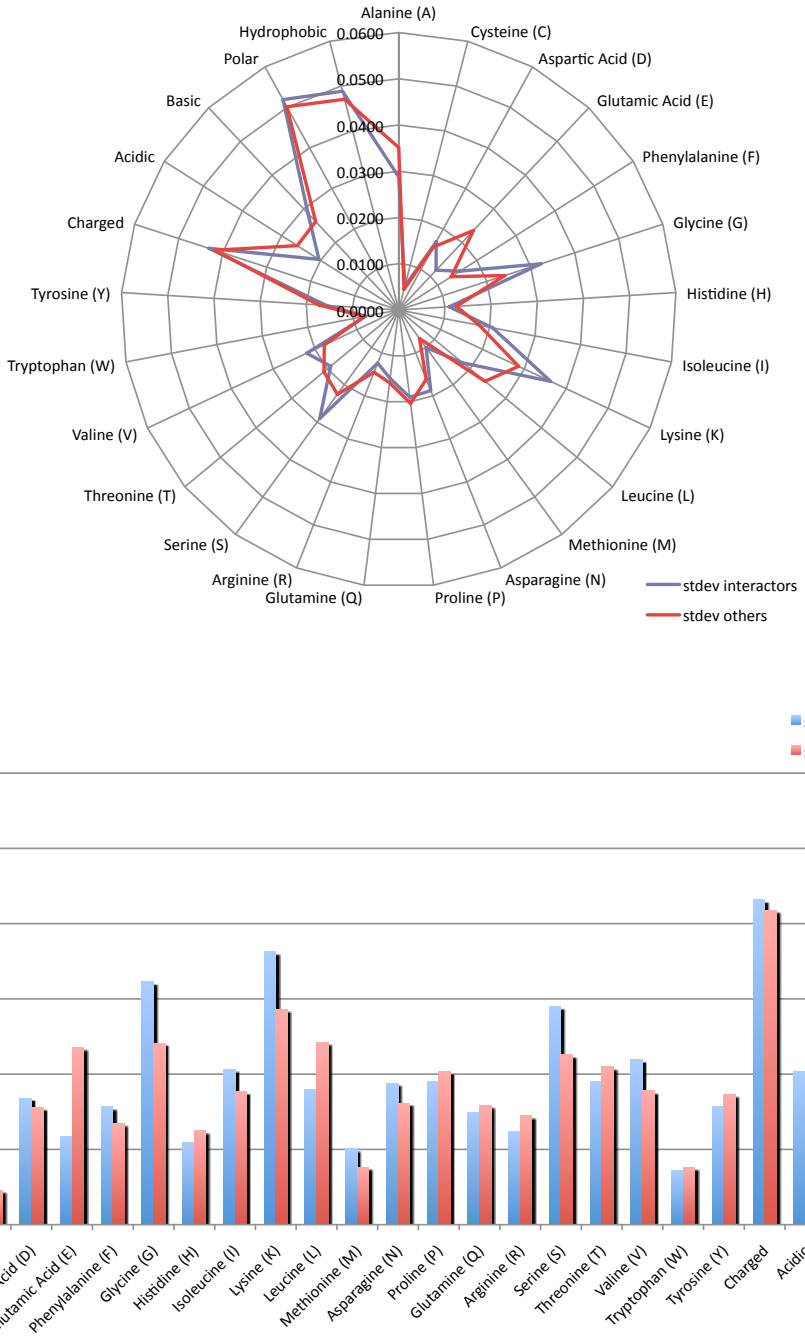


Figure 3.12. Sequence analysis of the SpeI interactors compared to the other GAS proteins present on the microarray. The standard deviation of sequence percentage of the single aminoacids and of the percentage of a specific aminoacid group is comparable among the two groups.

3.7 Fluorescence analysis

Interesting results were obtained from the fluorescence analysis of spotted controls. As mentioned before different forms of BSA were present on the microarray as 8 points of serial dilutions starting from a concentration of 1 mg/ml or 0.5 mg/ml reaching 0.008 mg/ml or 0.004 mg/ml.

