Surface Proteins of *Streptococcus agalactiae* and Related Proteins in Other Bacterial Pathogens

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INTRODUCTION

Gram-positive cocci cause some of the most common infections in humans (69, 96, 166, 237). Major efforts are therefore under way to find new ways to prevent and treat infections caused by these pathogens, to analyze pathogenetic mechanisms, and to develop efficient vaccines. While much work is devoted to studies of polysaccharide capsules (7, 186, 266), surface proteins are attracting increasing interest for studies of pathogenesis and as potential vaccine components.

Here, we review current knowledge about surface proteins of *Streptococcus agalactiae* (group B streptococcus [GBS]), the most important cause of invasive bacterial disease in newborns (60). This bacterium is part of the normal vaginal flora of many women and is therefore strategically located to cause serious infections in neonates, whose immune response is depressed compared to older children and adults (60, 63). In addition to its significance as a pathogen, *S. agalactiae* is a valuable model system for analysis of infections caused by encapsulated pathogens and for analysis of infections in the perinatal period.

Surface proteins of *S. agalactiae* are likely to play important roles during different stages of an infection and also hold promise as vaccine components (33, 140). Moreover, *S. agalactiae* surface proteins are of interest for use in the analysis of some major problems in medical microbiology, such as adhesion to epithelial cells, interactions with human extracellular matrix or plasma proteins, and escape from host immunity. This review focuses on the *S. agalactiae* surface proteins that have so far been purified and characterized at the molecular level, with emphasis on proteins that are known to elicit protective immunity and therefore are of interest for vaccine development. To provide the proper background, the first part of the review briefly describes the pathogenesis of *S. agalactiae* infections, the polysaccharide capsule, and current knowledge about *S. agalactiae* genomics. A detailed description of *S. agalactiae* infections is beyond the scope of this article, but excellent reviews are available (60, 179).

By tradition, *S. agalactiae* has most commonly been referred to as group B streptococcus or GBS, but in recent years there has been a tendency to use the Latin name for this pathogen, as for other pathogens, and this practice is followed here.

PATHOGENESIS OF *S. AGALACTIAE* INFECTIONS

Neonatal and Other Infections

S. agalactiae first received attention as a cause of bovine mastitis, a disease that gave the bacterium its name (119). During the last three decades, *S. agalactiae* has also emerged as an important cause of human disease and is now the most common cause of life-threatening invasive bacterial infections (septicemia, pneumonia, and meningitis) during the neonatal period (60, 221). The reason for this emergence of *S. agalactiae* as a cause of disease in human newborns is not known, but an interesting analysis indicates that the transfer of a lineage of bovine strains may have contributed (21). *S. agalactiae* is also an important cause of disease in parturient women, and in recent years it has emerged as a significant cause of serious disease in adults with underlying conditions such as diabetes and malignancy (64).

S. agalactiae is found in the vaginal and/or rectal flora of 15 to 40% of adult women, and children born to these women may develop disease due to exposure to the bacteria before birth or during the neonatal period (60, 220). These neonatal *S. agalactiae* infections are divided into early-onset and late-onset infections. Early-onset infection, which is the most common type of neonatal *S. agalactiae* disease, occurs within the first week of life, while late-onset infection occurs in infants between 1 week and 3 months of age (60, 220).

Early-onset and late-onset disease. In early-onset disease, the neonate is infected by exposure to *S. agalactiae* before or during birth (60). Recent mortality rates of 4 to 6% have been reported in the United States (218). Some early-onset infections may occur when the neonate is exposed to *S. agalactiae* during passage through the birth canal, but most early-onset infections are probably caused by ascending spread of the organism from the maternal genital tract through ruptured membranes into the amniotic fluid, in which the bacteria multiply, allowing them to colonize the respiratory tract of the fetus. In some cases, transmission of *S. agalactiae* into the amniotic fluid may even occur through intact membranes. After bacterial entry into the respiratory tract, pneumonia may develop and the bacteria may further spread to the bloodstream and cause septicemia. Bloodstream dissemination allows the bacteria to reach multiple body sites, where subsequent tissue penetration may result in manifestations such as meningitis and osteomyelitis (60, 181, 211). Thus, *S. agalactiae* probably has to adhere to, invade, and transcytose several epithelial/endothelial cell barriers to cause disease.

