Molecular studies on streptococcal surface proteins

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Abstract

Streptococcus agalactiae and Streptococcus pyogenes are two related human pathogens causing different diseases. This thesis focuses on a number of surface proteins expressed by S. agalactiae and S. pyogenes and, more specifically, the molecular and biological characterization of these proteins.

The first paper describes the novel Blr protein of S. agalactiae, which together with the previously described Slr protein of S. pyogenes identifies a family of streptococcal proteins with leucine-rich repeats (LRRs). Characterization of Blr and Slr revealed that the two proteins are efficiently camouflaged by other surface components. In S. agalactiae, exposure of Blr was increased ~100-fold in a mutant lacking the polysaccharide capsule, whereas S. pyogenes mutants lacking M protein and/or protein F displayed ~20-fold increased surface exposure of Slr. It seems possible that the camouflaging structures are downregulated during some parts of the infection process, thus exposing the Blr and Slr proteins on the streptococcal surface.

In the second paper, we analyzed a key problem in the vaccine field, the identification of antigens that elicit protective immunity. Our studies were focused on the immune response to the S. agalactiae proteins Rib and alpha. These proteins contain a unique N-terminal region and long repetitive C-terminal sequences. The immune response against pure Rib or alpha was almost exclusively directed against the repeat region, i.e. very few antibodies were directed against the N-terminal regions. Thus, the N-terminal region is nonimmunodominant in both Rib and alpha. Nevertheless, a fusion protein comprising the N-terminal regions of Rib and alpha elicited antibodies that were protective. Importantly, studies of the S. pyogenes M22 protein showed that the N-terminal region, which is targeted by opsonic antibodies, was also nonimmunodominant. Together, these results indicate that nonimmunodominant regions are of general interest for vaccine development.

The third paper addresses the role of fibrinogen (Fg) in bacterial virulence. We characterized the Fg-binding B repeat region of the S. pyogenes M5 protein. In a mouse model, a mutant lacking the Fg-binding domain was severely attenuated, indicating that binding of Fg plays a key role in virulence. Similarly, a bacterial mutant lacking the most N-terminal part of the M5 protein was avirulent. Together, these data indicate that two separate regions of M5 are important for virulence. The function of bacteria-bound Fg is not known, but our data support the notion that bound Fg protects the bacteria against complement deposition and thereby against phagocytosis.

Key words: Streptococcus agalactiae, Streptococcus pyogenes, leucine-rich repeat, fibrinogen, M protein, surface protein, Rib, alpha, nonimmunodominance, camouflaging, vaccine

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CONTENTS

LIST OF PAPERS

This thesis is based on the following papers, which are referred to in the text as **papers I-III**.

I: Waldemarsson, J., Areschoug T., Lindahl, G. and Johnsson, E. (2006). The streptococcal Blr and Slr proteins define a family of surface proteins with leucine-rich repeats: camouflaging by other surface structures. *J Bacteriol* 188:378-388[1]

II: Stålhammar-Carlemalm, M.*, Waldemarsson, J.*, Johnsson, E.*, Areschoug, T., and Lindahl, G. (2007). Nonimmunodominant regions are effective as building blocks in a streptococcal fusion protein vaccine. *Cell Host & Microbe* 2: 427-434[2]
* These authors made equal contributions

III: Waldemarsson, J., Stålhammar-Carlemalm, M., Sandin, C., and Lindahl, G. (2008). Critical role for bacteria-bound fibrinogen in *Streptococcus pyogenes* invasive infection. *Manuscript*

ABBREVIATIONS

ARF	Acute rheumatic fever
C1-inh	C1-inhibitor
C4BP	C4b-binding protein
CD	Cluster of differentiation
Fc	Fragment crystallizable
Fg	Fibrinogen
FH	Factor H
FHL-1	Factor H-like protein 1
HVR	Hypervariable region
Ig	Immunoglobulin
InlA	Internalin A
I.p.	Intraperitoneal
LRR	Leucine-rich repeat
MAC	Membrane attack complex
MASP	MBL-associated serine protease
MBL	Mannose-binding lectin
Mga	Multigene regulator of group A streptococcus
MLST	Multilocus sequence typing
OF	Opacity factor
PSGN	Post-streptococcal glomerulonephritis
SOF	Serum opacity factor
ST	Sequence type
SVR	Semivariable region
TT	Tetanus toxiod

SUMMARY

Streptococcus agalactiae and *Streptococcus pyogenes* are two related human pathogens causing different diseases. *S. agalactiae* (group B streptococcus; GBS) is the most important cause of pneumonia, sepsis and, in particular, meningitis in the neonatal period, whereas *S. pyogenes* (group A streptococcus; GAS) is responsible for mild infections such as pharyngitis and impetigo, but also in rarer cases severe and life-threatening conditions such as necrotizing fasciitis and the streptococcal toxic shock syndrome. This thesis focuses on a number of surface proteins expressed by *S. agalactiae* and *S. pyogenes* and, more specifically, the molecular and biological characterization of these proteins.

The first paper describes the novel Blr protein of *S. agalactiae*, which together with the previously described Slr protein of *S. pyogenes* identifies a family of streptococcal proteins with leucine-rich repeats (LRRs). The LRR motif is commonly found in proteins involved in different ligand interactions. Characterization of Blr and Slr revealed that the two proteins are efficiently camouflaged by other surface components. In *S. agalactiae*, exposure of Blr was increased ~100-fold in a mutant lacking the polysaccharide capsule, whereas *S. pyogenes* mutants lacking M protein and/or protein F displayed ~20-fold increased surface exposure of Slr. The physiological relevance of this phenomenom is unclear, but it is possible that the camouflaging structures are downregulated during some parts of the infection process, thus exposing the Blr and Slr proteins on the streptococcal surface.

In the second paper, we analyzed a key problem in the vaccine field, the identification of antigens that elicit protective immunity. Our studies were focused on the immune response to the *S. agalactiae* proteins Rib and α. These proteins contain a unique N-terminal region and long repetitive C-terminal sequences. The immune response against pure Rib or α was almost exclusively directed against the repeat region, i.e. very few antibodies were directed against the N-terminal regions. Thus, the N-terminal region is nonimmunodominant in both Rib and α. Nevertheless, a fusion protein comprising the N-terminal regions of Rib and α elicited antibodies that were protective and this fusion protein had properties much superior to one of similar size derived from the repeats. Importantly, studies of the *S. pyogenes* M22 protein showed that the N-terminal region, which is targeted by opsonic antibodies, was nonimmunodominant also in this protein. Together, these results indicate that non-immunodominant regions are of general interest for vaccine development.

The third paper addresses the role of fibrinogen (Fg) in bacterial virulence. Many human pathogens have been shown to bind Fg, and this interaction has been studied in detail

in vitro. However, the contribution of bacterial Fg binding to virulence is unclear. In this study we characterized the Fg-binding B repeat region of the *S. pyogenes* M5 protein. In a number of biochemical assays, the M5 protein was demonstrated to interact with mouse Fg. Thus, the mouse was a suitable model system to study the effect of the B repeat region on virulence. In the mouse model, a mutant lacking the Fg-binding domain was severely attenuated, indicating that binding of Fg indeed plays a key role in virulence. Similarly, a bacterial mutant lacking the most N-terminal part of the M5 protein was avirulent. Together, these data indicate that two separate regions of M5 are important for virulence. The effect observed with the mutant lacking the B repeat region was most likely due to binding of Fg and not to another mouse protein binding to this region, since a peptide derived from the B repeats only interacted with Fg among all proteins in mouse plasma. The function of bacteria-bound Fg is not known, but our data support the notion that bound Fg protects the bacteria against complement deposition and thereby against phagocytosis.

INTRODUCTION

The interplay between a bacterial pathogen and its host is often complex and multifaceted. An important part of these interactions relies on proteins located on the bacterial surface. As a consequence, studies of bacterial surface proteins and their role in pathogenesis have been the subject of intense research for a long time. In addition, surface proteins are crucial vaccine components, a research area that is attracting ever-growing attention due to the increasing number of antibiotic resistant bacterial strains.

This thesis focuses on surface proteins expressed by *Streptococcus agalactiae* and *Streptococcus pyogenes*, two important human pathogens causing infections ranging from relative mild to life-threatening ones. *S. agalactiae* is the most common cause of infections in the neonatal period, in which it is responsible for conditions like pneumonia, sepsis and meningitis. It has also attracted increasing attention as the cause of infections in adults (Schuchat, 1998; Edwards and Baker, 2001). *S. pyogenes* primarily infects children and young adults and commonly causes pharyngitis ('strep throat') and skin infections (impetigo). More severe diseases caused by *S. pyogenes* include necrotizing fasciitis, the streptococcal toxic shock syndrome, and the post-infectious sequelae acute rheumatic fever and acute glomerulonephritis (Cunningham, 2000; Carapetis *et al.*, 2005b).

The first two parts of this thesis will cover *S. agalactiae* and *S. pyogenes*, with emphasis on surface proteins relevant to my work. The following parts will deal with subjects that are of particular relevance to this thesis: proteins with leucine-rich repeats (LRRs), fibrinogen and the complement system.

STREPTOCOCCUS AGALACTIAE

Originally discovered as a common cause of bovine mastitis (Brown, 1920; Stableforth, 1938), *S. agalactiae* (group B streptococcus; GBS) was first demonstrated to be a human pathogen in 1938, when three cases of fatal infection were described (Fry, 1938). During the following decades, few infections caused by *S. agalactiae* were observed. It was not until the 1970s, when a dramatic increase in infection frequency was observed, that this organism attracted attention as a major neonatal pathogen. During the last 30 years *S. agalactiae* has remained the most important cause of pneumonia, sepsis and meningitis among newborns. Moreover, *S. agalactiae* is an important cause of infections among pregnant women and has received increasing attention as a cause of serious infections in nonpregnant adults with underlying medical conditions (Schuchat, 1998; Edwards and Baker, 2001). Of note, *S.*

agalactiae is part of the normal gastrointestinal and/or rectovaginal flora in many humans (Edwards and Baker, 2001).

Neonatal infections

Neonatal infections caused by *S. agalactiae* fall into two distinct categories, depending on the time when symptoms are observed: 1) early-onset disease (EOD), which affects the fetus *in utero* or newborns up to 7 days of age; and 2) late-onset disease (LOD), affecting children of the age of 8 days up to 3 months. Until recently, the absolute majority (~80%) of neonatal infections fell into the first category, but due to preventive antibiotic treatment of pregnant women in the United States, the frequency has dropped drastically (Schrag *et al.*, 2000). In contrast, the frequency of late-onset disease has not changed. In this category, the predominant disease is meningitis, a very serious condition which may cause death. In addition, among the survivors of neonatal meningitis a variety of sequelae are observed. Up to 50% of survivors suffer from neurological sequelae, such as mental retardation, cortical blindness, deafness, uncontrolled seizures, hydrocephalus, and speech as well as language delay (Schuchat, 1998; Edwards and Baker, 2001).

Early-onset disease

The primary infection route for early-onset disease is vertical transmission from asymptomatically colonized mothers. Most cases are believed to be due to an ascending spread of bacteria through ruptured membranes into the amniotic fluid, in which the bacteria can multiply and gain access to the respiratory tract of the fetus.

About 15-35% of all women of fertile age are colonized with *S. agalactiae* in the vagina. However, only a minor fraction (~1%) of children given birth by these women will develop disease. The reason for the vulnerability of these children is not understood, but several risk factors have been identified, such as premature delivery (<37 weeks' gestation), low birth weight, rupture of the amniotic membrane before labor onset and low maternal levels of type-specific IgG antibodies. Diseases frequently associated with early-onset disease include sepsis and pneumonia and, less frequently, meningitis. The attack rate is between 0.7 and 3.7 per 1000 live births and the mortality rate now ranges between 5 and 10% (Schuchat, 1998; Edwards and Baker, 2001).

Late-onset disease

Less is known about the pathogenesis of late-onset disease, but vertical transmission from the mother is thought to be of importance in many cases. This hypothesis is supported by studies showing that, in 50% of cases, bacterial isolates from the mothers have the same serotype as the bacteria isolated from the affected children. Horizontal transmission from hospital workers (i.e. nocosomial infections) and the community may also play a role, but the significance of these two factors remain largely unknown (Schuchat, 1998).

Clinical manifestations of late-onset disease include bacteremia without focus, meningitis and osteoarthritis. As noted above, the frequency of meningitis is considerable higher in late-onset disease than in early-onset disease. The attack rates range from 0.5 to 1.8 per 1000 live births (Edwards and Baker, 2001), comparable to the rates nowadays observed with early-onset disease (Schrag *et al.*, 2000). The mortality rate is lower compared to early-onset, between 2 and 6% (Edwards and Baker, 2001).

Infections in adults

S. agalactiae not only causes neonatal infections, but is also an important cause of infections in the adult population. Both pregnant women and nonpregnant adults are at risk.

As in the case of neonatal infections, very few cases of infections among pregnant women were observed before the 1970s (Edwards and Baker, 2001). Manifestations in pregnant women range from mild noninvasive infections to life-threatening conditions. Among the invasive presentations, bloodstream infections are the most common, but osteomyelitis, endocarditis and meningitis have also been observed (Schuchat, 1998).

In contrast to infections among pregnant women, *S. agalactiae* has been recognized as an occasional cause of invasive disease in nonpregnant adults since the 1940s (Rantz and Keefer, 1941; Rantz and Kirby, 1942). Among nonpregnant adults, elderly with underlying medical conditions are especially prone to infections caused by *S. agalactiae*. The association is particularly strong with diabetes mellitus, but liver cirrhosis and previous strokes have also been suggested to be predisposing factors. The most common clinical manifestation is bacteremia, but skin or soft tissue infections, pneumonia and meningitis are also seen. Population based surveillance studies in the United States have revealed an increasing incidence rate with 2.4 to 4.4 cases per 100.000 nonpregnant adults. The mortality is usually higher compared to neonatal infections, with rates as high as 21% (Schuchat, 1998, 1999).

Polysaccharide capsule structure and serotype classification

Pioneering work by Rebecca Lancefield in the 1930s revealed the presence of the group B carbohydrate (originally designated "C" substance), common to all *S. agalactiae* strains (Lancefield, 1933). This carbohydrate, which resides in the cell wall, is a complex structure composed of rhamnose, galactose, glucitol and *N*-acetylglucosamine (Michon *et al.*, 1987). Studies in animal models have indicated that it is not a target for protective antibodies (Lancefield *et al.*, 1975).

Lancefield also discovered the so-called type-specific polysaccharide antigen, originally designated the "S" substance (Lancefield, 1934, 1938). These early studies showed that strains of *S. agalactiae* can be divided into four distinct capsular serotypes based on the antigenic property of the type specific antigen. The four types were designated Ia, Ib, II and III, a classification still used today. After the discovery of these four classical serotypes, five additional serotypes have been described (Kogan *et al.*, 1996), making the total number of serotypes nine (Ia, Ib, II-VIII).