In the case of the biotinylated BSA, used as a positive control for binding detection, the 8 point serial dilutions ranged from 1 mg/ml to 0.008 mg/ml resulting in about 0.66 ng of protein in the highest concentration spot and about 0.012 ng in the lowest concentration spot. The fluorescence value of the biotinylated BSA spots obtained by subtracting the background value was analyzed in every experiment and the mean showed below in Figure 3.13.

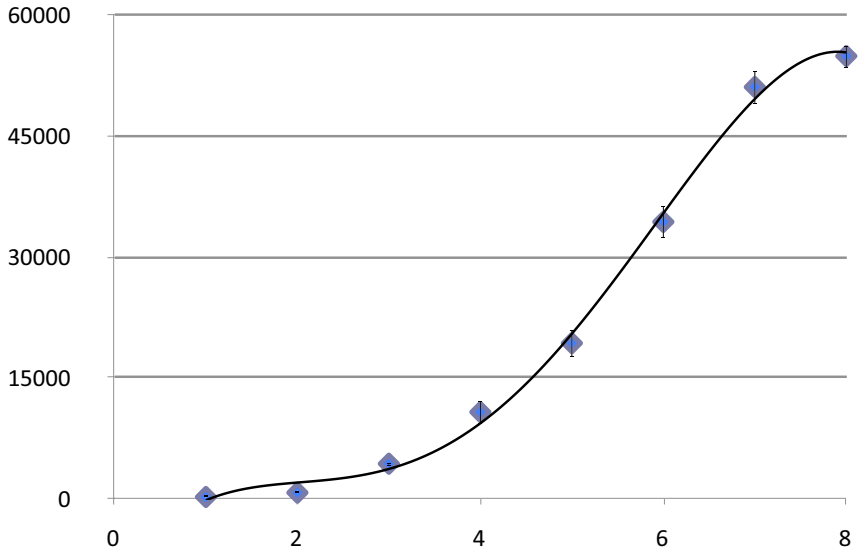


Figure 3.13. Mean fluorescence value of the biotinylated BSA curve of all the slides analyzed for this screening. The curve was spotted in triplicate on every slides since eventually spots with clear spotting anomalies are normally flagged out during fluorescence analysis. Very low standard deviation is observed on every spot.

The first fluorescence value above 5000 (the cutoff used in this screening) is the third point of the curve corresponding to 0.048 ng of biotinylated BSA on the spot. This gives an idea of the sensitivity of this approach, which is able to detect very low amount of biotinylated protein.

Another interesting result is the analysis of the control of every experiment which corresponds to the mean fluorescence value of every protein spot when incubated only with streptavidin-Cy3.

The values, shown below in Figure 3.14, never reach half of our cutoff value for a positive result, which is set at 5000.