In an important experimental study mimicking early-onset disease, *S. agalactiae* was used for intra-amniotic inoculation of pregnant *Macaca nemestrina* primates; this was followed by analysis of infected tissues (76, 211). Interestingly, bacteria were observed in vacuoles within fetal lung epithelial, endothelial, and fibroblast cells. In agreement with this observation, *S. agalactiae* has been demonstrated to invade human epithelial and endothelial cells (76, 118, 180, 212, 260, 271) and even macrophages (259) in tissue culture, and bacteria may also survive intracellularly (76, 212, 259). Thus, invasion of human cells may play an important role in pathogenesis. However, all available evidence indicates that *S. agalactiae* should be viewed

as an extracellular pathogen, although it might survive intracellularly at certain stages of an infection. In contrast to a typical intracellular pathogen such as *Listeria monocytogenes*, there is little evidence that *S. agalactiae* can replicate intracellularly (76, 212, 271). Moreover, it should be noted that the ability to invade human cells in vitro may not always reflect the in vivo situation. For example, *Staphylococcus aureus* readily invades human cells in tissue culture but was not found intracellularly in an animal model (161). However, the observations in the monkey model studied by Rubens et al. (211) indicate that the ability of *S. agalactiae* to invade cells in culture is indeed of relevance to human infections.

Late-onset neonatal disease is less common than early-onset disease but is becoming relatively more important because its incidence has not declined as a result of the prophylactic measures that have caused a decrease in the incidence of early-onset infections (see below). Little is known about the pathogenesis of late-onset infections, but vertical transmission of the organism from the mother to the infant probably explains most infections during this period. In some cases, late-onset disease occurs as nosocomial epidemics that primarily affect preterm neonates, who probably acquire the bacterium by horizontal transfer from nursery personnel. The two most common clinical manifestations of late-onset disease are meningitis and bacteremia without a focus of infection. Fatality ratios for late-onset disease are generally lower than those for early-onset infections and have been reported to range from 2 to 6% (60, 218, 220).

Importantly, a substantial proportion of neonates who survive *S. agalactiae* infection suffer from sequelae. After cases of neonatal meningitis, neurological sequelae occur in up to 50% of the survivors and include mental retardation, cortical blindness, deafness, uncontrolled seizures, hydrocephalus, hearing loss, and speech and language delay (60).

Because illness develops in only a small fraction of neonates colonized by *S. agalactiae* during birth, many studies have tried to identify factors that may increase the risk for development of disease. Rupture of membranes before labour onset and increased interval between membrane rupture and delivery are considered to be major risk factors. Other predisposing factors include premature delivery, low birth weight, dense vaginorectal colonization, and intrapartum fever (60). The infant's susceptibility to *S. agalactiae* infection may also be enhanced by low levels of maternal antibodies against the polysaccharide capsule (8) or against surface proteins (C. Larsson and G. Lindahl, unpublished data).

Incidence of neonatal disease. The overall incidence of invasive neonatal *S. agalactiae* disease has recently been estimated to be 0.6 to 1.8 cases per 1,000 live births in the United States (60, 218) and 0.7 cases per 1,000 live-births in the United Kingdom and Ireland (90). Mortality rates were reported to be between 4 and 6% in the United States (218) and ~10% in the United Kingdom (90). Interestingly, it has been suggested that the incidence of neonatal disease is considerably greater than reported, because the requirement for positive cultures from blood or cerebrospinal fluid may underestimate the true burden of disease (151). Of note, neonatal *S. agalactiae* disease is more common than some other well-known diseases affecting newborns, such as rubella and spina bifida.