The structure and components of the polysaccharide capsule have been studied in great detail. The individual components are galactose, glucose, *N*-acetylglucosamine and sialic acid, juxtaposed in unique combinations, thereby creating antigenically distinct structures. Importantly, sialic acid is always present as a terminal residue of the polysaccharide side chain (Edwards and Baker, 2001).

The capsule elicits protective immunity to *S. agalactiae* infections (Lancefield *et al.*, 1975; Edwards and Baker, 2001). The importance of anti-capsular antibodies in the mother was shown in studies by Baker and Kasper (1976) and Hemming *et al.* (1976), who concluded that neonates born by mothers with a low concentration of type-specific antibodies were at higher risk of developing a bacterial infection.

The role of the capsule in bacterial virulence has been addressed in several studies. Strains producing large amounts of the type III polysaccharide capsule are more virulent in mice compared to strains with lower capsular levels (Durham *et al.*, 1981; Yeung and Mattingly, 1984). Importantly, Rubens *et al.* (1987) showed that an acapsular mutant derived from a type III strain was attenuated for virulence in a neonatal rat model. Further studies using a mutant lacking the sialic acid residue of the capsule showed that this mutant was as attenuated as a mutant lacking the entire capsule (Wessels *et al.*, 1989), demonstrating that the terminal sialic acid component is essential for bacterial virulence.

Antibodies directed against the type III capsule opsonize *S. agalactiae* bacteria in the presence of complement (Edwards *et al.*, 1980). In the absence of type specific antibodies the

sialic residue prevents consumption of C3 and factor B (Edwards *et al.*, 1982), suggesting that interference with the alternative pathway of the complement system may explain the virulence properties of the sialylated capsule (Marques *et al.*, 1992).

The division into distinct capsular types is very useful from an epidemiological point of view. Studies in the 1970s and 1980s demonstrated that the four classical serotypes were uniformly distributed among strains carried by asymptomatically colonized women and neonates. Very similar serotype distribution patterns were described in strains isolated from neonates with early-onset disease without meningitis. However, in late-onset disease, the predominant serotype is type III. In particular, >90% of the strains causing meningitis belong to this serotype (Edwards and Baker, 2001). These findings focus interest on serotype III.

During the early 1990s, a serological shift occurred in North America with the emergence of type V strains as a cause of some invasive infections among neonates and as a common cause of infections in nonpregnant adults (Blumberg *et al.*, 1996; Lin *et al.*, 1998). Epidemiological studies from England, Wales and Sweden mirror those findings (Berg *et al.*, 2000; Persson *et al.*, 2004; Weisner *et al.*, 2004). Of note, the serotype distribution in Japan may be quite different compared to Western Europe and the United States. In this country, types VI and VIII were reported to be the most frequently occurring serotypes in colonized women (Lachenauer *et al.*, 1999).

Sequence types

In addition to the classification system based on the polysaccharide capsule, strains of *S. agalactiae* can be divided into so-called sequence types (STs) using a technique designated MLST (multilocus sequence typing). This technique is based on the DNA sequence of internal fragments of seven housekeeping genes and has been used to study the population structure of several pathogens, including *Neisseria meningitidis* and *Streptococcus pneumoniae* (Enright and Spratt, 1998; Maiden *et al.*, 1998). The first study using MLST to classify *S. agalactiae* described 29 STs, of which ST-17 was the most common one (Jones *et al.*, 2003). Importantly, ST-17 was strongly associated with invasive neonatal disease and all ST-17 strains were of serotype III, further highlighting the importance of this serotype as a cause of meningitis among neonates. A study using clinical isolates from Sweden came to a similar conclusion (Luan *et al.*, 2005), supporting the conclusion that ST-17 strains have enhanced virulence. Interestingly, ST-17 is identical to ET-1, a putative high-virulence clone identified in 1989 through an electrophoretical method (Musser *et al.*, 1989). Of note, a comparative study of bovine and human isolates reported that ST-17 strains may be more

related to bovine strains than to other human strains, suggesting that ST-17 strains may be of bovine origin (Bisharat *et al.*, 2004).

Genomics

To date, three complete and five draft genome sequences of *S. agalactiae* are available. The strains studied represent the four classical serotypes (Ia, Ib, II and III) and the emerging serotype V. Of note, only one of these strains belongs to the clinically important ST-17 lineage (see above). Analysis of the eight genomes revealed a genome size of ~2.1 Mbp and the number of predicted genes ranged between 2,034 and 2,481. Furthermore, the core genome, i.e. the number of genes shared by all strains, consists of ~1,800 genes. The variable parts of the genome are commonly flanked by insertion elements, suggesting that horizontal gene transfer events have occurred in these regions (Glaser *et al.*, 2002; Tettelin *et al.*, 2002; Tettelin *et al.*, 2005).

Two of the completely sequenced genomes have been analyzed in detail for the presence of genes encoding putative surface proteins. In strain NEM316, belonging to serotype III, 30 open reading frames containing the LPXTG motif were identified (Glaser *et al.*, 2002), and in the type V strain 2603V/R the corresponding number was 24 (Tettelin *et al.*, 2002). These genomes were also analyzed for presence of genes encoding putative lipoproteins, a distinct class of surface proteins, which in contrast to LPXTG proteins are tethered to the cell membrane via the N-terminal part (Sutcliffe and Russell, 1995). This analysis revealed 36 and 51 such genes in strains NEM316 and 2603V/R, respectively (Glaser *et al.*, 2002; Tettelin *et al.*, 2002).

Surface proteins

The presence of a *S. agalactiae* surface component of proteinaceous nature was first described in 1971 (Wilkinson and Eagon, 1971). Designated Ibc or the C antigen, this surface antigen was shown to be composed of two distinct parts, one trypsin resistant fraction (TR) and one fraction sensitive to trypsin (TS). These fractions were later shown to correspond to distinct proteins, which were designated α and β (Bevanger and Maeland, 1979). The two proteins are expressed, together or separately, by many type Ia and type Ib strains, occasionally by type II strains and very rarely by type III strains (Bevanger, 1983; Johnson and Ferrieri, 1984). Instead, most type III strains express another surface protein, designated Rib (Stålhammar-Carlemalm *et al.*, 1993), which is also present in some type II strains. As described below,

Rib and α belong to the Alp family of proteins, which are characterized by their extremely repetitive structure (Michel *et al.*, 1992; Wästfelt *et al.*, 1996; Lindahl *et al.*, 2005).

Several early studies investigated whether surface proteins expressed by *S. agalactiae* elicit protective immunity. Using rabbit antiserum raised against whole bacteria, Lancefield *et al.* showed that antibodies against the C antigen were protective in a mouse model (Lancefield *et al.*, 1975). This finding was corroborated in a study by Bevanger and Naess (1985), who showed that antiserum against partially purified α and β protected mice against lethal infection. Similar protection data were obtained with recombinant proteins (Michel *et al.*, 1991). These studies attracted rather limited attention, because α and β are almost never found in the clinically very important serotype III strains. However, subsequent work demonstrated that almost all type III strains express the Rib protein, which elicits protective immunity (Stålhammar-Carlemalm *et al.*, 1993). This finding focused interest on surface proteins as components in *S. agalactiae* vaccines. The initial characterization of Rib indicated that it is related to α, but the two proteins do not show antigenic cross-reactivity (Stålhammar-Carlemalm *et al.*, 1993).

As described above, analysis of the *S. agalactiae* genome have revealed several known and putative surface proteins. However, to describe all of them is beyond the scope of this thesis, which is focused on surface proteins which elicit protective immunity. Below I will summarize the current knowledge of *S. agalactiae* surface proteins with such properties.

The Alp family

In 1991, Michel *et al.* purified recombinant α protein from *E. coli* and showed that the protein gave rise to a regular ladder pattern when analyzed by Western blot (Michel *et al.*, 1991). Sequence analysis of the gene (designated *bca*) encoding α revealed the presence of 9 repeats, each identical even at the nucleotide level (Michel *et al.*, 1992). Interestingly, the distance between the bands in the ladder pattern in the blots corresponded to the size of one repeat. It was initially suggested that this size heterogeneity was due to the synthesis of α proteins with different number of repeats (Michel *et al.*, 1992), but subsequent work indicated that the ladder pattern is an artifact and is due to the presence in each repeat of an acid labile Asp-Pro bond that is hydrolyzed during the Western blot analysis (Wästfelt *et al.*, 1996). Similarly, the repeats of Rib contain two Asp-Pro bonds, explaining the appearance of a ladder pattern (Wästfelt *et al.*, 1996). Because of this property, Rib and α have been referred to as “laddering proteins”.

The structural similarity between Rib and α became apparent when the sequence of the *rib* gene was determined, demonstrating that Rib contained 12 repeats, identical at the nucleotide level and sharing extensive similarity to α. Thus, Rib and α are members of a novel protein family with members that have extremely repetitive structure (Wästfelt *et al.*, 1996). This family was later designated the Alp family, for α-like protein family (Baron *et al.*, 2004; Lindahl *et al.*, 2005). Of note, the members of the Alp family are encoded by allelic genes, i.e. each *S. agalactiae* strain encodes a single member of the family.

While the size of each Alp family protein is constant within a strain, there is considerable size difference between strains. For instance, the molecular size of the α protein has been reported to vary between 62.5 and 167 kDa (Madoff *et al.*, 1991), whereas the corresponding interval for Rib is 65-125 kDa (Stålhammar-Carlemalm *et al.*, 1993). The size difference is due to variation in the number of repeats. Rare intragenic recombination events during DNA replication might explain this phenomenon (Puopolo *et al.*, 2001).

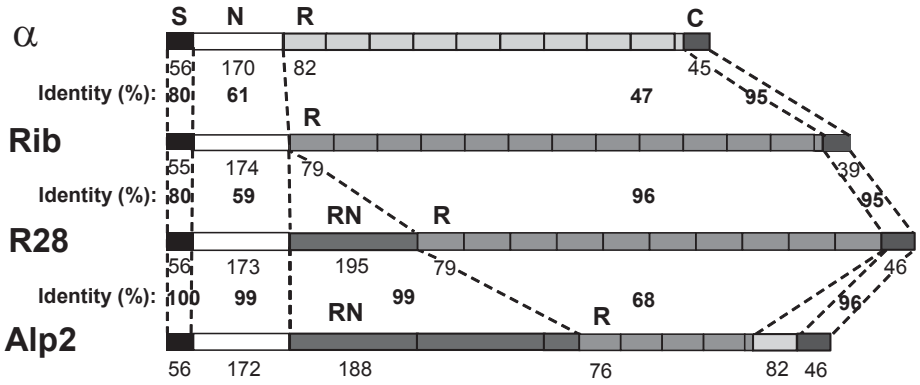

Figure 1. Sequence comparion of the four members of the Alp family. The number of amino acids in each protein subregion is indicated. Residue identity (%) between protein sections are indicated in bold. S, signal sequence; N, nonrepeated N terminal region; RN, repeat region present in only R28 and Alp2; R = repeat region present in all four members; C, C terminal region. Adopted from Lindahl *et al.* (2005).

The genetic organization of Rib and α is similar (Figure 1). Following an unusually long signal sequence (55 amino acids in Rib; 56 amino acids in α (Wästfelt *et al.*, 1996)) there is an N-terminal region of about 170 residues and a tandemly arranged repeat region (described above). The C-terminal region contains an LPXTG motif, which is used to covalently attach the mature protein to the cell wall (Navarre and Schneewind, 1999). The signal sequences are 80% identical at the amino acid level, the N-terminal regions show 61% residue identity,

whereas the corresponding figure for the repeats is 47%. Finally, the short C-terminal regions are almost identical (95% residue identity).

Following the discovery of Rib and α, two additional proteins belonging to the Alp family have been described. These two proteins are designated R28 and Alp2. The R28 protein was originally discovered in *Streptococcus pyogenes* (Lancefield and Perlmann, 1952), and was shown to be a member of the Alp family (Stålhammar-Carlemalm *et al.*, 1999). Subsequent studies demonstrated that it was also present in many *S. agalactiae* strains of serotypes V and VIII, where it was designated Alp3 (Lachenauer *et al.*, 2000). Because R28 and Alp3 are nearly identical (98% residue identity) the name R28 will be used here for both proteins. Interestingly, the N-terminal region of R28 is longer than those of Rib/α and part of this region shows homology to a region of the β protein (see below), which is not a member of the Alp family (Stålhammar-Carlemalm *et al.*, 1999). In contrast, the repeat region of R28 is very similar to that of Rib (96% identity).

The fourth member of the family, designated Alp2, was first described in a type V strain (Lachenauer and Madoff, 1996) and was shown to be almost identical to R28 in its N-terminal part. The Alp2 protein occurs rather infrequently among type Ia, III and V strains (Lachenauer *et al.*, 2000; Kong *et al.*, 2002).

TABLE 1. Relationship between capsular serotype and expression of Alp proteins

Capsular serotype	*Commonly expressed Alp protein*
Ia	α or ε
Ib	α
II	α or Rib
III	Rib
V	R28
VIII	R28

Adopted from Lindahl *et al.* (2005) and Brimil *et al.* (2006)

Two further genes have been postulated to encode members of the Alp family. However, these genes have not been characterized in detail and will only be briefly mentioned here. The atypical, non-typeable strain Prague 25/60 was shown to harbor a gene designated *alp4*, which was only partially sequenced. This gene was not present in any other strain analyzed (Kong *et al.*, 2002). A putative Alp family member designated ε (epsilon) or Alp1

has also been described (Puopolo and Madoff, 2003; Creti *et al.*, 2004). This protein may be expressed by many type Ia strains previously considered to express α (Brimil *et al.*, 2006).

The distribution of Alp family members among different serotypes is summarized in table 1. Importantly, there is a strong correlation between the expression pattern of members of the Alp family and the capsular serotype, a correlation which is particularly strong among serotype III strains, which almost exclusively express the Rib protein.

Biological function. The biological function of the Alp family proteins remains unclear, but some studies indicate that they may promote adhesion or invasion of human cells. For instance, the R28 protein of *S. pyogenes* has been demonstrated to mediate adhesion to a cervical epithelial cell line (Stålhammar-Carlemalm *et al.*, 1999). Furthermore, the α protein has been reported to mediate invasion of epithelial cells (Bolduc *et al.*, 2002), possibly by interacting with glycosaminoglycans or integrins on the host cell surface (Baron *et al.*, 2004, 2007; Bolduc and Madoff, 2007). A protein construct derived from α, consisting of the first repeat and the adjacent third of the N-terminal region, was shown to be sufficient for heparin binding, suggesting that this region of α may be responsible for the glycosaminoglycan interaction (Auperin *et al.*, 2005). Considering that most strains harbor a member of the Alp family, one might speculate that a common function of all Alp family proteins may be to interact with host cells. However, no study to date has demonstrated that this is the case. In addition, the role in virulence of the Alp family proteins is unclear (Lindahl *et al.*, 2005), but they are likely to play a role given the fact that all *S. agalactiae* strains probably express a member of the Alp family.