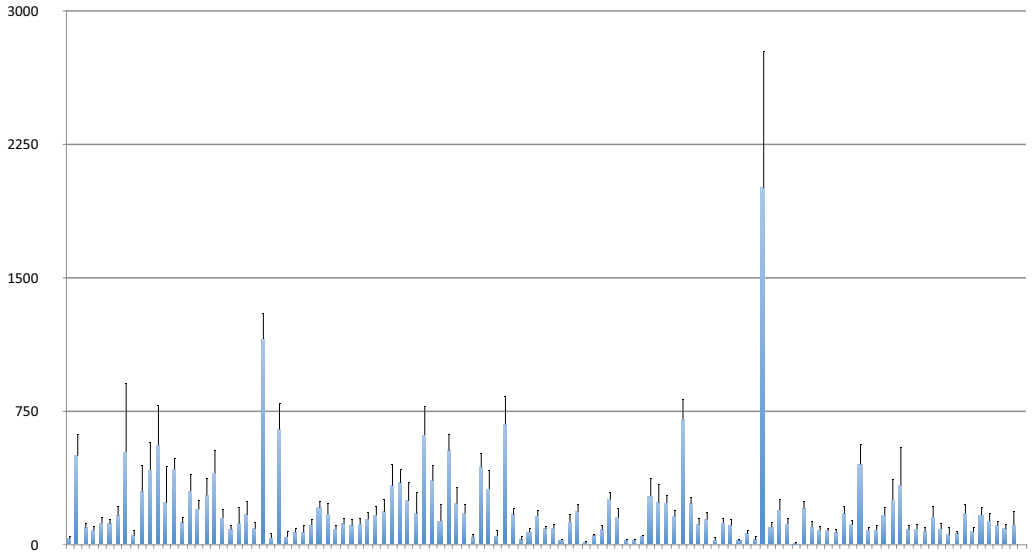


Figure 3.14. Mean fluorescence value of protein spots of all the slides analyzed for this screening when incubated with only streptavidin. One slide incubated only with streptavidin-Cy3 was included into every experiment as hybridization and spotting control. The proteins are spotted in quadruplicates onto every slide and signals with clear anomalies are flagged out during fluorescence analysis and excluded. Every fluorescence signal detected is less than half our chosen cutoff of 5000 (background fluorescence intensity plus 3 times its standard deviation).

It has already been mentioned how it was expected to observe a different behavior for a spotted protein compared to its binding properties when the same protein is in solution. A comparative fluorescence analysis was performed for all the interactions showing the reciprocal fluorescence value when available (Figure 3.15).

It's clear how in most of the cases the fluorescence value of the interaction for a given protein is highest when the protein is in solution. This is due to the technique that has one of the two interacting proteins immobilized on a nitrocellulose matrix and, thus, far from being flexible.