The incidence of neonatal *S. agalactiae* disease declined considerably in the United States during the 1990s, probably because of the introduction of surveillance programs and intrapartum antibiotic prophylaxis (169, 217, 218). However, the widespread use of antibiotic prophylaxis to inhibit *S. agalactiae* disease may be problematic, because it has been accompanied by an increase in the incidence of early-onset sepsis caused by *Escherichia coli* (239), a finding that stresses the need for an *S. agalactiae* vaccine (63).

Animal models. Like many other pathogens, strains of *S. agalactiae* isolated from human infections appear to be adapted to their human host, and there is no good animal model. Most *S. agalactiae* strains isolated from cases of bovine mastitis even have properties distinct from human isolates (21, 60). For example, molecular analysis has shown that most bovine strains lack genes for the surface proteins ScpB and Lmb, which are found in all human isolates, and lack the gene for the immunoglobulin A (IgA)-binding β protein, which is common among human isolates (55, 73). Moreover, human isolates may express components that specifically interact with the human host. For example, the surface-localized C5a peptidase of *S. agalactiae* (ScpB) may degrade human C5a but not mouse C5a (23, 28) and the IgA-binding β protein binds human IgA but probably not rat IgA (146, 214). Nevertheless, the mouse and the rat are valuable model systems for analysis of *S. agalactiae* infections, and several bacterial components are known to act as virulence factors in these models. However, results obtained with animal models must be interpreted with caution, and lack of evidence that a bacterial component contributes to virulence in an animal model does not exclude the possibility that it is of major importance in human infections. In the future, the use of transgenic mice may facilitate studies of some aspects of the infectious process, as described in other systems (100, 143, 207).

Of note, infection of mice (or rats) is usually performed by the intraperitoneal (i.p.) route, a situation thought to at least partially reflect invasive infection in humans. Moreover, several investigators have used mouse or rat pups for i.p. infection, because *S. agalactiae* is of particular importance as a pathogen in the neonatal period (209). However, all i.p. infections bypass the adhesion step that probably is essential in natural infections, so a bacterial mutant affected in adhesion may have normal virulence in an i.p. infection model.

Known virulence factors. Several *S. agalactiae* virulence factors have been identified in the mouse and rat models. In particular, the polysaccharide capsule and the secreted hemolysin are of major importance for virulence (57, 178, 213, 263). Moreover, superoxide dismutase and D-alanylated lipoteichoic acid play significant roles (199, 200). In contrast, the use of animal models has provided little evidence that surface proteins contribute to virulence, although a recent report indicates that the surface-localized protease CspA makes a contribution (87). This situation may at least partially reflect species specificity in the interactions between surface proteins and the infected host.

In an interesting study of a strain of capsular type Ia, Jones et al. (113) used signature-tagged mutagenesis (STM) and a neonatal rat model of i.p. infection to identify novel *S. agalactiae* genes implicated in virulence. Most of the genes identified were predicted to affect transport, regulation, intermediate metabolism, and cell wall metabolism, stressing the importance of such functions for virulence. In contrast, only one typical surface protein was identified, a result that may be due to the inherent limitations of the animal model used. Moreover,

many surface proteins may contribute to adherence and colonization, steps that were bypassed in the STM model (113). The surface protein identified was the laminin-binding Lmb protein or a closely related protein recently identified in the *S. agalactiae* genome (113, 242). In addition, the STM screening identified a mutant with strongly decreased virulence that lacks the penicillin-binding protein PBP1a (113), which may be surface exposed and contribute to phagocytosis resistance (114).

THE POLYSACCHARIDE CAPSULE AND SEROLOGICAL TYPES

All clinical isolates of *S. agalactiae* express a polysaccharide capsule; nine different capsular types have been identified so far (123). From the early days of *S. agalactiae* research, much interest has been focused on this capsule, because anticapsular antibodies confer protective immunity in an animal model and because capsular typing is epidemiologically important (60, 133, 134). The capsule is a major virulence factor with antiphagocytic function (158, 213, 263), and attempts are in progress to develop a multivalent capsular conjugate vaccine (9, 192).