Immune response. As mentioned above, Rib and α elicit protective immunity (Michel *et al.*, 1992; Stålhammar-Carlemalm *et al.*, 1993) when administrated without adjuvant or with alum (Larsson *et al.*, 1999). Because these proteins were studied as potential vaccine components in **paper II**, the present knowledge concerning the immune response will be briefly summarized here. Studies of α have demonstrated a complex antibody response to this protein. It was reported that most antibodies elicited by intact α are directed against conformational epitopes present only in proteins with a higher number of repeats (Gravekamp *et al.*, 1996). However, protective epitopes are present in the repeat region and in the N-terminal region of α (Kling *et al.*, 1997). Moreover, the repeat region of α reduces the antibody response to all parts of the intact protein (Gravekamp *et al.*, 1997). During an experimental infection in the presence of anti-α antibodies, strains with fewer repeats arose spontaneously, suggesting that these variants could escape host immunity (Madoff *et al.*, 1996). However, the situation under non-immune conditions is less clear, since there was no

difference with regard to virulence when mice were pretreated with preimmune serum. Thus, the biological relevance of these findings is unclear. Moreover, naturally occurring α molecules have high number of repeats (Madoff *et al.*, 1991), implying that the repeats confer a selective advantage.

Pili

Pili are long, proteinaceous structures extending from the bacterial surface. Extensively studied in Gram-negative bacteria, and in *Escherichia coli* in particular, pili play an important role in attachment to host cells. The mechanism of pili assembly in Gram-negative bacteria has been elucidated and involves non-covalent interaction between the pilus subunits (Soto and Hultgren, 1999; Sauer *et al.*, 2000). Considerably less is known about pili of Gram-positive organisms. Early studies from the 1960s and the 1970s identified pili in the genus *Corynebacterium* (Yanagawa *et al.*, 1968; Yanagawa and Honda, 1976) and in *Actinomyces* species (Girard and Jacius, 1974; Cisar and Vatter, 1979), but whether this was a general feature of Gram-positive organisms was not investigated.

Work in the *Actinomyces* system suggested that assembly of pilus subunits required a sortase-like gene and may involve polymerization of precursor proteins (Yeung and Ragsdale, 1997). However, the details of the assembly mechanism remained largely unknown until 2003, when Ton-That and Schneewind, using *Corynebacterium diphtheriae* as a model system, demonstrated that pilus subunits were, in contrast to Gram-negative organisms, covalently attached to each other and that this attachment was mainly dependent upon sortase A, tethering the growing pili to the peptidoglycan layer (Ton-That and Schneewind, 2003). Importantly, this study also identified the presence of a so-called pilin motif in predicted surface proteins of other Gram-positive organisms, among them *S. agalactiae* and *S. pneumoniae*, indicating that these pathogens have pili. Indeed, Lauer *et al.* (2005) demonstrated that a type VIII strain of *S. agalactiae* expresses pili on the bacterial surface. However, neither the assembly mechanism nor the function was described.

Detailed analysis using an *S. agalactiae* type III strain revealed the presence of three pilus subunits: Gbs1474, Gbs1477 and Gbs1478 (Dramsi *et al.*, 2006). The spatial distribution of these three proteins in the mature pilus was examined by electron microscopy, an analysis which showed that Gbs1474 was located at the base of the pilus, Gbs1478 was unevenly interspersed along the pilus base and shaft, whereas Gbs1477 showed abundant staining along the entire pilus structure. Thus, Gbs1477 is apparently the major pilus subunit. In contrast to pili of *C. diphtheriae*, the assembly of the *S. agalactiae* pilus relied primarily on two class C

sortases, designated sortase C3 and sortase C4, whereas the anchoring of the pilus to the cell wall was suggested to be dependent upon sortase A. A similar study demonstrated that genes encoding pili are present in all eight sequenced genomes of *S. agalactiae* (Rosini *et al.*, 2006). Furthermore, this study corroborated the essential function of the class C sortases with regard to pilus polymerization. Of note, the crystal structure of GBS52, one of the minor pilus subunits in the type V strain 2603V/R, was recently solved, a study that demonstrated the presence of two Ig-like domains in this protein (Krishnan *et al.*, 2007). Similarly, studies of the major pilus subunit of an *S. pyogenes* strain demonstrated the presence of two Ig-like domains. Interestingly, the latter study showed that the domains in various pilins are stabilized by isopeptide bonds, which may confer resistance to proteases (Kang *et al.*, 2007).

Regarding the biological function of pili of *S. agalactiae*, Dramsi *et al.* (2006) demonstrated that a mutant lacking Gbs1478 was impaired in its ability to adhere to epithelial cells. A similar study showed that pili expressed by a serotype V strain contributed to adhesion to and invasion of brain microvascular endothelial cells (Maisey *et al.*, 2007). These studies suggest a functional similarity between pili of Gram-positive and Gram-negative pathogens, despite completely different assembly mechanisms.

Three major pilus subunits together with the previously described Sip protein (Brodeur *et al.*, 2000) were shown to be protective when administrated with complete Freund's adjuvant (Maione *et al.*, 2005), indicating that pili are potential vaccine candidates. However, in a subsequent study no protection was observed when the major pilus subunit was administered in the absence of adjuvant (Buccato *et al.*, 2006), suggesting that this pilus component might not be a suitable vaccine candidate.

Other proteins eleciting protective antibodies

β protein. As described above, the C antigen was demonstrated to be composed of two antigenically distinct parts, one trypin-resistant and one trypsin-sensitive. The latter component was designated β (Bevanger and Maeland, 1979) and was shown to elicit protective immunity in mice (Bevanger and Naess, 1985). The gene for the β protein, *bac* (not to be confused with *bca*, encoding the α protein) was sequenced from two different strains (Hedén *et al.*, 1991; Jerlström *et al.*, 1991). The sequence analysis revealed the presence of a proline rich region, designated the XPZ region, in the C terminal domain. In this region, every third amino acid is a proline. Each proline is flanked by a residue with low hydrophilicity (X) and a residue with high hydrophilicity (Z), hence the name XPZ. Moreover, the third residue (Z), is alternatively of positive and negative charge. The XPZ region varies in size between 90

and 150 amino acids (Areschoug *et al.*, 2002a). Although biochemically characterized in detail, no specific function has been attributed to the XPZ region. The C-terminal region of the β protein contains the sorting motif LPXTG (Hedén *et al.*, 1991).

The β protein binds serum IgA and secretory IgA of human origin (Russell-Jones *et al.*, 1984; Lindahl *et al.*, 1990) via a 73-residue domain located in the N-terminal region (Hedén *et al.*, 1991; Jerlström *et al.*, 1996). Of note, the affinity for secretory IgA is much lower than for serum IgA (Lindahl *et al.*, 1990). This finding was surprising and puzzling, because *S. agalactiae* colonizes mucosal surfaces, where secretory IgA is the dominating Ig class. The binding site in IgA is located at the Cα2 and Cα3 domain interface in the Fc part. Interestingly, the same binding site interacts with the human IgA receptor CD89 (Pleass *et al.*, 2001), implying that the β protein may interfere with the binding of IgA to CD89, thereby interfering with IgA effector function. The β protein also binds human factor H (Areschoug *et al.*, 2002b), a negative regulator of the complement system, suggesting that recruitment of factor H may be of importance in avoiding complement mediated killing. The binding site for factor H is located in the C-terminal part of β, but has not been mapped in detail. Thus, β has separate binding sites for IgA and factor H.

The β protein is expressed by almost all strains belonging to serotype Ib, but also by a minority of type Ia, II and V strains (Johnson and Ferrieri, 1984; Areschoug *et al.*, 1999; Kong *et al.*, 2002). However, β is almost never found in the clinically important serotype III strains.

Sip protein. The Sip protein (for surface immunogenic protein) is present in all nine serotypes. This protein is localized on the bacterial surface, but it is also secreted (Brodeur *et al.*, 2000; Rioux *et al.*, 2001). Moreover, the degree of conservation is very high, with isolates from six different serotypes showing 98% residue identity. This protein was discovered after immunological screening of a genomic library using a pool of normal human sera reactive with whole cell extracts of *S. agalactiae*. The Sip protein was found to elicit protective immunity, as judged by passive protection studies in mice (Brodeur *et al.*, 2000). The protective ability of Sip was further analyzed in a neonatal mouse model and it was shown that passive and active vaccination of pregnant mice protected pups against strains of several serotypes (Martin *et al.*, 2002). Thus, Sip may be a promising vaccine candidate. However, it is unclear whether Sip elicits protective immunity when administrated together with an adjuvant accepted for human use. Moreover, antibodies against Sip may not protect against all *S. agalactiae* strains (Maione *et al.*, 2005).

C5a peptidase. Another highly conserved surface protein is the C5a peptidase. Originally discovered in *S. pyogenes* (Wexler *et al.*, 1985), an almost identical protein, designated ScpB, was later identified in *S. agalactiae* (Hill *et al.*, 1988; Bohnsack *et al.*, 1991; Cleary *et al.*, 1992). The *scpB* gene was shown to be present in all human isolates (Franken *et al.*, 2001) and was found to be highly conserved (Chmouryguina *et al.*, 1996; Bohnsack *et al.*, 2000; Glaser *et al.*, 2002). The extensive similarity between the two streptococcal C5a peptidases indicates a horizontal gene transfer event between *S. pyogenes* and *S. agalactiae* (Chmouryguina *et al.*, 1996). ScpB cleaves C5a, an important anaphylatoxin of the complement system. Of note, the species specificity of ScpB seems to be restricted, since it cleaves human C5a, but not that of the mouse (Bohnsack *et al.*, 1993).

The *in vivo* role of ScpB was addressed using C5a-deficient mice and exogenously supplied human C5a. In this model, neutrophil infiltration was more pronounced in mice infected with a ScpB mutant compared to mice infected with a wildtype strain, suggesting that ScpB contributes to virulence by inactivating C5a (Bohnsack *et al.*, 1997). However, certain strains express a truncated version of ScpB, which is unable to inactivate C5a (Bohnsack *et al.*, 2000). This finding led investigators to hypothesize that ScpB has a second function, unrelated to cleavage of C5a. Indeed, it was later shown that ScpB binds to fibronectin (Beckmann *et al.*, 2002; Cheng *et al.*, 2002b), an interaction which may promote invasion of epithelial cells (Cheng *et al.*, 2002b). Immunization studies have demonstrated that mice immunized with purified ScpB cleared a subsequent bacterial challenge (Cheng *et al.*, 2002a), suggesting that ScpB may be a suitable vaccine component. However, this protection study employed a mycobacterial phospholipid adjuvant, which cannot be used in humans.

Disease prevention

More than 35 years after its emergence as a neonatal pathogen, there is still no very effective prevention against infections caused by *S. agalactiae*. The two strategies used to prevent such infections are chemoprophylaxis (i.e. use of antibiotics) and immunoprophylaxis (i.e. development of a vaccine). From a long-term perspective, immunoprophylaxis holds the most promise for prevention of *S. agalactiae* infections, especially among neonates and pregnant mothers (Baker, 1990).

Antibiotics

In the United States consensus guidelines were issued in 1996, offering prophylactic antibiotics to pregnant women colonized by *S. agalactiae* at the time of labor. A follow-up

study to these guidelines demonstrated that the incidence of early-onset infections decreased by 65 percent from 1993 to 1998 (Schrag *et al.*, 2000). A similar decline was reported from Australia, after the introduction of prevention strategies (Isaacs and Royle, 1999). In contrast, the number of late-onset infections was unaffected by these preventive methods. Moreover, the use of antibiotics raises concerns with regard to the emergence of resistant strains of *S. agalactiae* and other bacterial species (Stoll *et al.*, 2002). Although still highly susceptible to penicillin, resistance to both erythromycin and clindamycin has been reported among certain *S. agalactiae* isolates (Schrag *et al.*, 2000). Since these two antibiotics are recommended to penicillin-allergic women (Moore *et al.*, 2003), it is of importance to continuously monitor the resistance pattern of *S. agalactiae*.

Vaccines

Capsule. The best way to substantially decrease the number of infections caused by *S. agalactiae* would be the development of a vaccine. The type specific polysaccharide capsule has attracted much attention because it elicits protective immunity (Lancefield *et al.*, 1975) and because low antibody levels against the capsule may be a risk factor for developing neonatal infection (Baker and Kasper, 1976). However, immunization with the capsule alone fails to elicit a good immune response among adults, suggesting that conjugation with a carrier protein might overcome this problem by inducing a T-cell dependent response (Baker, 1990). Indeed, high titers of IgG were observed when mice were immunized with the type III capsule conjugated to tetanus toxiod (TT) (Lagergård *et al.*, 1990). A similar construct elicited a good antibody response in rabbits and protected mice against a lethal dose of a type III strain (Wessels *et al.*, 1990). Similar protection levels were observed with conjugates consisting of the type II capsule and TT (Paoletti *et al.*, 1992), and the Ia polysaccharide and TT (Wessels *et al.*, 1993). However, due to the lack of cross reactivity between capsules of the different serotypes, capsules of several serotypes must be included in a vaccine to achieve broad protection.

In a study by Paoletti *et al.* (1994), pregnant mice were immunized with a mixture of the four polysaccharides Ia, Ib, II and III, each conjugated to TT, after which the neonatal pups were challenged with bacteria corresponding to the serotypes used in the immunization. This tetravalent conjugate protected the pups against all four serotypes. Because serotype V has emerged as an important cause of infection (Blumberg *et al.*, 1996), a TT conjugate based on the type V polysaccharide was constructed and shown to elicit protective immunity (Wessels *et al.*, 1995). To achieve protection against strains predominately occurring in Japan,

polysaccharides from serotypes VI and VIII must probably be included in a future vaccine. Immunization studies with these two serotypes using TT as the carrier elicited good protection in mice (Paoletti *et al.*, 1999). Thus, to achieve broad protection against *S. agalactiae* infections, polysaccharides from at least seven serotypes must be included in a conjugate vaccine.

In addition to the problem concerning multiple serotypes, excessive use of TT as a carrier protein might decrease the immune response to the conjugated polysaccharide (Schutze *et al.*, 1985; Dagan *et al.*, 1998). Since TT is used in many licensed vaccines, this issue raises questions whether this carrier is suitable for use in conjugate vaccines aimed at *S. agalactiae*. To avoid such immunomodulatory effects, an alternative approach would be to use other proteins than TT as carriers. One such alternative carrier is the B subunit of cholera toxin. A conjugate consisting of this carrier coupled to the type III capsule induced a good immune response on mucosal surfaces (Shen *et al.*, 2000). Several *S. agalactiae* proteins have also been used as carriers. A study by Madoff *et al.* (1994) used the β protein coupled to the type III capsule and showed that immunization of pregnant mice with this conjugate protected the pups against a subsequent challenge with a serotype III strain and a β-expressing type Ia strain. The α protein has also been used as a carrier protein in a conjugate with the serotype III polysaccharide capsule and this conjugate was shown to elicit protective immunity against strains expressing α or the type III capsule (Gravekamp *et al.*, 1999).