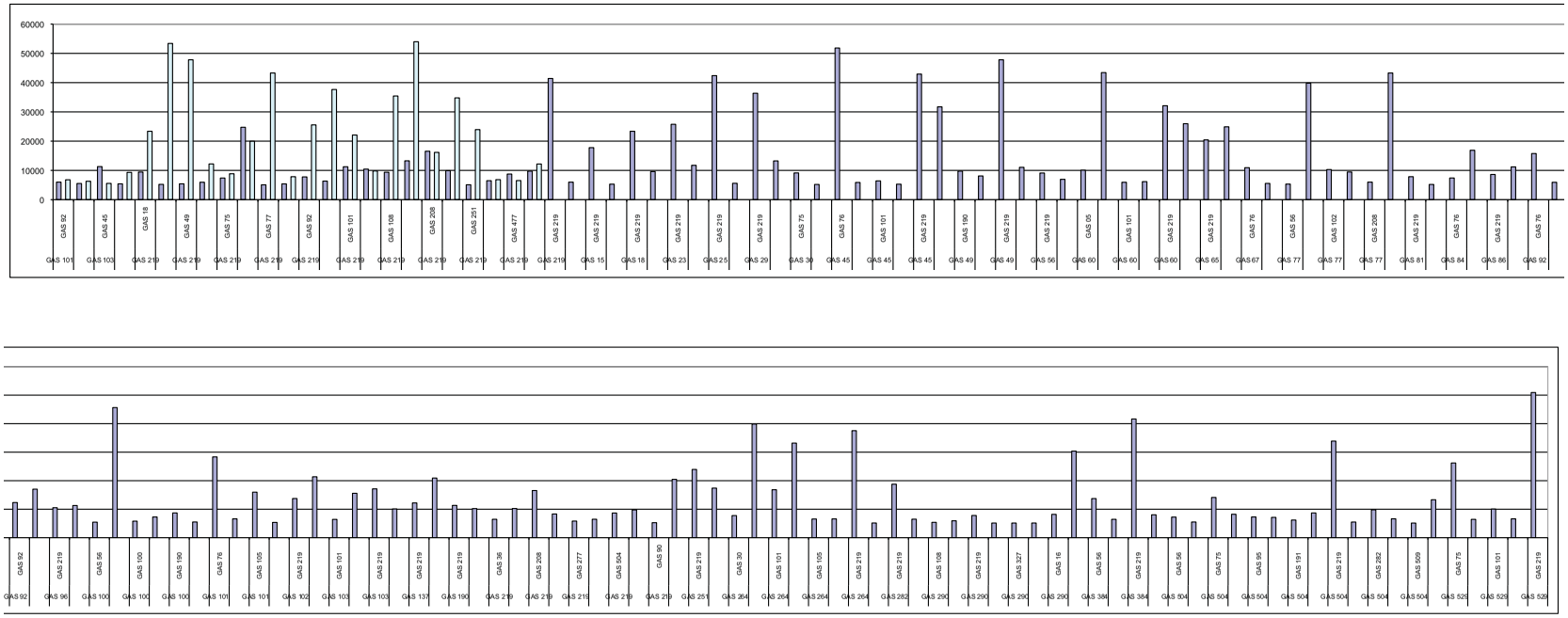


Figure 3.15. Fluorescence analysis of all interactions. Fluorescence values of reciprocal interactions are also reported when available. The difference in fluoresce intensity depending on which of the two interactors is immobilized or in solution suggest that high or low binding affinity can't be determined by this particular approach.

4. Discussion

The screening of about 100 proteins of Group A Streptococcus described in this work is considered as a first step into understanding the composition of protein complexes present on the bacterial cell surface. As of today, efforts are made in studying the cell surface architecture and in developing more advanced tools for protein complexes analysis (Gavin, Bosche et al. 2002; Ho, Gruhler et al. 2002; Yu, Braun et al. 2008).

Knowing the exact composition of the surface of the bacteria and its organization would provide an invaluable tool for developing an effective vaccine against the pathogen. Antigens that are part of a vaccine composition may exist as part of a complex *in vivo* on the bacterial surface.

Focusing the immune response against a complex instead of a single protein will generate a more effective protective antibody response and will avoid the elicitation of "junk" antibodies that occurs when immunizing with only one component of a whole protective protein complex (Figure 4.1).

A protein microarray based experimental approach for high-throughput protein-protein interaction screening was successfully set up involving high throughput protein expression and purification and microarray hybridization under straightforward experimental conditions.

The technique was validated confirming known interactions (such as fibronectin and fibronectin binding proteins or SpeI dimerization) and using Biacore T100 analysis that confirmed all the 14 interaction tested. Nevertheless, even after successful confirmations, this approach must still be considered a screening for protein-protein interactions, where false positives and false negatives have to be expected. Moreover the resulting interactions