The four "classical" capsular serotypes, identified by Lancefield, are types Ia, Ib, II, and III. When *S. agalactiae* emerged as an important neonatal pathogen, strains of serotype III predominated as the cause of serious infections, in particular as the cause of meningitis (60). However, in some (but not all) recent surveys, type Ia strains have been equally common among isolates from invasive infections, although type III remains the most important cause of late-onset infection and meningitis (88, 117, 145). In addition, strains of type V have recently emerged as a significant cause of *S. agalactiae* infection (22) and strains of types VI and VIII are the most common strains isolated from healthy women in Japan (131).

With regard to surface proteins, the subject of this review, the capsule is of particular relevance for at least two reasons. First, expression of a given surface protein is often correlated with the capsular type (6, 89, 112, 124, 130, 163, 236). Such associations may represent evolutionary lineages and could lack functional significance, but an interesting hypothesis predicts that immune selection has favored strains with certain combinations of surface structures because of their greater fitness (83). Second, the capsule might have been expected to interfere with the function of antibodies directed against surface proteins, either by blocking the access of antibodies or by interfering with antibody effector functions. However, this does not appear to be the case, because antibodies to surface proteins do protect against infection, at least in an animal model, a situation that encourages efforts to develop a protein-based vaccine. It is possible that the capsule does not block opsonization because it is thinner in vivo than in vitro, because it is expressed only in certain growth phases, and/or because it is very loose and flexible.

GENOMICS

Genome Sequences and Comparative Genomics

The rapidly increasing knowledge about genome sequences is transforming studies of *S. agalactiae*. So far, sequences have been published for one serotype III strain and one serotype V
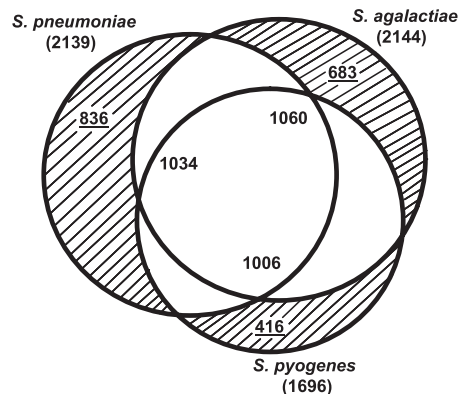

FIG. 1. Comparison of protein-encoding genes in the genomes of three streptococcal species: *S. agalactiae*, *S. pyogenes*, and *S. pneumoniae*. The total number of predicted protein-encoding genes in a bacterial species is given below the name of that species. The numbers of proteins unique to each species are given in the corresponding shaded areas, while the number of proteins shared by all three species is indicated in the innermost part of the figure. For example, *S. agalactiae* was predicted to have 2,144 protein-encoding genes, of which 683 are unique to this species, while 1,060 protein-encoding genes have homologs in both of the two other streptococcal species. In addition, 401 *S. agalactiae* proteins have homologs in only one of the two other species (not shown). The comparison is based on three published genome sequences (66, 252, 253). Modified from reference 252 with permission of the publisher.

strain (78, 252). These low-G+C genomes have a size of ~2 Mb and were predicted to have genes encoding ~2,100 proteins.

Comparison of the *S. agalactiae* genome with the genomes of two other pathogenic streptococci, *Streptococcus pyogenes* and *Streptococcus pneumoniae* (66, 253), showed that most of the predicted *S. agalactiae* proteins have homologs in at least one of the two other species (78, 252). As summarized in Fig. 1, the genome of the sequenced type V strain is predicted to comprise 2,144 protein-encoding genes, of which 683 are unique to *S. agalactiae* while 1,060 are shared with both of the other genomes. In addition, 401 putative proteins of this *S. agalactiae* strain are shared with one, but not both, of the other species (not indicated in Fig. 1). Moreover, the chromosomal order of genes is highly conserved between *S. agalactiae* and *S. pyogenes*, stressing the relatedness between these two species, while the gene order showed low conservation between *S. agalactiae* and *S. pneumoniae*, possibly reflecting the importance of transformation and recombination in the evolution of *S. pneumoniae* (78).