Proteins. Recent studies have focused on the use of pure proteins as vaccine components. Early immunization studies demonstrated that both the α and the β proteins are protective (Bevanger and Naess, 1985; Michel *et al.*, 1991). However, from a vaccine point of view, the implications of these findings were limited because α and β are not expressed by all strains, especially not by the clinically important type III strains. As mentioned above, the possibility to develop a protein-based vaccine received only limited attention until the discovery of protein Rib, which is expressed by almost all type III strains and elicits protective immunity (Stålhammar-Carlemalm *et al.*, 1993). The available evidence suggests that a vaccine containing Rib and α would protect against a majority of *S. agalactiae* strains (Larsson *et al.*, 1996, 1999). However, a vaccine based on a single protein would facilitate administration. With the long-term goal to develop a vaccine based on a single protein, we have therefore constructed a fusion protein based on the N-terminal parts of Rib and α. As described in **paper II**, this fusion protein has interesting properties and elicits protective immunity to strains expressing Rib or α.

STREPTOCOCCUS PYOGENES

S. pyogenes (group A streptococcus; GAS) is responsible for a wide variety of diseases, which is a reflection of its many and diverse virulence factors. This bacterium causes common and often mild infections in primarily children and teenagers, but in rarer cases also life-threatening conditions among people of all ages. It is a strict human pathogen; hence, no good animal models are available (Cunningham, 2000; Stevens and Kaplan, 2000). In classical studies in the 1920s and 1930s, Rebecca Lancefield developed a serological system to classify *S. pyogenes* (Lancefield, 1928, 1933), a system that is still used today. Furthermore, these important studies paved the way for subsequent, more molecular studies.

Diseases

More than 500,000 deaths are estimated to occur annually due to *S. pyogenes* infections, which makes this bacterium one of the most important pathogens from a global point of view. Approximately 18.1 million people are suffering from severe infection at any given time and 1.78 million new such cases occur each year (Carapetis *et al.*, 2005b).

The disease most commonly associated with *S. pyogenes* is pharyngitis ('strep throat') with ~616 million new cases occurring each year (Carapetis *et al.*, 2005b). Clinical symptoms include sore throat, fever and swelling of the tonsils. Pharyngitis is often self-limiting and resolves within 3-4 days. However, there is a risk of developing acute rheumatic fever or poststreptococcal glomerulonephritis (see below), which may be a reason for antibiotic treatment (Stevens and Kaplan, 2000). It should also be noted that in some settings more than 20% of school-age children can be asymptomatic carriers of *S. pyogenes*.

Scarlet fever is characterized by a "strawberry" tongue, erythema and a fine rash over the upper part of the body. It was very common at the end of 19th century, with mortality rates ranging between 25 and 35% in the United States and Western Europe. Complications among children suffering from scarlet fever included obstructions of the upper airways and otitis media. Since the introduction of antibiotics, the rate of scarlet fever has dramatically decreased. Moreover, the symptoms seen today are usually quite mild (Stevens and Kaplan, 2000).

Impetigo (also known as pyoderma) is a superficial skin infection, occurring on the face or extremities, and is characterized by lesions filled with pus, which later rupture and form thick honey-colored crusts. Individuals affected by impetigo are primarily children below school-age, especially those living in a warm, humid climate. Interestingly, there is a strong

correlation between impetigo and poststreptococcal glomerulonephritis (see below) (Bisno and Stevens, 1996; Cunningham, 2000).

S. pyogenes is the most common cause of erysipelas and cellulitis, two skin infections affecting mainly adults and elderly (Stevens and Kaplan, 2000). Erysipelas occurs primarily in the most superficial layers of the skin and cutaneous lymphatics, whereas cellulitis extends more deeply into the subcutaneous tissue, although the distinction is not always clear-cut. Both conditions are manifested by local inflammation and fever (Bisno and Stevens, 1996).

From a historical point of view, the most important and serious infection caused by *S. pyogenes* is puerperal fever (also known as childbed fever). At its peak in the 18th and the first part of the 19th century, mortality rates of 50% or more were not unusual. Seminal work by the Hungarian physician Semmelweis in the 1840s demonstrated that infections were transmitted by doctors, that failed to observe appropriate hand hygiene. When stricter hygiene was enforced, the mortality rates dropped significantly (Semmelweis, 1861; Stevens and Kaplan, 2000). However, it was not demonstrated until the 1930s that *S. pyogenes* indeed was the cause of puerperal fever (Lancefield and Hare, 1935). Nowadays, this disease is very rare (Stevens and Kaplan, 2000).

S. pyogenes is also responsible for two invasive conditions, nectrotizing fasciitis (NF) and the streptococcal toxic shock syndrome (STSS). The hallmark of NF is a subcutaneous infection with destruction of fascia and fat, whereas STSS is characterized by shock and multiple organ failure. Both conditions have a very rapid onset and are associated with high mortality rates (Bisno and Stevens, 1996).

Two non-suppurative sequelae are associated with *S. pyogenes*: acute rheumatic fever (ARF) and poststreptococcal glomerulonephritis (PSGN). ARF is characterized by arthritis, carditis and/or chorea, and believed to be of autoimmune nature. The pathogenesis of ARF is unknown, but antibodies directed against streptococcal surface proteins are assumed to cross-react with human tissue. Immunological factors such as the HLA type may also play a role. The clinical symptoms of PSGN include edema, hypertension and hematuria. Little is known about the mechanism behind PSGN, but several hypotheses have been proposed, one being deposition of immune complexes in the glomerulus (Cunningham, 2000; Stevens and Kaplan, 2000). It is commonly believed that ARF may develop after pharyngitis, whereas PSGN is associated with both pharyngitis and impetigo. Interestingly, strains causing ARF do not usually cause PSGN and vice versa. Thus, it has been proposed that two distinct types of strains exist; rheumatogenic strains and nephritogenic strains (Bisno and Stevens, 1996; Stevens and Kaplan, 2000). However, this distinction is not always clear-cut, as judged by

epidemiological studies of strains causing disease in the aboriginal population in Australia. Despite the fact that the incidence of impetigo by far exceeds that of pharyngitis (Bessen *et al.*, 2000), the frequency of ARF is very high in this population (Carapetis *et al.*, 2005a). A similar observation was made among ethnic Polynesians in Hawaii (Erdem *et al.*, 2007).

Even though extensively used since its original discovery in the 1930s, penicillin remains the drug of choice with regard to infections caused by *S. pyogenes*. Remarkably, no penicillin resistance has been observed, an observation which has puzzled many scientists in the field. Alternative drugs include clindamycin, which has been shown to be more effective against cases of severe streptococcal disease. However, antibiotic treatment is inefficient against certain invasive infections, leaving no choice but to surgically remove the infected tissue (Stevens and Kaplan, 2000).

Classification

S. pyogenes is also known as the group A streptococcus, a designation based on the presence of the species-specific group A carbohydrate localized in the bacterial cell wall (Lancefield, 1933). To subdivide different *S. pyogenes* strains, Rebecca Lancefield developed a classification system based on the surface localized M protein, defined as a type-specific protein antigen. Antisera were raised by immunization with crude hot-acid extracts of *S. pyogenes* bacteria and these antisera were absorbed with heterologous strains to remove non-strain-specific antibodies. Using this approach, a large collection of M-typing sera was obtained (Lancefield, 1928). This system, which is ~80 years old, laid the framework for serotyping *S. pyogenes* strains and helped to eventually define >100 different M types (Kehoe, 1994). Today, the sequence of the 5' end of the *emm* gene is used to define new types, the so-called *emm*-types (Beall *et al.*, 1996). This sequence of the M protein gene encodes the highly variable N-terminal region, which elicits the type-specific antibodies used the in Lancefield classification system.

All *S. pyogenes* strains can be assigned to one of two major groups, based on the expression of a protein designated serum opacity factor (SOF). Strains expressing this protein are referred to as OF^+, whereas strains lacking SOF are designated OF^-. Like M protein, SOF varies extensively in sequence between strains, allowing the identification of different OF types. Interestingly, there is a strong correlation between M type and OF type in OF^+ strains, although the corresponding genes are not closely linked. The explanation for this correlation is not known (Top and Wannamaker, 1968; Widdowson *et al.*, 1970).

Treatment of *S. pyogenes* with trypsin degrades the M protein, but exposes a distinct set of antigens, the T-antigens. Unrelated to M proteins, these antigens are used as an alternative typing system. About 25 different T-antigens have been described using techniques similar to those mentioned above. There is a correlation between T-antigens and M types; i.e., certain T antigens are associated with specific M types. On the other hand, strains of some M types may express several distinct T-antigens (Kehoe, 1994). Until recently, no detailed studies on the T-antigens were available. However, in 2005 it was reported that the T-antigens form pilus structures on the bacterial surface. The assembly process was dependent upon a sortase C enzyme, as previously observed for both *C. diphteriae* and *S. agalactiae* (see above). Moreover, the T-antigens were shown to elicit protective immunity in mice (Mora *et al.*, 2005). The biological function of the *S. pyogenes* pili was addressed in a recent report which demonstrated that the pilus of an M5 strain mediated adhesion to both human tonsillar epithelium and primary human keratinocytes (Abbot *et al.*, 2007). Furthermore, pili of an M1 strain were shown to promote bacterial aggregation and biofilm formation (Manetti *et al.*, 2007). Of note, the crystal structure of the major pilus structure of an M1 strain revealed a modified variant of the Ig fold (Kang *et al.*, 2007).

Virulence factors

Virulence is generally defined as the ability of an organism to cause disease. The components involved in this process are referred to as virulence factors. Analysis of the first sequenced *S. pyogenes* genome (an M1 strain) identified more than 40 known or putative virulence factors (Ferretti *et al.*, 2001). This multitude probably reflects the ability of *S. pyogenes* to cause a very wide array of infections. Moreover, different virulence factors may be expressed at different stages or sites of infection, i.e. these genes are probably temporarily and/or spatially regulated, most likely by environmental factors (Cunningham, 2000). Here I will only briefly describe some of the most extensively studied structures that have been implicated in virulence.

M protein. The most extensively studied virulence factor of *S. pyogenes* is the M protein, which has a very strong antiphagocytic effect when bacteria are grown in whole blood in the absence of type-specific antibodies (Lancefield, 1962; Fischetti, 1989). Early studies suggested that M protein exerts its inhibitory effect on phagocytosis by downregulating C3 deposition on the bacterial surface, i.e. by inhibiting opsonization (Jacks-Weis *et al.*, 1982). Extensive molecular studies have indicated that the ability of M protein to block complement deposition depends on its ability to recruit a human plasma protein, either

fibrinogen (Whitnack and Beachey, 1982; Carlsson *et al.*, 2005) or the complement regulator C4BP (Thern *et al.*, 1995; Johnsson *et al.*, 1996; Berggård *et al.*, 2001). M protein has also been implicated in adhesion to epithelial cells (Okada *et al.*, 1995; Courtney *et al.*, 2002).

Most studies of M protein have been performed *in vitro*, e.g. by analyzing bacterial phagocytosis resistance in human blood. These studies have strongly indicated that M protein plays a key role in virulence. However, results obtained *in vitro* may not reflect the *in vivo* situation (Virgin, 2007). It is therefore important that a number of studies have addressed the role of M protein *in vivo*. One study showed that M protein deficient bacteria were rapidly cleared from the pharynx of baboons (Ashbaugh *et al.*, 2000). Another study showed that an M negative mutant was severely attenuated in virulence in a soft tissue infection mouse model (Ashbaugh *et al.*, 1998). These important studies indicate that M protein is crucial with regard to both colonization and virulence. However, no study has analyzed which part(s) of the M protein that mediate(s) these properties. In **paper III**, we show that the fibrinogen-binding domain of the M5 protein most likely plays a major role in virulence in the mouse model.

Capsule. The hyaluronic acid capsule of *S. pyogenes* inhibits phagocytosis and affects virulence in several mouse models (Wessels *et al.*, 1991; Wessels and Bronze, 1994; Wessels *et al.*, 1994; Ashbaugh *et al.*, 1998). The capsule has also been implicated in bacterial binding to CD44 on epithelial cells, implying that an additional function of the capsule may be to mediate adhesion of *S. pyogenes* to host cells although not to the same extent as M protein (Schrager *et al.*, 1998). Interestingly, there is a correlation between the degree of capsule expression and the incidence of rheumatic fever and invasive diseases, suggesting that mucoid strains may be more likely to cause these conditions (Johnson *et al.*, 1992).

C5a peptidase. As described above, the C5a peptidase is expressed by both *S. agalactiae* and *S. pyogenes*, and it is designated ScpA in the latter species. The two proteins show extensive sequence homology (~95%), which is reflected in their shared ability to inactivate C5a. A mutant lacking ScpA was cleared more rapidly than the wildtype strain in an intranasal colonization mouse model (Ji *et al.*, 1996), suggesting that ScpA may act as a virulence factor.

Putative adhesins. Several surface structures expressed by *S. pyogenes* have been implicated in adhesion to epithelial cells, such as lipotechoic acid (Beachey and Ofek, 1976), protein F (Hanski and Caparon, 1992), FBP54 (Courtney *et al.*, 1994), SOF (Kreikemeyer *et al.*, 1995), and R28 (Stålhammar-Carlemalm *et al.*, 1999), suggesting that these proteins may contribute to virulence. As noted above, pili have recently been demonstrated to function in adhesion (Abbot *et al.*, 2007).

Extracellular proteins. S. pyogenes also expresses a number of extracellular proteins secreted into the environment. Streptolysin S and streptolysin O, produced by most strains, are two cytolytic proteins capable of damaging the membranes of polymorphonuclear leukocytes and platelets (Bisno *et al.*, 2003; Wessels, 2005). Streptokinase binds to human plasminogen and confers plasmin activity, which is believed to facilitate bacterial spread and virulence by dissolving fibrin clots (Sun *et al.*, 2004). *S. pyogenes* is also known for its production of numerous superantigens (most of them known as streptococcal pyrogenic exotoxins or Spes), which are believed to be associated with the toxic shock syndrome (Kotb *et al.*, 2002; Bisno *et al.*, 2003). Superantigens are exotoxins capable of non-specific activation of a large subset of T-cells, an interaction which results in massive T-cell proliferation and extensive cytokine production (Kotb, 1995). The Spe family comprises a vast number of members, of which SpeA, SpeB and SpeC have been the most studied. Of note, SpeB also functions as a cysteine protease, capable of degrading vitronectin, fibronectin and other proteins (Cunningham, 2000). Other enzymes suggested to affect the virulence properties of *S. pyogenes* include IdeS/Mac-1, which may inhibit opsonophagocytosis and cleaves IgG in the hinge region (Lei *et al.*, 2001; von Pawel-Rammingen *et al.*, 2002), and EndoS, which deglycosylates IgG (Collin and Olsén, 2001).