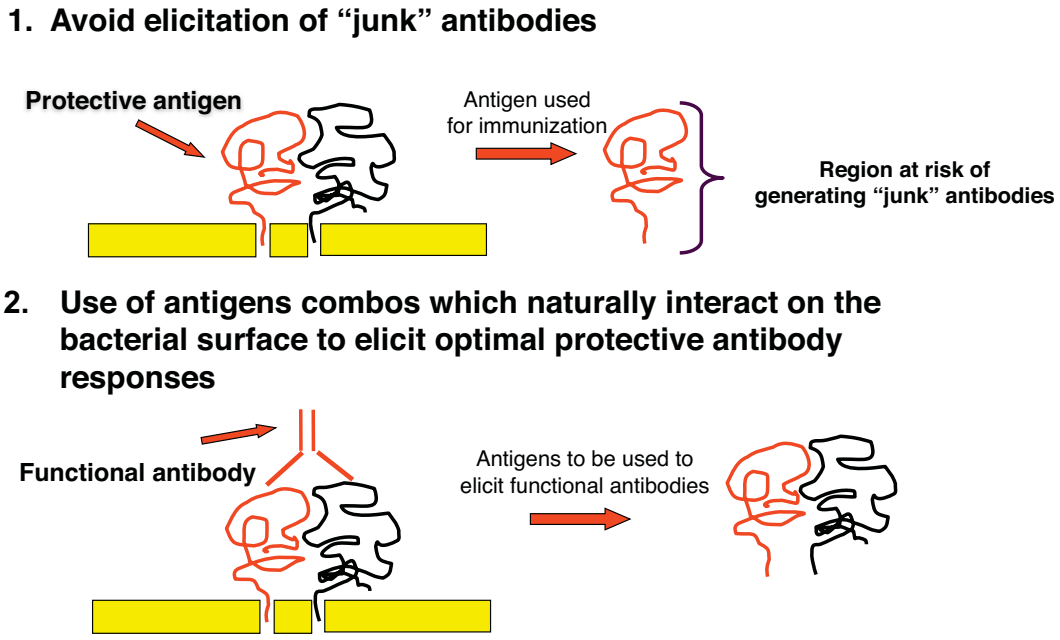


Figure 4.1. Screening for surface protein complexes applied to vaccine research. Immunizing with a complex will avoid elicitation of junk antibodies and will elicit more functional ones.

need to be verified using different *in vitro* techniques and need to be assayed *in vivo* with other methodologies. Another limitation emerged regarding protein behavior, which can be very different when the protein is immobilized or in solution. This would result in a unilateral interaction, instead of a reciprocal one, and become a false negative.

Even though a small number of proteins was included in this screening, interesting interactions emerged mostly regarding an important exotoxin of Group A Streptococcus probably unraveling the mechanism that leads to the maturation and activation of the protein with zinc ions (Proft, Moffatt et al. 1999; Arcus, Proft et al. 2000) via the interaction with two substrate binding proteins of the transition metal sABC transporter family (Chandra, Yogavel et al. 2007; Desrosiers, Sun et al. 2007).

Another interesting interaction of SpeI, confirmed by Biacore analysis, is the one with DppA, another protein belonging to the ABC transporters family in this case specific for

the uptake of dipeptides (Podbielski and Leonard 1998). Podbielski and Leonard described also the influence of this protein on SpeB production, another superantigen with cysteine protease functions. This interaction supports the theory that many proteins participate in the maturation of superantigens during their secretion.

The finding of a unique domain for secretion in *S. pyogenes*, the ExPortal, and the knowledge that it contributes to the secretion of another SAg family protein (SpeB) (Rosch and Caparon 2005) suggest that a protein exported through this domain may have many interactions with other proteins secreted by the bacterium during its transport to the outside and maturation. As reported before, immobilization of the protein samples on solid supports has drawbacks. Possible distortion of the immobilized proteins as well as inconsistent orientation of spotted proteins on the arrays can lead to erroneous results (Washburn 2003). Recently, a new method adopting mass spectrometry techniques has been developed as an alternative to protein microarrays, but it is time consuming and can be used only for a small set of proteins (Ouyang, Takats et al. 2003). Nevertheless, considering all the interactions found during this screening, both reciprocal and unilateral ones, it is possible to start to unravel the major interaction networks of *S. pyogenes* as part of a much broader picture. Even though in this study only reciprocal interactions have been taken into account, several interesting ones may be present in the univocal interactions that may be verified with different approaches (Figure 4.2).

In conclusion it has been shown how protein microarrays could be used for high throughput screening of protein-protein interactions *in vitro* under straightforward experimental conditions, revealing several interactions and opening new perspectives on the current understanding of how proteins are modified by the bacterial cell in order to become major players in causing disease.