Each of the two sequenced *S. agalactiae* genomes contains ~15 regions, dispersed around the genome, that share some properties with pathogenicity islands (PAI) (56, 216). Interestingly, these regions contain genes for many predicted surface proteins and putative virulence factors and include many of the genes that are unique to *S. agalactiae* (78, 252). In several cases, the regions are related to mobile elements such as prophages, plasmids, and conjugative transposons, suggesting that they have been laterally transferred. These "islands" apparently exhibit broad diversity among different *S. agalactiae* isolates and were predicted to undergo rapid evolution (78, 252).

Predicted Surface Proteins

Two types of putative surface proteins can readily be identified in the *S. agalactiae* genome: proteins with an LPXTG

anchoring motif (177) and lipoproteins, which have a characteristic lipobox sequence (241). While all LPXTG proteins are commonly thought to be surface exposed, many lipoproteins may not be accessible on the surface because they are masked by other cell envelope components and perform their main function as part of ABC transport systems (242, 252).

The two *S. agalactiae* genomes were predicted to encode 21 or 24 LPXTG proteins, respectively (78, 252), and may also encode some proteins with LPXTG-related motifs (78). The type III genome (78) was predicted to encode 36 lipoproteins, while as many as 51 putative lipoproteins were identified in the type V genome (252). A separate bioinformatic analysis of the two genomes identified a core of 39 common putative lipoproteins, along with 2 proteins unique to the type III strain and 5 proteins unique to the type V strain (242). For many of these predicted surface proteins, hypothetical functions can be predicted from comparisons with similar proteins in other bacteria. The predicted LPXTG proteins include putative adhesins and enzymes, but most of the LPXTG proteins identified from genome sequences have no obvious function (78, 252). The predicted lipoproteins include components of ABC transport systems, putative adhesins, cation-binding proteins, enzymes, and components involved in protein localization and folding (78, 242, 252).

Concerning surface proteins that have been purified and characterized, proteins with an LPXTG-like motif have so far been most extensively studied, as described in this review. Among these proteins are putative or known adhesins and several enzymes. Of note, many of the LPXTG proteins of *S. agalactiae* are not present in *S. pyogenes* or *S. pneumoniae*, implying that each pathogen possesses a specific repertoire of LPXTG proteins (78).

In addition to the predicted LPXTG-containing proteins and lipoproteins mentioned above many proteins might be located on the surface, as suggested by immunological analysis and by comparison with known surface proteins identified in other bacterial species (78, 252). Indeed, an immunological analysis reported by Tettelin et al. (252) indicated that the type V strain studied by them expressed more than 55 different surface proteins, of which only a minority were LPXTG proteins or lipoproteins. Some of these putative surface proteins may be anchored to the bacterial cell wall by novel mechanisms (41, 46). For example, analysis of the type V genome identified a putative secreted metalloprotease containing so-called GW repeats, which have been implicated in the binding of some *Listeria monocytogenes* surface proteins to lipoteichoic acid (116). Moreover, it has been reported that several enzymes usually considered to be intracellular may also be expressed on the *S. agalactiae* surface (98). It will be of obvious interest to analyze all of these predicted surface proteins to determine their role in pathogenesis and evaluate their suitability as possible vaccine components.

A Putative High-Virulence Clone

A number of groups have analyzed whether collections of *S. agalactiae* strains isolated from infections have a clonal structure. Characterization of many isolates by multilocus enzyme electrophoresis, restriction fragment length polymorphism, pulsed-field gel electrophoresis, *infB* allele analysis, and presence of mobile genetic elements, or multilocus sequence typing has provided strong evidence that such *S. agalactiae* populations indeed are clonal (20, 25, 53, 89, 91, 93, 115, 172, 174, 210, 245, 247). Interestingly, clones identified by restriction fragment length polymorphism correspond to a large degree to the different capsular serotypes (89).