The M protein family

Originally described in 1928, the surface-localized M protein is one of the most well-characterized bacterial virulence factors. It is mainly known for its ability to inhibit phagocytosis by human granulocytes, an ability which is well documented (Lancefield, 1962; Fischetti, 1989; Thern *et al.*, 1998; Carlsson *et al.*, 2003). For a long time it was believed that *S. pyogenes* only expresses one single M protein. However, it is now known that strains can express one, two or three members of the M protein family. These proteins are designated M, Enn and Mrp, and are encoded by the *emm*, *enn* and *mrp* genes, respectively, all of which are part of the Mga regulon (see below). The Mrp and Enn proteins are referred to as “M-like proteins”, whereas the term “M protein” is reserved for the protein encoded by the *emm* gene. Of note, the M protein is not the only member of the family with antiphagocytic properties; the Mrp protein has been demonstrated to possess a similar function (Podbielski *et al.*, 1996; Thern *et al.*, 1998; Courtney *et al.*, 2006). However, the majority of studies on phagocytosis resistance have been focused on the M protein.

The Mga regulon

Mga was the first regulator of virulence factors identified in *S. pyogenes*. This protein positively regulates expression of the M, Enn and Mrp proteins, and also several other proteins, including ScpA, SOF, the streptococcal inhibitor of complement (Sic) and the fibronectin-binding protein Fba. The genes encoding members of the M protein family and ScpA are closely linked and constitute the core Mga regulon. These genes are tandemly arranged on the bacterial chromosome and located immediately downstream of *mga*, which is thus closely linked to many of the genes it is regulating (Figure 2). Of note, all *S. pyogenes* strains harbor the *mga* gene (Kreikemeyer *et al.*, 2003).

Mga is activated by increased CO_2 concentration, increased temperature and iron limitation. It has been suggested that Mga is part of a two-component system, but no sensor component has been identified and no phosphorylation of Mga has been demonstrated. Thus, the molecular details of the environmental sensing capabilities of Mga remain largely unknown (Kreikemeyer *et al.*, 2003).

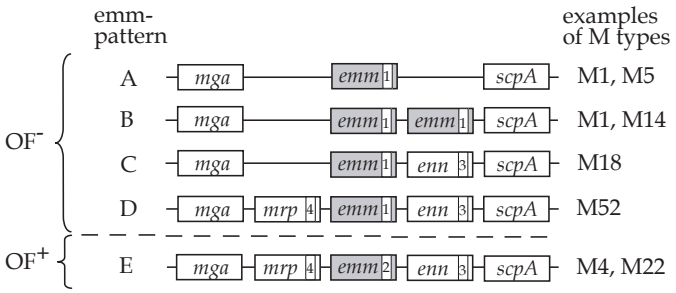

Figure 2. Organization of the core Mga regulon. Genes encoding members of the M protein family (*mrp*, *emm* and *enn*) are flanked by the *mga* gene and the *scpA* gene, encoding Mga and the C5a-peptidase, respectively. The *emm* pattern (A-E) is indicated to the left and examples of M types from each pattern are indicated to the right. The *emm* gene, encoding the M protein, is shown in grey. The subfamilies (1-4), which the *emm* patterns are based on, are indicated in each gene encoding a member of the M protein family. Adopted from Bessen *et al.* (2000) and Carlsson (2005).

As noted above, an individual strain may express one, two or all three members of the M protein family. Interestingly, there is correlation between these expression patterns and the expression of SOF. Strains that are OF^+ invariably express all three members, whereas OF^- strains can express the M protein alone or together with the Mrp and/or Enn proteins (Kehoe, 1994; Navarre and Schneewind, 1999).

Sequence analysis of the conserved 3' region of the genes encoding the M protein family revealed four distinct subfamilies, designated SF1-4, which allowed the division of the core Mga regulon into five different so-called *emm* patterns, designated A-E (Hollingshead *et al.*, 1993; Hollingshead *et al.*, 1994). Interestingly, strains belonging to pattern A-C mainly cause throat infections, category D strains may be primarily responsible for skin infections, whereas category E strains are evenly distributed among both conditions (Bessen *et al.*, 1996).

M protein structure

The M protein is a dimeric, coiled-coil protein with fibrillar structure extending 50-60 nm from the bacterial surface. The coiled-coil homodimeric structure is a direct result of the seven residue repeat pattern (also called heptad repeats) present in the amino acid sequence, allowing formation of hydrophobic interactions between the two polypeptides, thus stabilizing the α-helical structure (Fischetti, 1989). Of note, the overall organization of an M protein is similar between different serotypes (Figure 3).

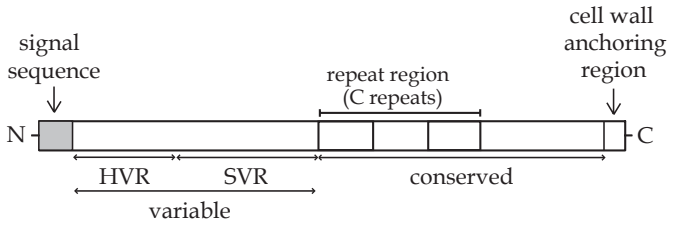

Figure 3. Schematic representation of an M protein (encoded by the *emm* gene). The signal sequence is cleaved upon secretion. The variable region located in the N-terminal part can be subdivided into a semivariable region (SVR) and a hypervariable region (HVR), the latter being located most distally to the bacterial surface in the mature protein. The C-terminal part is conserved and is largely composed of so-called C repeats. The M protein is anchored to the cell wall via the LPXTG motif (Navarre and Schneewind, 1999), located in the most C-terminal part of the protein. Modified from Persson (2006).

Following secretion of M protein through the cytoplasmic membrane, the signal peptide is cleaved off, and the mature protein is covalently attached to the cell wall via the LPXTG motif (Navarre and Schneewind, 1999). The C-terminal part of the protein, which is located closest to the cell wall, shows limited sequence variation between different M types and is designated the conserved region. This region contains 2-4 so-called C repeats (Hollingshead *et al.*, 1986). Minor sequence variations in the conserved region allow the division of M proteins into two different classes; class I or class II (Bessen *et al.*, 1989). The first category is

associated with OF^- strains, whereas class II M proteins are found in OF^+ strains (Navarre and Schneewind, 1999).

The N-terminal region of the M protein displays considerable sequence variation between serotypes and is therefore referred to as the variable region, a region which can be further divided into the semivariable region (SVR) and the hypervariable region (HVR). In spite of the extensive sequence variability, the SVR and the HVR of different M proteins may specifically bind to the same ligand. The SVR of some strains binds human IgA (Johnsson *et al.*, 1994), and the HVR in M proteins of OF^+ strains binds the complement regulator C4b-binding protein, C4BP (Thern *et al.*, 1995; Johnsson *et al.*, 1996). Interestingly, when comparing the amino acid sequence of HVRs from seven different C4BP binding M proteins, no sequence identity was found, implying that the ligand-binding properties of the HVR was retained despite extreme sequence variability (Persson *et al.*, 2006). Of note, in the mature M protein, the HVR is located most distally to the surface of *S. pyogenes* (Kehoe, 1994).

Binding of human plasma proteins

All M proteins bind one or more human plasma proteins. Some of these proteins, such as C4BP, IgA and fibrinogen, have been implicated in phagocytosis resistance when bound to the surface-localized M protein (Whitnack and Beachey, 1982; Berggård *et al.*, 2001; Carlsson *et al.*, 2003, 2005). Interactions with other plasma components, such as plasminogen, have been suggested to facilitate bacterial spread (Sun *et al.*, 2004). Thus, *S. pyogenes* has evolved the ability to recruit and exploit a variety of plasma proteins to promote virulence. Below, I will briefly summarize current knowledge regarding M protein-mediated binding to human plasma proteins. As examples, the ligand binding properties of two extensively studied M proteins, M5 and M22, which are expressed by strains of the OF^- and OF^+ lineage, respectively, are shown in Figure 4.

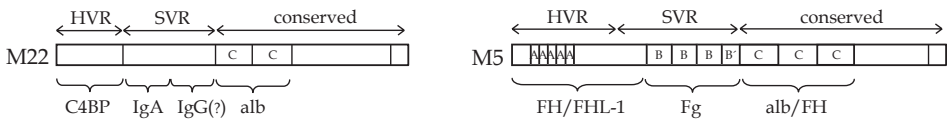

Figure 4. Binding of human plasma proteins to the M22 and M5 proteins. M22 belongs to the OF^+ lineage and M5 to the OF^- lineage. HVR, hypervariable region; SVR, semivariable region; C4BP, C4b binding protein; FH, factor H; alb, albumin; FHL-1, factor H-like protein 1. Adopted from Persson (2006).

Fibrinogen. Binding of Fg to *S. pyogenes* was first demonstrated more than 70 years ago (Tillett and Garner, 1934). Importantly, all *S. pyogenes* strains bind Fg (Kronvall *et al.*, 1979a), a binding which for a long time was assumed to be attributed to the M protein. This has indeed been shown to be the case for OF^- strains. However, in OF^+ strains the Fg interaction is mediated by the Mrp protein and not the M protein (O'Toole *et al.*, 1992; Stenberg *et al.*, 1992). The binding of Fg has been localized to the B repeat region of the M protein (Kehoe, 1994; Ringdahl *et al.*, 2000; Carlsson *et al.*, 2005). An early observation suggested that bacteria-bound Fg may inhibit phagocytosis through interference with complement deposition via the alternative pathway (Whitnack and Beachey, 1982), but a recent study indicates that Fg exerts its inhibitory effect on the classical pathway (Carlsson *et al.*, 2005). It is widely assumed that the Fg binding contributes to the virulence properties of *S. pyogenes*, but no *in vivo* study has addressed this issue. In **paper III** we demonstrate that binding of Fg to the surface-localized M5 protein is important for virulence in a mouse model.

C4BP. Many M proteins bind C4BP, a regulator of complement activation via the classical pathway (Thern *et al.*, 1995; Persson *et al.*, 2006). This interaction occurs within the ~50 aa residue HVR (Johnsson *et al.*, 1996). Binding of C4BP contributes to phagocytosis resistance by inhibiting deposition of complement on the bacterial surface (Berggård *et al.*, 2001; Carlsson *et al.*, 2003). These results can be explained by the fact that C4BP bound to the surface of *S. pyogenes* retains its regulatory effect on the classical pathway (Thern *et al.*, 1995). However, the finding that many M proteins bind C4BP was initially puzzling, because it has been generally assumed that bacteria activate complement via the alternative pathway. This apparent paradox was resolved by the finding that *S. pyogenes* activates complement via the classical pathway (Carlsson *et al.*, 2003).

Factor H/FHL-1. Many strains of *S. pyogenes* bind factor H (FH) and its smaller splice-variant factor H-like protein 1 (FHL-1), both of which are complement regulators of the alternative pathway (Horstmann *et al.*, 1988; Johnsson *et al.*, 1998). This finding has led investigators to hypothesize that these two plasma proteins, when bound to the M protein, are important for avoiding a complement attack initiated by the alternative pathway. However, at present there is no evidence supporting this hypothesis. In fact, all available evidence indicates that binding of FH and FHL-1 is not important with regard to phagocytosis resistance (Perez-Casal *et al.*, 1995; Kotarsky *et al.*, 2001). Thus, the biological role of the FH/FHL-1 binding is unclear.

Albumin. Many *S. pyogenes* strains bind human serum albumin (Kronvall *et al.*, 1979b), which binds to the C repeat region of the M protein (Retnoningrum and Cleary, 1994;

Åkesson *et al.*, 1994). Little is known about the biological function of this interaction, but albumin bound to the M protein has been implicated in preventing antibodies from binding to the C repeat region (Sandin *et al.*, 2006).

IgA and IgG. The ability of many *S. pyogenes* strains to bind the Fc-parts of IgA or IgG was first demonstrated in the 1970s (Kronvall, 1973; Christensen and Oxelius, 1975). A subsequent study demonstrated that binding to IgA and/or IgG is a common property of many clinical isolates (Lindahl and Stenberg, 1990). The binding to both of these immunoglobulins was later attributed to members of the M protein family (Frithz *et al.*, 1989; Heath and Cleary, 1989). The IgA binding M proteins have been studied in most detail. The IgA binding region in these proteins is well-defined and can be studied in isolated form as a synthetic peptide (Johnsson *et al.*, 1999; Sandin *et al.*, 2002). Importantly, binding of the Fc-part of IgA to an M protein contributes to phagocytosis resistance (Carlsson *et al.*, 2003), possibly by interfering with the binding of IgA to the human IgA-Fc receptor CD89 on neutrophils (Pleass *et al.*, 2001).

Kininogen. Some M proteins have been demonstrated to bind kininogen. The binding site in M protein has not been precisely defined, but it has been proposed that it does not overlap with those of other plasma proteins interacting with the M protein studied (Ben Nasr *et al.*, 1995). Kininogen can be cleaved by the streptococcal cysteine protease SpeB, resulting in release of proinflammatory kinin, which in turn has been suggested to increase vascular permability (Herwald *et al.*, 1996).

Plasminogen. Certain *S. pyogenes* strains express an M protein capable of binding plasminogen via a site in the semivariable region (Ullberg *et al.*, 1989; Berge and Sjöbring, 1993; Wistedt *et al.*, 1995). Bound plasminogen can subsequently be activated into plasmin, a very potent serine protease with broad specificity, by secreted streptokinase or by host factors (Boyle and Lottenberg, 1997; Ringdahl *et al.*, 1998). The presence of plasmin activity on the bacterial surface has been implicated in bacterial virulence (Khil *et al.*, 2003; Sun *et al.*, 2004; Walker *et al.*, 2005).

Fibronectin. Several M proteins bind fibronectin (Frick *et al.*, 1995), an interaction which has been suggested to mediate invasion of epithelial cells (Cue *et al.*, 2001). In addition to the M protein, several other *S. pyogenes* proteins, including the structurally unrelated protein F, have also been implicated in fibronectin binding (Hanski and Caparon, 1992).

LRR PROTEINS

Proteins with leucine rich repeats (LRRs) comprise a large group of proteins with characteristic repeats, which usually are arranged in a tandem fashion. Each individual repeat is 20-29 aa long and includes the 11 residue consensus sequence LXXLXLXXNXL (X = any amino acid). The leucines can be replaced by other amino acids with hydrophobic side chains, most frequently isoleucine or valine but also phenylalanine, whereas the asparagine is sometimes replaced with a cysteine or a threonine. LRR proteins have been identified in both prokaryotes and eukaryotes. Their function is diverse, ranging from RNase inhibition to lipopolysaccharide binding, but the common theme is believed to be involvement in different ligand recognitions (Kobe and Deisenhofer, 1995b; Buchanan and Gay, 1996).