Major interaction networks

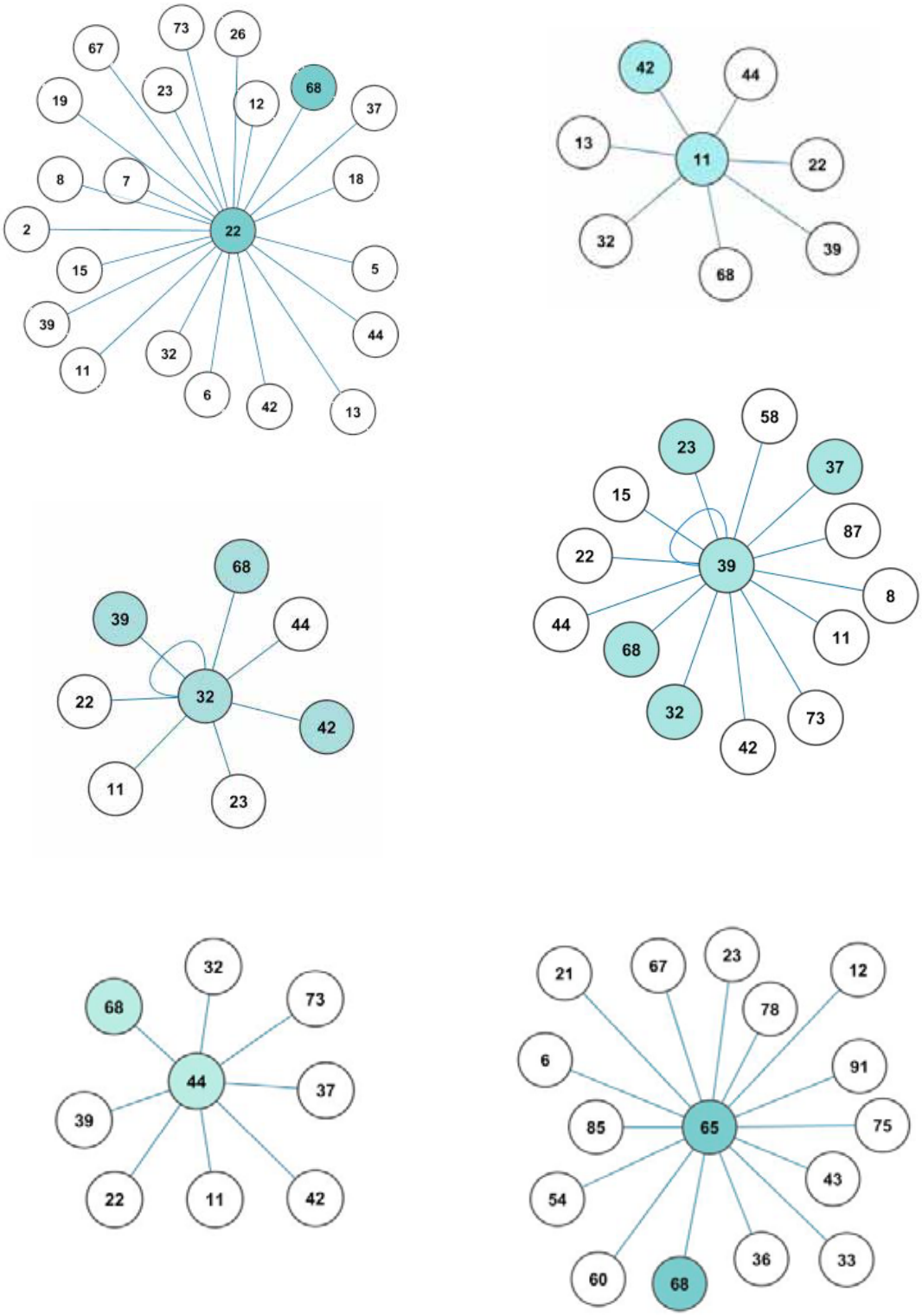
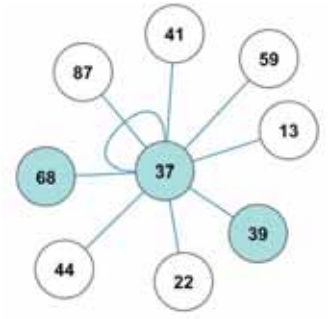
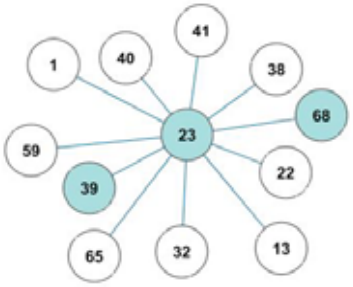
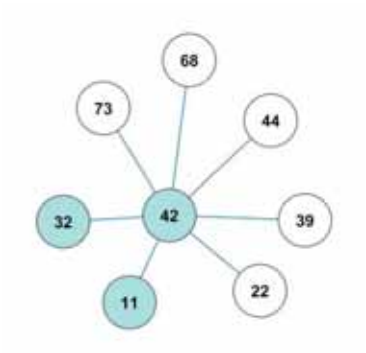
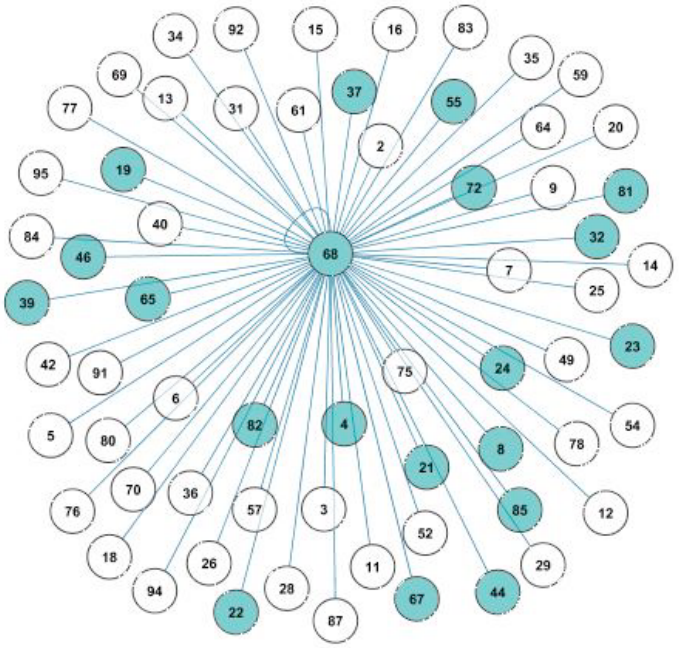