Several reports indicate that serotype III strains in one clone are strongly enriched among invasive-disease isolates, implying that strains in this clone have increased pathogenic potential and are of particular relevance for studies of pathogenesis (20, 115, 172, 245). This putative high-virulence clone was first described by Musser et al. (172), and the available evidence indicates that their clone ET-1 is identical to clones designated III-3 (245), ST-17 (115), or GIII (20, 70). Interestingly, a recent analysis employing MLST indicated that this putative high-virulence clone (ST-17) corresponds to a bovine lineage introduced among humans (21). However, analysis of two strain collections did not support the conclusion that this type III clone has increased virulence (53, 89, 93). Thus, the situation is unclear, possibly reflecting different epidemiological settings in the different geographical areas where the strains were collected (93). Of note, neither of the two *S. agalactiae* genome sequences published so far represents the putative high-virulence type III clone (78, 252).

There is some evidence that strains in the putative high-virulence clone have unique surface properties. These strains have significantly increased sialic acid content, a property that might contribute to increased virulence (245, 246). Moreover, subtractive hybridization of DNA from a strain in the III-3 clone has allowed the identification of a novel surface protein, Spbl, found only in strains of that clone (1, 27). With regard to surface properties, it may also be of relevance that most strains in the putative high-virulence clone carry the group II intron GBSi1 inserted between the genes encoding the two surface proteins ScpB and Lmb, suggesting that expression of these proteins could be altered (79, 247).

SURFACE PROTEINS

Historically, the first surface protein antigen identified in *S. agalactiae* was the c antigen, which was detected with antisera raised against whole bacteria (268). The c antigen (also designated Ibc) has been found in many strains but not in the clinically important type III strains (67, 163). Characterization of this antigen subsequently showed that it is composed of two unrelated protein components, the trypsin-resistant α protein and the trypsin-sensitive β protein (18), both of which have now been extensively characterized, as described below. Of note, a strain that is reported to express c antigen may express either or both of the α and β proteins. Because α and β together constitute the c (or C) antigen, the designations alpha C and beta C, and other related designations, have also been used for these proteins. Moreover, the designation ACP (alpha C protein) was recently used for the α protein (11). It is not known why the α and β proteins are commonly expressed by the same *S. agalactiae* strain (16, 112, 163).

Lancefield et al. (136) made the important observation that antibodies to the c antigen confer protection against *S. agalactiae* infection in a mouse model, implying that not only the capsule but also surface proteins may elicit protective immu-

nity. As pointed out by Ferrieri (67), this finding remained relatively ignored, possibly because the c antigen is expressed by only some strains and not by the clinically important type III strains. However, early work by Bevanger and Naess (19) demonstrated that each of the α and β proteins, the two components of the c antigen, elicit protective immunity. Subsequent work on type III strains identified Rib, a novel surface protein that elicits protective immunity and is expressed by most strains not expressing α (236). This finding suggested that an *S. agalactiae* vaccine based solely on surface proteins might be developed and focused interest on surface proteins for analysis of *S. agalactiae* pathogenesis.

Below, we summarize work on those *S. agalactiae* surface proteins that have so far been studied most extensively. Of note, the genes encoding these different surface proteins are widely distributed over the *S. agalactiae* chromosome, except for the closely linked *scpB* and *lmb* genes, which are located on a putative composite transposon (73).

THE Alp FAMILY OF PROTEINS

A Family of Highly Repetitive Proteins That Elicit Protective Immunity

Sequencing of the α protein, one of the two components of the c antigen, revealed the presence of an extended region composed of long, completely identical repeats (165). Remarkably, these repeats were found to be identical even at the nucleotide level. Characterization of the Rib protein showed that it has similar overall structure and sequence, identifying a novel family of bacterial surface proteins (261). In this review we adopt a nomenclature already used by other investigators and refer to this family as the Alp protein family, where Alp stands for α-like protein (11).