The first LRR protein to be described was the leucine-rich α_2-glycoprotein, a human serum protein with unknown function (Takahashi *et al.*, 1985). Since then, a vast number of LRR proteins have been identified, such as adenylyl cyclase, G-protein coupled receptors and proteins involved in the splicing machinery (Buchanan and Gay, 1996). Importantly, the toll-like receptors (TLRs) and the nucleotide-binding oligomerization domain (NOD) receptors, which are crucial members of the immune system and part of the first line of defense against pathogens, both contain LRR domains recognizing conserved structures located on the microbial surface (Medzhitov, 2001; Inohara and Nuñez, 2003). In this context, it is interesting to note that several plant species express LRR proteins as part of the defense system against invading pathogens (Buchanan and Gay, 1996). Moreover, the recent discovery in jawless vertebrates of variable lymphocyte receptors containing highly diverse LRR domains further highlights the importance of LRR proteins with regard to protein-protein interactions (Pancer *et al.*, 2004, 2005). Of note, mutations in certain human LRR proteins have been implicated in diseases such as Crohn's and Parkinson's (Ogura *et al.*, 2001; Taylor *et al.*, 2006).

The first crystal structure of a LRR protein was that of the porcine ribonuclease inhibitor. In this protein, the tandemly arranged repeats form a horseshoe shaped molecule, in which each repeat is composed of an α-helix, facing the convex side, and a β-strand, facing the concave side (Figure 5) (Kobe and Deisenhofer, 1993). Later it was shown that the β-strands are responsible for binding to the ligand, ribonuclease A (Kobe and Deisenhofer, 1995a). A number of subsequent crystal studies of other LRR proteins have shown great overall structural similarity to that of the ribonuclease inhibitor (Kajava and Kobe, 2002). It should be noted that the curvature may vary between different proteins (Kajava, 1998). In

addition, the number of repeats is variable; the *Drosphila* protein chaoptin has been demonstrated to contain as many as thirty repeats (Krantz and Zipursky, 1990).

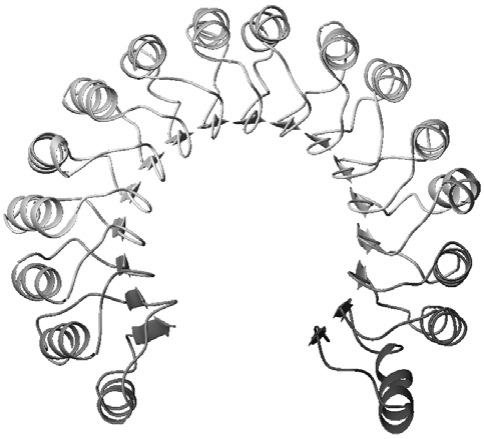

Figure 5. Top view of porcine ribonuclease inhibitor (PDB accession code 2BNH), showing its horseshoe shape. The outer layer is composed of α-helices and the inner layer of parallel β-strands. Adopted from Wikipedia (http://en.wikipedia.org/Ribonuclease_inhibitor).

Although most extensively studied in eukaryotes, there are several LRR proteins expressed by bacterial pathogens, including Internalin A (InlA) of *Listeria monocytogenes* (Gaillard *et al.*, 1991), the YopM protein of *Yersinia pestis* (Evdokimov *et al.*, 2001), the IpaH protein family in *Shigella flexneri* (Venkatesan *et al.*, 1991) and the SspH proteins in *Salmella enterica* serovar Typhimurium (Miao *et al.*, 1999).

The most well-studied bacterial LRR protein is InlA of *L. monocytogenes*. InlA mediates bacterial invasion of cells of the intestinal epithielium by binding to its human receptor E-cadherin. The binding domain of InlA consist of 15 LRRs, each 22 amino acids long with the exception of the sixth repeat which is one amino acid shorter (Gaillard *et al.*, 1991; Mengaud *et al.*, 1996; Schubert *et al.*, 2002). Interestingly, the interaction between InlA and E-cadherin is species specific, as demonstrated by studies showing that InlA is able to bind to human, but not mouse E-cadherin, a property due to a single proline residue crucial for the interaction with InlA. This residue is present in human E-cadherin, whereas the corresponding position in mouse E-cadherin is occupied by a glutamatic acid residue (Lecuit *et al.*, 1999). The importance of the species specific interaction was demonstrated in a mouse model, using transgenic mice expressing human E-cadherin. These mice were permissive for

Listeria, as opposed to wild type mice (Lecuit *et al.*, 2001). The crucial interaction with the proline residue is mediated by a hydrophobic pocket located in the sixth repeat in InlA, as judged by a study of InlA in complex with human E-cadherin (Schubert *et al.*, 2002).

A homolog of InlA was identified in *S. pyogenes* using *in silico* analysis (Reid *et al.*, 2001). Molecular characterization of this protein, designated Slr (for streptococcal leucine-rich), revealed the presence of 10½ LRRs (Reid *et al.*, 2003). In **paper I** we identify Blr (for group B, leucine-rich), a protein with sequence similarity to both Slr and InlA, expressed by *S. agalactiae*. Together, Blr and Slr define a family of streptococcal surface proteins with LRRs.

FIBRINOGEN

Upon blood vessel injury, the coagulation process is rapidly activated. A key component of this process is fibrinogen (Fg), which is converted into fibrin monomers that polymerize to form a stable clot, effectively sealing off the site of injury. The conversion of Fg to fibrin is mediated by thrombin, which exposes the polymerization sites, enabling the formation of a fibrin network. During the wound healing process the fibrin clot is dissolved by plasmin, an enzyme which cleaves the fibrin molecule at several distinct sites (Nieuwenhuizen, 2001).

Fg is synthesized in the liver and has a plasma concentrations of ~3 mg/ml. It is a large (~340 kDa) glycoprotein and consists of six polypeptide chains; 2 Aα, 2 Bβ and 2 γ chains, which are covalently bound via 29 disulfide bonds (Weisel, 2005). The Fg molecule may be divided into a central E domain, composed of the N-terminal parts of the six chains, and two flanking globular D domains (Figure 6). The so-called αC domains extend from the D domains and stabilize the fibrin clot (Weisel and Medved, 2001). Of note, Fg is present not only in plasma, but also in exudates on mucosal surfaces (Persson *et al.*, 1998).

In addition to its vital function in haemostasis, Fg has been shown to bind to leukocytes, such as neutrophils, monocytes, macrophages and certain lymphocytes. The interaction is mediated by surface localized integrins, such as $\alpha_M\beta_2$ (also known as CD11b/CD18 or Mac-1) and $\alpha_X\beta_2$ (CD11c/CD18). The binding to $\alpha_M\beta_2$ has been localized to the γ chain in the D domain of Fg (Ugarova and Yakubenko, 2001). Transgenic mice lacking the $\alpha_M\beta_2$ binding site of Fg were severely compromised with regard to clearing a bacterial infection, implying that leukocyte interactions with Fg play an important role in the inflammation process (Flick *et al.*, 2004).

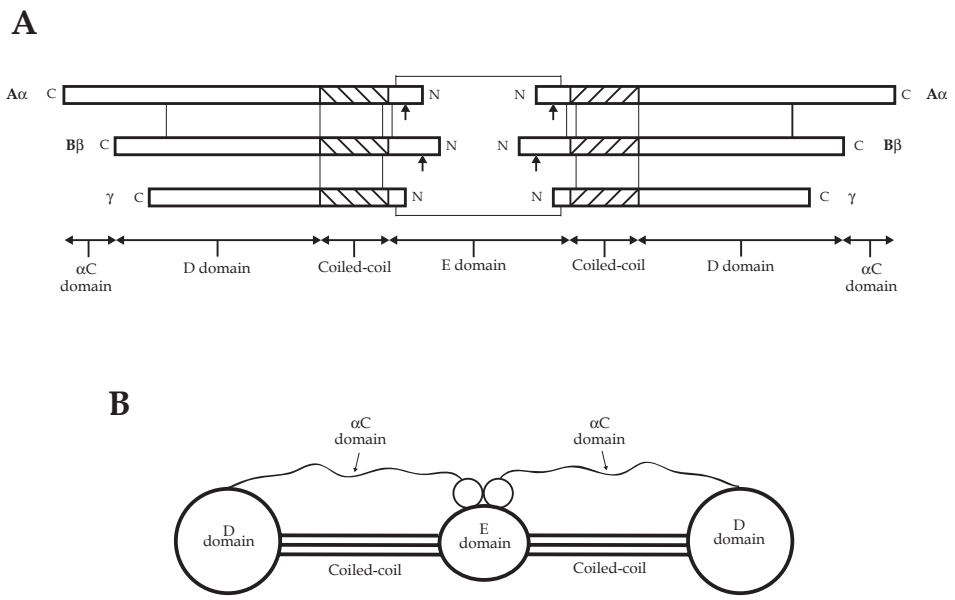

Figure 6. Schematic representation of fibrinogen (Fg). A. Polypeptide organization of Fg. The thrombin cleavage sites in the Aα and the Bβ chains are indicated with arrows. The coiled-coil regions are depicted with hatched lines. Disulphide bonds are indicated with thin black lines. For the sake of clarity, some of the 29 disulphide bonds have been omitted. B. Domain organization of Fg. Based on information available in Weisel and Medved (2001) and Weisel (2005).

THE COMPLEMENT SYSTEM

An important part of innate immunity, the complement system consists of >35 proteins, primarily synthesized in the liver. This system is activated through a number of proteolytical steps where each component activates the next one in a cascade pattern. Three distinct activation pathways exist: the classical pathway, the lectin pathway and the alternative pathway. A common characteristic of all pathways is the formation of C3b, the central and most important component of the complement system which functions as an opsonin "labeling" pathogens for phagocytosis. The three pathways converge in the formation of the membrane attack complex (MAC). A brief overview of the complement system is presented in Figure 7. Of note, in addition to its important function in innate immunity, complement has also been demonstrated to assist in the removal of immune complexes and apoptotic bodies. Moreover, the adaptive branch of the immune system relies on the complement system for an efficient humoral response (Prodinger *et al.*, 2003; Verschoor and Carroll, 2004).

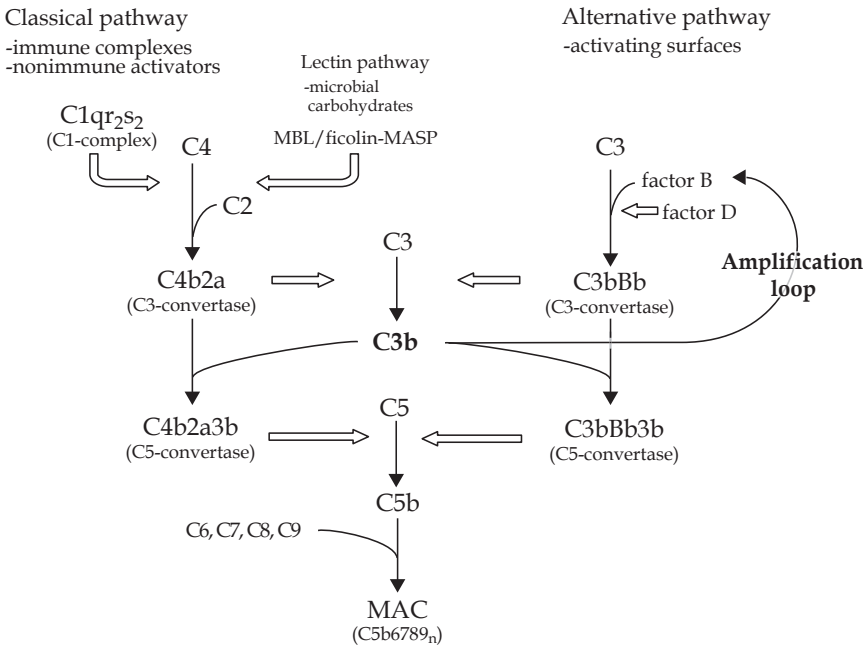

Figure 7. Schematic overview of the complement system. Adopted from Persson (2006).

The classical pathway was the first branch of the complement system to be described. However, phylogenetic analysis has revealed that this pathway is the most recently evolved one. Activation via the classical pathway is initiated by antibodies, predominately IgM and IgG, the former being the most potent activator. Other activators include C-reactive protein (CRP), bacterial lipopolysaccharides and viral envelopes. When one of these activators interacts with C1q of the C1 complex (consisting of C1q, C1r and C1s in a 1:2:2 ratio), activation via the classical pathway is initiated. C1r is rapidly activated and cleaves C1s, events which expose a serine protease domain in C1s. Subsequently, C1s cleaves C4 into C4a and C4b, the latter of which covalently attaches to the surface of the pathogen. The membrane-bound C4b molecule binds C2 and the C1 complex then splits bound C2 into C2a and C2b. The C2b molecule diffuses away, whereas C2a, together with C4b forms the classical pathway C3 convertase (also known as C4b2a). This convertase cleaves C3, resulting in formation of C3a and C3b. The C3a molecule diffuses away and functions as a so-called anaphylatoxin, attracting phagocytes to the site of infection, whereas C3b covalently binds to the surface of the pathogen via a reactive thioester group. Importantly, C3b is a central component of the complement system and functions as an opsonin. An important

additional function of C3b is its association with the classical pathway C3 convertase, an interaction which results in the formation of the classical pathway C5 convertase, C4b2a3b, which cleaves C5 into C5a, a potent anaphylatoxin, and C5b, the starting point for the formation of the MAC (see below) (Prodinger *et al.*, 2003).

The lectin pathway, although being discovered relative recently, is considered to be the most ancient pathway from an evolutionary point of view, indicating that the complement system evolved from a lectin-based opsonizing system (Holmskov *et al.*, 2003). Interestingly, the components of the lectin pathway are structurally similar to those of the classical pathway (Gadjeva *et al.*, 2001). Initiating events include binding of mannan binding lectins (MBL) or ficolins to conserved, repetitive structures on the surface of a pathogen, such as lipotechoic acid of Gram-positive bacteria and lipopolysaccharides of Gram-negative bacteria. Following the initial binding, MBL associated serine proteases (MASPs) 1, 2 and 3 may be recruited. This complex cleaves C4 and C2 into their respective split products, resulting in formation of the classical pathway C3 convertase C4b2a. Thus, the lectin pathway and the classical pathway converge at this point (Holmskov *et al.*, 2003; Prodinger *et al.*, 2003).

Activation via the alternative pathway begins with the spontaneous hydrolysis of C3, a slow event which results in C3(H_2O), which has properties similar to C3b. The C3(H_2O) molecule can bind factor B, one of the central components of the alternative pathway, and the protease factor D then cleaves the complex, resulting in fluid phase C3(H_2O)Bb. This complex can cleave C3 into C3b, which then can interact with adjacent surfaces, as described above. In this fashion, low amounts of C3b are constantly produced. The fate of the newly formed C3b depends upon the nature of the surface. If C3b is deposited on the surface of a host cell (also known as a non-activator surface), C3b will be rapidly degraded. In contrast, C3b bound to the surface of a pathogen may interact with factor B, which in turn recruits factor D, resulting in the formation of the alternative pathway C3 convertase C3bBb, which is stabilized by properdin. A rapid formation of numerous C3b molecules follows, which bind covalently to the activator surface. Thus, the alternative pathway functions as an important amplification loop for the formation of C3b. Some of the C3b molecules will be associated with C3bBb, forming the alternative pathway C5 convertase C3bBbC3b, which cleaves C5 into C5a and C5b (Morgan and Harris, 1999; Prodinger *et al.*, 2003).