Figure 4.2. Major interaction networks. Reciprocal interactions (in **blue**) and the unilateral interactions are shown resulting in a much broader picture regarding *S. pyogenes* surface protein complexes.


41
87
59
13
68
37
39
44
22


41
1
40
38
68
23
59
39
22
65
32
13


68
73
44
32
42
39
11
22


34
92
15
16
83
35
59
69
13
31
61
37
55
64
20
77
2
95
19
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81
40
84
46
68
32
7
14
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65
25
23
42
91
75
24
49
6
5
80
82
4
54
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11
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94
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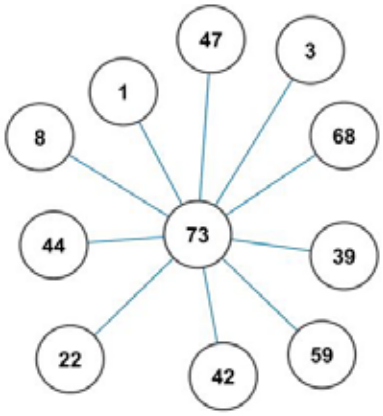
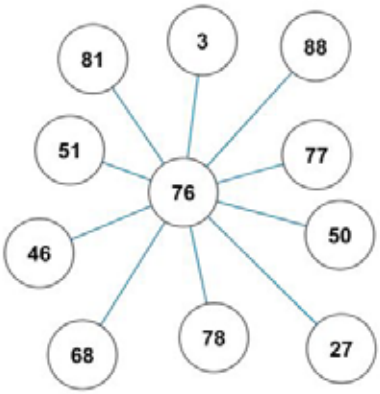
67
44
22
87


47
3
1
8
68
73
44
39
22
42
59


81
3
88
51
77
76
50
46
68
78
27

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Appendix

Spotting program:

VersArray ChipWriter Pro system

Data file about Arraying run settings

Created: 01/11/08-10:48:28

[Source Plate]

Source Plate Type(Number of Wells)= 384(24x16)

Number of Source Plate Positions= One

Sample Picking Direction= Top-down, Left-right

Barcode Reading= No

[Print Head]

Pin Type= Quill

Use Every Second Hole= No

Number of Pins in Y Axis= 2

Number of Pins in X Axis= 6

[Slide/Membrane]

S/M Mode= Slides

Number of Slides in Y= 16

Number of Slides in X= 4

Slide Size(X by Y)= 75x25

Number of Slides between Redips= 64

Dwell Time for Printing= 0.010 (Sec)

[Grid]

Top Left Grid Margin in X= 9.000 (mm)

Top Left Grid Margin in Y= 6.000 (mm)

Bottom Right Grid Margin in X= 2.000 (mm)

Bottom Right Grid Margin in Y= 9.000 (mm)

Distance between Dots in X= 260.000 (micron)

Distance between Dots in Y= 260.000 (micron)

No. of Dots Per Grid in X= 10

No. of Dots Per Grid in Y= 12

No. of Duplicate Dots Per Grid in X= 1

No. of Duplicate Dots Per Grid in Y= 1

No. of Reprints of the Same Dot= 1

Grid Printing Direction= Left-right, Top-down

No. of Different Supergrids in X= 1

No. of Different Supergrids in Y= 1

Distance between Supergrids in X= 4.500 (mm)

Distance between Supergrids in Y= 5.670 (mm)

Supergrid Printing Direction= Left-right, Top-down

No. of Duplicate Supergrid Clusters in X= 1

No. of Duplicate Supergrid Clusters in Y= 1

Distance between Duplicate Supergrid Clusters in X= 4.500 (mm)

Distance between Duplicate Supergrid Clusters in Y= 4.500 (mm)

Use Full Plates= No

No. of Full Plates= 3

No. of Wells in Last Plate= 288

Total No. of Spots in a Grid= 120

[Stacker/Blot]

Use Stackers= Yes

Number of Stackers Used= 5

Use Lid Option= Each plate has lid

Total number of plates in stackers per loading cycle per stacker= 1

Use Blots= Yes

Number of blot slides in blot adapters BLOT1= 2

Number of blot slides in blot adapters BLOT2= 0

Number of blot slides in blot adapters BLOT3= 0

Number of blot slides in blot adapters LONG BLOT= 0

Number of blots per sample= 12

All blotting slides are sufficient for= 5 (plates)

[Washing]

No. of Wash Cycles= 5

Wash Time Per Cycle= 3.000 (sec)

Vacuum Time= 1.000 (sec)

Last Vacuum Time= 2.500 (sec)

UltraSonic Clear Time= 12.000 (sec)

No. of Dips between Sonications= 1

No. of Wash Sessions between Water Bath Refillings= 5

Water Bath Fill Time= 10.700 (sec)

Include Re-dips into No. of Dips(Sonication)= No

Oscillate Pins in Water= Yes

Wash Selection= Always Wash

[Options]

Source Plate Travel Time Down= 1.000 (Sec)

Source Plate Travel Time Up= 1.000 (Sec)

Source Plate Wait Time In= 1.500 (Sec)

Water-bath Oscillation Distance in X= 2.000 (mm)

Water-bath Number of Oscillations in Bath= 4

Blot Slides Blot Distance= 400 (micron)

Blot Slides Blot Dwell Time= 0.030 (sec)

Slide/Membrane/Blot Approach Speed= 6.000 (mm/sec)

****

Run Start:

Plate No.	FirstDip Time	Last Dip Time	Last Dip No.	Last Slide
1	10:51:15	12:27:06	32	(1,4)
2	12:28:58	14:04:48	32	(1,4)
3	14:06:39	15:42:29	32	(1,4)
4	15:44:21	16:57:41	24	(1,4)

Run End

****

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Rino Rappuoli