Following formation of C5b by any of the three pathways described above, a series of non-enzymatic cascade steps commence, resulting in the formation of the membrane attack complex (MAC). The first step in this process involves the association of C5b with C6, following rapid recruitment of C7. This complex, designated C5b67, inserts into the

phospholipid membrane, enabling sequential interaction with C8 and C9. Upon binding to the C5b6-8 complex, C9 undergoes a conformational change which enables polymerization and formation of a pore in the membrane, causing cell lysis. As many as 18 C9 molecules can participate in the formation of a pore. Of note, due to the presence of the thick cell wall, Gram-positive bacteria are resistant to MAC mediated lysis (Morgan and Harris, 1999; Prodinger *et al.*, 2003; Verschoor and Carroll, 2004).

Because of its potent effector mechanism, the complement system is tightly regulated by a number of fluid-phase and surface-bound proteins (Figure 8). The C1 complex of the classical pathway is under the control of the C1 inhibitor (C1-inh), which associates with C1r and C1s and is not released until C1 is activated by interaction with an antibody-antigen complex. In addition, C1-inh may also inactivate already activated C1. Similarly, C1-inh also inactivates the MASP proteins of the lectin pathway.

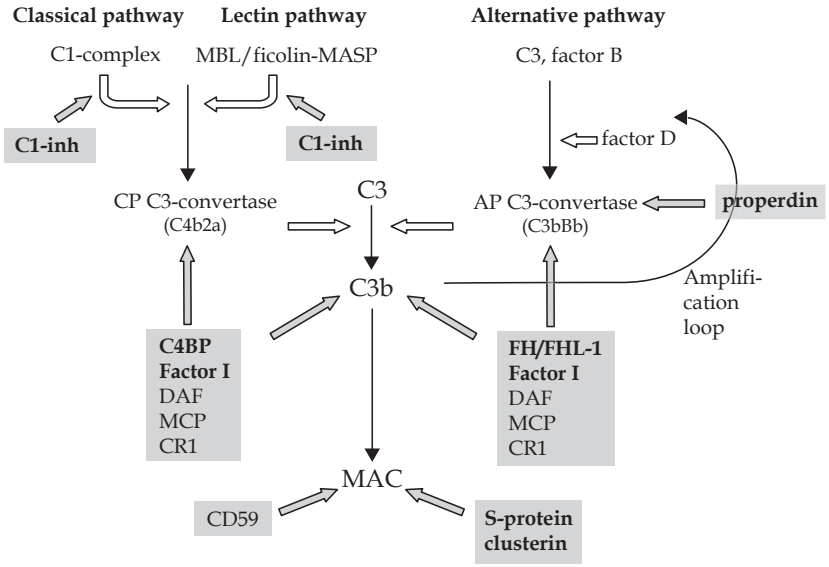

Figure 8. Schematic representation of the regulation of the complement system. Complement regulators (grey boxes) are either soluble plasma proteins (bold) or cell membrane proteins. All regulators except properdin (which stabilizes the AP C3 convertase) inhibit activation at the step indicated. Adopted from Persson (2006).

However, the major regulatory control in the complement system is exerted at the level of C3 formation, more specifically the two C3 convertases. The plasma protein C4b-binding protein (C4BP) causes disassociation of the classical pathway C3 convertase into C4b and C2a and serves as a co-factor for factor I, which subsequently degrades C4b into the smaller fragments

C4c and C4d. The plasma protein factor H (FH) binds to and dissociates the alternative C3 convertase. Moreover, FH serves as a co-factor for factor I in the degradation of C3b into the enzymatically inactive iC3b. Thus, factor I is a central player in the regulation of the complement system (Morgan 1999, Prodinger 2003).

In addition to the soluble regulators C4BP and FH, several important complement regulators are present on cell surfaces. These include membrane cofactor protein (MCP; CD46) and decay accelerating factor (DAF; CD55), which are present on virtually every cell. In addition, complement regulator 1 (CR1; CD35) is also an important surface-bound regulator, but the expression of CR1 is restricted to cells of the immune system, such as erythrocytes, monocytes, macrophages and neutrophils. Importantly, MCP, DAF and CR1 inhibit both the alternative and classical C3 convertase (Morgan 1999, Prodinger 2003).

The terminal membrane attack complex is regulated by the plasma proteins S-protein and clusterin, which bind to and inhibit C5b67 from insertion into the plasma membrane. In addition, the cell-bound regulator CD59 binds to C8 and prevents polymerization of C9 (Morgan 1999, Prodinger 2003).

PRESENT INVESTIGATION

PAPER I: The streptococcal Blr and Slr proteins define a family of surface proteins with leucine-rich repeats: camouflaging by other surface structures

Proteins with leucine-rich repeats (LRRs) occur among both eukaryotes and prokaryotes. Among bacteria, the most well-studied LRR protein is Internalin A (InlA) of *Listeria monocytogenes*. This protein mediates invasion of intestinal epithelial cells by binding to its human ligand E-cadherin (Gaillard *et al.*, 1991; Mengaud *et al.*, 1996). Recently, a protein with sequence homology to Internalin A was identified in *S. pyogenes* (Reid *et al.*, 2001). This protein, designated Slr (for streptococcal leucine-rich), contains 10½ LRRs in its C-terminal part. The Slr protein has the characteristics of a lipoprotein and is thus most likely covalently attached to the cell membrane via its N-terminal part (Reid *et al.*, 2003). In contrast, InlA is a LPXTG protein and is anchored to the cell wall via the C-terminal part. The LRR region of InlA is located in the N-terminal part and interacts with E-cadherin (Bierne and Cossart, 2007).

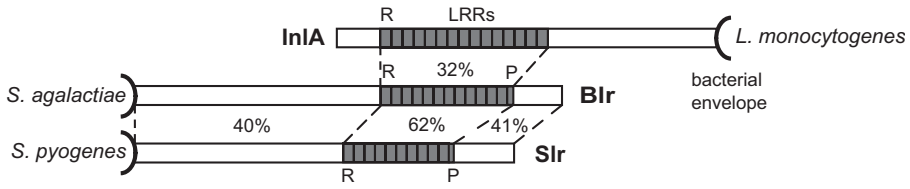

Figure 9. Schematic comparison between the InlA, Blr and Slr proteins. Amino acid residue identities of different regions are indicated in percents. In all three proteins, the LRR region is probably located most distally to the cell surface. There are 12.5 LRRs in Blr, 10.5 LRRs in Slr and 15 LRRs in InlA. R, repeat; P, partial repeat.

We have identified an InlA homologue in *S. agalactiae*. This homologue, designated Blr (for group B, leucine-rich), also shows considerable residue identity and overall similarity to Slr (Figure 9). Thus, Blr and Slr define a family of streptococcal proteins with LRRs. Recombinant proteins were constructed, which were used to raise antisera. Analysis with these antibodies revealed that Blr is present on the bacterial surface, but is largely camouflaged by the capsule, as judged by binding assay analysis with an acapsular mutant. Indeed, surface exposure of Blr was increased >100 times in this mutant compared to the wild-type strain. This effect was not due to upregulation of Blr synthesis in the capsule-

negative mutant, because very similar amounts of Blr were present in the wild-type strain and the acapsular mutant. In contrast, the capsule did not mask the unrelated surface protein Rib, demonstrating that Blr is selectively camouflaged.

In *S. pyogenes*, analysis with anti-Slr antibodies demonstrated that the hyaluronic acid capsule of this pathogen did not camouflage Slr. However, the antiphagocytic M protein and the fibronectin-binding protein F interfered with surface exposure of Slr. Indeed, the surface exposure of Slr was increased >20-fold in mutants lacking either or both of these surface proteins. Thus, both Blr and Slr are camouflaged, but by different surface structures (Figure 10). The physiological relevance of this phenomenon is unclear, but is it possible that the camouflaging structures are downregulated during some parts of the infection process, thus exposing the Blr and Slr proteins on the streptococcal surface. Such dynamic changes could play an important role during an infection.

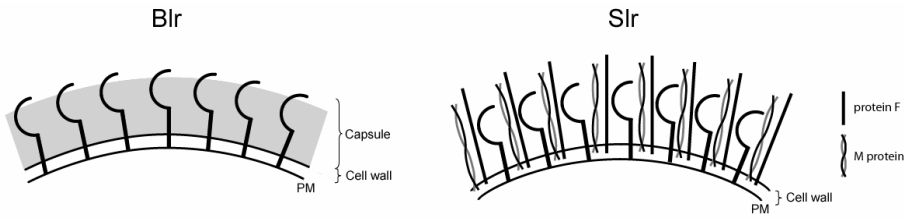

Figure 10. Schematic representation of the camouflaging phenomenon. The Blr protein (left) is camouflaged by the capsule, whereas Slr (right) in camouflaged by the M protein and protein F.

We also analyzed the immunological cross-reactivity of Blr and Slr. The result of such analysis was not obvious, because studies with α and Rib, two members of the Alp family, demonstrated that these proteins did not cross-react, despite showing ~50% sequence identity (Stålhammar-Carlemalm *et al.*, 1993; Wästfelt *et al.*, 1996). Analysis with purified proteins in an ELISA demonstrated a strong cross-reactivity between Blr and Slr. It was of interest to analyze whether a similar result would be obtained using whole bacteria, i.e. when the two proteins were present on the bacterial surface. Because Blr and Slr are camouflaged, mutants lacking the camouflaging structure were employed in this analysis. Surprisingly, this analysis with whole bacteria demonstrated a limited cross-reactivity. These data suggest that those regions of the two proteins, which are exposed in the absence of camouflaging, have been under selective pressure to diverge antigenically.

It was previously reported that Slr contributes to *S. pyogenes* virulence in a mouse model of i.p. infection (Reid *et al.*, 2003). In contrast, InlA is not a virulence factor in the

mouse, because the interaction with E-cadherin is species-specific (Lecuit *et al.*, 1999, 2001). It was therefore of interest to analyze the role of Blr in virulence. In this analysis, a Blr-negative mutant was as virulent as the wild-type strain in an i.p. infection model, indicating that Blr does not contribute to virulence in this model. Importantly, antibodies against Blr were produced during the course of an infection, demonstrating that Blr is immunogenic under these conditions.

In summary, we have identified a family of streptococcal proteins with LRRs, the members of which are camouflaged by other surface structures. The two proteins, designated Blr and Slr, show only limited cross-reactivity when exposed on the bacterial surface, despite substantial residue identity.

PAPER II: **Nonimmunodominant regions are effective as building blocks in a streptococcal fusion protein vaccine**

At present, there is no available vaccine against *S. agalactiae*. Research in this area has been focused either on the capsule or on surface proteins. An apparent disadvantage with constructing a vaccine based on the polysaccharide capsule is the existence of multiple serotypes, which must be included in order to achieve broad coverage. As a consequence, most studies during recent years have focused on surface proteins (Larsson *et al.*, 1996, 1999; Brodeur *et al.*, 2000; Lindahl *et al.*, 2005; Maione *et al.*, 2005).

The Rib and α proteins belong to the Alp family, a protein family in which the members have a long and extremely repetitive structure (Lindahl *et al.*, 2005). Most strains of *S. agalactiae* express either Rib or α and it has been estimated that a vaccine based on these two proteins may protect against ~90% of all *S. agalactiae* strains (Larsson *et al.*, 1999). From a vaccine point of view, it would be advantageous if a single protein could be administered. It was therefore of interest to derive a fusion protein from Rib and α. The large size of Rib and α and the instability of the repeat regions suggested that use of subregions would be the best approach. Which subregions to include was not obvious, because a number of studies have indicated that the immune response to the α protein is complex (Gravekamp *et al.*, 1996, 1997; Kling *et al.*, 1997).

Initial experiments indicated that almost all of the antibodies against intact Rib and α were directed against the repeat region. Thus, the repeat regions of the Rib and α proteins are immunodominant. The reason for this is presently unknown. However, it raises important questions regarding vaccine development. Since the immune response is directed almost

exclusively against the repeats, this region in Rib and α would seemingly be the most attractive one as a component in a fusion protein vaccine. However, this argument does not exclude that the nonimmunodominant N-terminal region would also be suitable for the construction of a fusion protein. Indeed, antibodies raised against the N-terminal part of Rib (designated RibN) protected mice against a lethal infection. The level of protection was similar to or better than that seen when mice were passively vaccinated with antiserum against a construct consisting of two repeats originating from Rib (designated Rib2R). Together, our data showed that protective epitopes are present in both the N-terminal region and the repeat region of Rib. A similar situation prevails for the α protein (Kling *et al.*, 1997).

Because protective epitopes are located in the N-terminal region and the repeat region of Rib and α, it was of interest to compare these regions with regard to ability to elicit protective immunity. Therefore, we constructed a fusion protein consisting of the N-terminal regions, designated RibN-αN, and a fusion protein consisting of two repeats from each protein, designated Rib2R-α2R. Antisera against these two constructs were raised and shown to be protective against Rib- and α-expressing strains in passive vaccination experiments.

The fusion protein RibN-αN was equally immunogenic in mice when administrated with CFA, alum or PBS. In contrast, antibodies against Rib2R-α2R were detected in only one out of four CFA mice. In addition, when this antigen was administered with alum or PBS no antibodies were detected. Thus, RibN-αN was much more immunogenic compared to Rib2R-α2R under these conditions. Importantly, active immunization experiments demonstrated that RibN-αN, using alum as an adjuvant, protected mice against a subsequent challenge with Rib- or α-expressing strains.

A previous study has suggested that α mediates invasion of epithelial cells *in vitro* (Bolduc *et al.*, 2002). Therefore, we compared the role of Rib and α in invasion of the human cervical cell line ME180, employing mutants lacking either protein. These mutants demonstrated significantly lower level of invasion compared to the wild type strains. Thus, Rib and α share the ability to promote bacterial invasion of human epithelial cells, an ability which was efficiently inhibited with anti-(RibN-αN) antibodies.

The observation that the N-terminal regions of Rib and α were nonimmunodominant, but targeted by protective antibodies, prompted us to investigate whether this was a feature of other bacterial surface proteins. This analysis was focused on the extensively studied M22 protein of *Streptococcus pyogenes*, in which the N-terminal region is a target for opsonizing antibodies. Three long synthetic peptides (M22-N, Sap22 and C22) corresponding to different regions of M22 were employed (Figure 11). Strikingly, antiserum raised to the entire M22

protein reacted well with C22, whereas the same antiserum showed little or no reactivity against the M22-N and Sap22 peptides, which are both derived from the N-terminal region. Thus, the N-terminal region of the M22 protein of *S. pyogenes* is nonimmunodominant, in analogy to Rib and α of *S. agalactiae*.

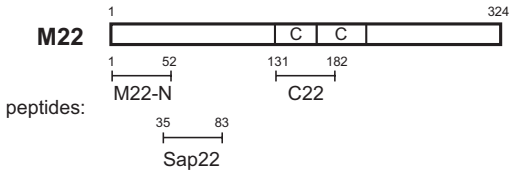

Figure 11. Schematic representation of the mature M22 protein and three synthetic peptides derived from M22. The numbers above each peptide refers to the corresponding amino acid position in M22. C, C repeat region. Modified from Carlsson *et al.* (2003).

In summary, we show that the antibody response to the Rib and α proteins is largely directed against the repeat regions, i.e. the N-terminal regions of these proteins are non-immunodominant. Nevertheless, a fusion protein derived from the N-terminal parts of Rib and α was immunogenic even without adjuvant and conferred good protective immunity in an animal model. Moreover, antibodies raised against the fusion protein inhibited bacterial invasion of epithelial cells. In agreement with the result obtained with Rib and α, analysis of the M22 protein of *S. pyogenes* demonstrated the N-terminal part of this protein was also non-immunodominant. These data focus interest on nonimmunodominant protein regions as vaccine components.

PAPER III: Critical role for bacteria-bound fibrinogen in *Streptococcus pyogenes* invasive infection

Many bacterial pathogens express surface proteins which have the ability to bind fibrinogen (Fg), a major component of human plasma. It is commonly assumed that this interaction contributes to virulence and *in vitro* studies suggest that bound Fg may promote adhesion, affect quorum sensing, trigger vascular leakage or interfere with complement deposition (Foster and Höök, 1998; Rothfork *et al.*, 2003; Herwald *et al.*, 2004; Carlsson *et al.*, 2005). However, the *in vivo* importance of the Fg interaction is unclear.

All clinical isolates of *S. pyogenes* bind Fg (Kronvall *et al.*, 1979a). In many strains, this interaction is mediated by M protein (Kehoe, 1994), but in many other strains, in particular OF^+ strains, the binding is mediated by the related Mrp protein (O'Toole *et al.*, 1992; Thern *et*

al., 1998). In paper III we studied the Fg-binding B repeat region of the M5 protein and its effect on virulence in a mouse model, using well-defined internal deletion mutant strains.

It was not obvious that the M5 protein binds mouse Fg. Therefore, this interaction was characterized in detail. The M5 protein was able to bind to Fg of both human and mouse origin. Moreover, human and mouse Fg were equally effective in blocking the interaction between human Fg and pure M5 protein. Finally, M5 expressed on the bacterial surface interacted with mouse Fg in a plasma absorption assay. Thus, the mouse system is relevant with regard to studying the biological importance of the Fg interaction.

Next, we used mixed infection experiments to analyze the *in vivo* role of the Fg binding domain. Mice were infected i.p. with a 1:1 mixture of wild-type and mutant bacteria and sacrificed after 48 hours, when spleens and livers were analyzed for presence of the two strains in the inocolum. When wild-type bacteria and the ΔM5 mutant, which lacks the entire M protein, were used in such experiments, almost all of the recovered bacteria were wild-type. Thus, the M5 protein is a virulence factor in this model. To specifically analyze the role of the Fg-binding domain, we performed mixed infection experiments using wild-type bacteria and a mutant, designated ΔB, lacking this domain. The results were similar to those obtained with the ΔM5 mutant. Thus, the Fg-binding domain B repeat region is crucial with regard to virulence. Furthermore, a mutant designated ΔN1, which lacks the N-terminal region in M5, was also analyzed. Interestingly, this mutant was also strongly attenuated. Thus, both the N-terminal region and the B repeat region of M5 contribute to virulence under the conditions used. Importantly, these effects of the deletions were not unspecific because a mutant lacking the conserved C repeat region was only slightly attenuated. The role of the Fg-binding domain was further analyzed in a mouse model using a lethal bacterial dose. Mice infected with wild-type bacteria succumbed, whereas all mice given the ΔB mutant survived, confirming that the Fg-binding domain is important in virulence.

To analyze whether the Fg-binding domain has other ligands than Fg, a fusion protein consisting of the entire B repeat region of M5 and GST was constructed. The construct, designated GST-M5B was coupled to a glutathione column, after which whole plasma, of human or mouse origin, was applied. Under these conditions, the eluted fractions contained only Fg, implying that of all proteins present in plasma, the B repeat region specifically interacts with Fg. A recent study reported that the surface protein FnBPA, expressed by *Staphylococcus aureus*, contains a binding site that interacts with both Fg and elastin (Keane *et al.*, 2007). Therefore, we analyzed whether the M5 protein binds elastin in a dot-blot. No such binding was observed, indicating that Fg is the sole ligand of the B repeat region.

In vitro studies using human blood have demonstrated that Fg bound to the surface-localized M protein promotes phagocytosis resistance by inhibiting complement deposition (Carlsson *et al.*, 2005). To investigate whether mouse Fg bound to M5 also inhibits complement deposition, M5-expressing wild-type bacteria were incubated in mouse serum with and without the addition of pure mouse Fg. In the presence of Fg, complement deposition was reduced ~6-fold, indicating that mouse Fg indeed interferes with complement deposition. These results may explain the reduced virulence of the ΔB mutant in mixed infection experiments.

In summary, we have analyzed the *in vivo* role of the Fg binding B repeat region of the M5 protein. This region binds mouse and human Fg equally well, allowing detailed analysis in a mouse infection model. Using mixed infection experiments, we show that the B repeat region is crucial with regard to virulence. Furthermore, Fg is most likely the sole ligand of this region. Thus, bacteria-bound Fg contributes to virulence, possibly by inhibiting complement deposition.

CONCLUSIONS

- The novel Blr protein of *S. agalactiae* and the previously described Slr protein of *S. pyogenes* identify a family of streptococcal proteins with leucine-rich repeats (LRRs), repeats present in proteins that are mainly responsible for different ligand interactions. Both Blr and Slr display sequence homology to Internalin A of *Listeria monocytogenes*, a protein responsible for bacterial invasion of epithelial cells. Remarkably, Blr and Slr were efficiently camouflaged by other surface components, as judged by antibody binding experiments Access to Blr was inhibited by the capsule, whereas Slr was masked by the antiphagocytic M protein and the fibronectin-binding protein F. We propose that the camouflaging structures may be downregulated during a certain infection stage, thus exposing Blr and Slr on the bacterial surface. Moreover, despite displaying a high degree of sequence identity, Blr and Slr showed a low degree of immunological cross-reactivity when exposed on the bacterial surface, suggesting that the two proteins have been under evolutionary pressure to diverge antigenically.

- Traditionally, vaccine development has been focused on proteins or protein regions that elicit a good immune response. However, few studies have addressed the vaccine potential of protein regions which are poorly immunogenic within the intact protein. We hypothesized that such regions are interesting as vaccine components. In this study, we found that the immune response to Rib and α, two surface proteins expressed by *S. agalactiae*, is mainly directed against the C-terminal repeat region. Thus, this region is immunodominant. Nevertheless, a fusion protein derived from the nonimmunodominant N-terminal regions of Rib and α was as effective as a fusion protein of similar size derived from the repeats in generating protective antibodies. Moreover, the N-terminal fusion protein was immunogenic even without adjuvant. Antibodies against this fusion protein inhibited bacterial invasion of an epithelial cell line. Importantly, the N-terminal region of the M22 protein of *S. pyogenes*, which is targeted by opsonic antibodies, was also nonimmunodominant. Together, these data focus interest on nonimmunodominant regions for vaccine development.

- Many pathogenic bacteria have the ability to bind fibrinogen (Fg). However, despite being extensively characterized *in vitro*, little is known about the *in vivo* relevance of

this interaction. We studied the Fg-binding B repeat region of the M5 protein of *S. pyogenes*. A mutant lacking this region was severely attenuated for virulence in a mouse model. This effect was most likely due to the lack of Fg binding, since a peptide derived from the B repeat region exclusively bound Fg among all plasma proteins. *In vitro* experiments demonstrated that complement deposition was inhibited ~6-fold in the presence of mouse Fg, potentially explaining the lack of virulence of the mutant lacking the Fg binding domain. Together, our studies provide the first clear evidence that Fg-binding to a major bacterial pathogen contributes to virulence.

SAMMANFATTNING PÅ SVENSKA

Patogena mikroorganismer har många olika mekanismer att orsaka sjukdomar. Flera av dessa mekanismer är beroende av ytproteiner, som interagerar med värdorganismen. Ytproteiner är även viktiga komponenter i vaccin, ett forskningsområde som blir allt viktigare med tanke på den oroande ökningen av antalet antibiotikaresistenta bakteriestammar i samhället. I denna avhandling studeras ett flertal ytproteiner hos två viktiga humanpatogener, *Streptococcus pyogenes* (grupp A streptokocker) och *Streptococcus agalactiae* (grupp B streptokocker).

S. pyogenes ger upphov till milda infektioner som halsfluss och svinkoppor, men kan även i mer sällsynta fall ge upp till livshotande infektioner och efterföljande komplikationer, och kallas därför ibland ”mördarbakterien”. Uppskattningsvis dör c:a 500 000 människor varje år p.g.a. infektioner orsakade av *S. pyogenes*, främst i utvecklingsländer där tillgången till sjukvård är begränsad. Denna bakterie uttrycker en mängd olika virulensfaktorer, varav ett flertal är ytproteiner. Det mest välstuderade av dessa är M-proteinet, som med sina antifagocytära egenskaper tillåter bakterien att undvika en attack från immunförsvaret.

S. agalactiae är besläktad med *S. pyogenes*, men ger upphov till helt andra sjukdomstillstånd. Denna bakterie är den viktigaste orsaken till infektioner hos nyfödda barn, såsom lunginflammation, blodförgiftning och hjärnhinneinflammation. Bakterien överförs under förlossningen till fostret från vaginan hos koloniserade mödrar. Ungefär 25% av samtliga kvinnor är koloniserade och barn födda av dessa kvinnor kommer således att bli exponerande för bakterien. Dock drabbas endast en liten del av dessa barn av infektioner orsakade av *S. agalactiae*, vilket bl.a. beror på nivån skyddande antikroppar hos modern. *S. agalactiae* kan även ge blodförgiftning och diverse hudinfektioner hos äldre och personer med nedsatt immunförsvar och/eller övriga sjukdomar, exempelvis diabetes. Relativt lite är för tillfället känt om de sjukdomsframkallande mekanismerna hos *S. agalactiae*, men liksom hos *S. pyogenes* är förmodligen flera ytproteiner inblandade i denna process.

I **delarbete I** beskriver vi det nyupptäckta ytproteinet Blr som uttrycks av *S. agalactiae*. Sekvensanalyser visar att Blr innehåller s.k. leucin-rika regioner (på engelska leucine-rich repeats eller LRR). Proteiner innehållande LRR är vanligt förekommande hos både eukaryoter och prokaryoter, och är involverade i diverse protein-protein-interaktioner. Vidare uppvisar Blr sekvenslikheter med det tidigare beskrivna Slr-proteinet hos *S. pyogenes*, som även det innehåller LRR. Således har vi identifierat en ny LRR-familj med medlemmar hos två olika streptokock-arter. Blr och Slr uppvisar även sekvenslikheter med Internalin A, ett protein som uttrycks av humanpatogenen *Listeria monocytogenes* och medierar invasion av epitelceller.

Vid molekylär karakterisering av Blr och Slr upptäckte vi att dessa proteiner mycket effektivt kamoufleras av andra komponenter på bakteriecellsytan, ett fenomen som tidigare inte beskrivits i litteraturen. Den fysiologiska relevansen av detta fynd är oklar, men det kan tyda på att de kamouflerande strukturerna nedregleras någon gång under infektionsprocessen, vilket i sin tur leder till att Blr och Slr kommer exponeras på bakterieytan. Trots omfattande likheter mellan Blr och Slr på aminosyranivå uppvisar de endast en svag immunologisk korsreaktivitet under fysiologiska förhållanden, något som kan reflektera att de två proteinerna under evolutionens gång utvecklats för att undvika korsreagerande antikroppar.

Det finns för närvarande inget vaccin mot *S. agalactiae*, men i USA pågår vaccinförsök baserade på polysackaridkapseln. Eftersom ett flertal distinkta kapseltyper existerar och därför måste inkluderas i ett eventuellt vaccin, vore det bättre att använda vanligt förekommande ytproteiner som huvudsakliga komponenter i ett vaccin. I **delarbete II** studerar vi Rib och α, två ytproteiner som tillsammans uttrycks av majoriteten kliniska isolat. Dessa proteiner tillhör en familj, vars medlemmar karakteriseras av extremt repeterade sekvenser. Våra analyser visade att antikroppssvaret mot Rib och α huvudsakligen är riktat mot den C-terminalt belägna repetitiva regionen, d.v.s. denna region är ”immunodominant”. Däremot var få antikroppar riktade mot de N-terminala delarna, som således är ”icke-immunodominanta”. Trots detta ger ett fusionsprotein bestående av de N-terminala delarna av Rib och α upphov till ett bra immunsvar och skyddande immunitet i en musmodell. Antikroppar mot detta fusionsprotein hämmar även Rib- och α-medierad invasion av en epitelcellslinje. Slutligen visade analyser med en annan bakterie, *S. pyogenes*, att den N-terminala delen hos det välstuderade M-proteinet var ”icke-immunodominant”. Eftersom den N-terminala delen i M-proteinet är mål för skyddande antikroppar tyder detta på att bakterier utvecklat en generell mekanism för att undvika ett immunsvar mot vissa delar av ytproteiner. Vidare visar denna studie på ett nytt koncept inom vaccinforskningen, nämligen att icke-immunodominanta regioner paradoxalt nog är intressanta som vaccinkomponenter.

Ett flertal bakteriella patogener har förmågan att binda fibrinogen (Fg), ett protein som finns i hög koncentration i blodet och spelar en central roll i koagulationsprocessen. Denna interaktion mellan Fg och bakterier är välstuderad *in vitro*, men dess roll *in vivo* är oklar. I **delarbete III** analyserar vi den Fg-bindande B-regionen i M5-proteinet hos *S. pyogenes* och hur den bidrar till virulensen i en musmodell. En bakteriemutant som saknar B-regionen visade sig ha kraftigt nedsatt virulens i denna djurmodell, liksom en mutant där den mest N-terminala delen av M5 är deleterad. Detta resultat visar att två oberoende regioner i M5-proteinet har stor betydelse för dess virulensegenskaper. Egenskaperna hos mutanten som

saknar den Fg-bindande domänen studerades i mer detalj. Denna region visade sig endast binda Fg av samtliga proteiner närvarande i musplasma, vilket tyder på att B-regionen hos M5 selektivt binder Fg. Detta resultat tyder i sin tur på att den nedsatta virulensen i musmodellen förmodligen beror på avsaknad av Fg-bindning. *In vitro*-försök visade att Fg bundet till ytan av *S. pyogenes* nedreglerar deposition av komplement, en viktig del av det medfödda immunförsvaret. Detta resultat kan ge en molekylär förklaring till den uteblivna virulensen hos mutanten som saknar förmåga att binda Fg. Delarbete III bidrar således med viktig kunskap gällande den biologiska betydelsen av interaktionen med Fg hos *S. pyogenes*.

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