Four members of the Alp family have so far been identified: the α, Rib, R28, and Alp2 proteins (Fig. 2A). In addition to their relevance for vaccine development and for analysis of *S. agalactiae* pathogenesis, these proteins are of interest as models for other repetitive proteins, which are common in gram-positive bacteria (120, 177) and in higher organisms (162, 240, 262). Because Alp-related proteins have been identified in several bacterial pathogens, studies of Alp family proteins are also of general interest for the analysis of pathogenetic mechanisms.

The available genetic and immunological evidence indicates that the different Alp proteins are encoded by allelic genes, implying that a strain of *S. agalactiae* expresses only one member of the family (27, 124, 130, 236). Indeed, studies of flanking regions in various strains showed that these regions are highly conserved (130). Moreover, analysis of the two available genome sequences (78, 252) indicates that the corresponding Alp family members (Rib and Alp2) are encoded by allelic genes. Of note, the expression of a given Alp family protein is strongly correlated with capsular type (Table 1).

The sequence organization in Alp family proteins (Fig. 2A) is similar to that in many surface proteins of gram-positive bacteria (177). An N-terminal signal sequence is followed by a nonrepeated N-terminal region, a repeat region, a wall-anchoring region with an LPXTG motif, a short hydrophobic region that may span the cellular membrane, and a charged tail

TABLE 1. Members of the Alp protein family commonly found in *S. agalactiae* strains with different capsular serotypes[a]

Capsular serotype	Alp family protein commonly expressed
Ia	α
Ib	α
II	α, Rib
III	Rib
V	R28
VIII	R28

[a] This table includes only the Alp family proteins that are commonly found in the serotypes indicated; it does not include the uncommon serotypes IV, VI, and VII, because strains of those serotypes have not been characterized with regard to expression of proteins in the Alp family. See the text for references.

(165, 261). Like many other surface proteins of gram-positive bacteria, the Alp family members lack cysteine residues. Interestingly, proteins in the Alp family have exceptionally long signal sequences, 55 to 56 amino acid residues, as demonstrated by N-terminal sequencing of proteins purified directly from streptococci (236, 261).

Except for the Alp2 protein (which appears to be rare, as described below) there is only one type of repeat in each protein. This type of repeat, designated R in Fig. 2A, is present in all four proteins. Within a given protein, these repeats are identical or virtually identical, but they vary in sequence among different Alp proteins. The residue identity between different repeats varies from 95% for the pair Rib-R28 to 40% for the pair α-Alp2. These repeats have a length of 76 to 82 amino acid residues, and the number of repeats in a protein varies among strains (see below). The Alp2 protein differs from other members in the family in having a second type of tandem repeat (here designated RN) in the N-terminal half of the protein (Fig. 2A).

Interestingly, it has been predicted that the repeats in Alp family proteins have a structure related to the Ig-like fold (35), a structure identified also in some other repetitive prokaryotic proteins (86, 152, 160, 198). In agreement with this sequence analysis, computer modeling of the Rib repeat predicts that it contains at least four β-sheets in a compact structure (E. Lindahl and G. Lindahl, unpublished data). Because domains with the Ig-like fold are often implicated in molecular recognition, these data suggest that Alp family proteins may have such a function (35). Alternatively, the repeat region may act as a rod needed for exposure of a unique ligand-binding region, by analogy to the situation in intimin from *E. coli* and invasin from *Yersinia*, two proteins in which the repeat regions have Ig-like fold (86, 152).

Four Members of the Family: the α, Rib, R28, and Alp2 Proteins

The large majority of *S. agalactiae* strains studied so far have the gene for one of the four Alp family proteins, as demonstrated by PCR analysis with specific primers (124), but rare strains may encode a related protein. For example, the atypical reference strain Prague 25/60 may express a protein (tentatively designated Alp4), which was not found in any other strain among 224 *S. agalactiae* strains analyzed (124). Moreover, it has been suggested that some strains express an Alp-
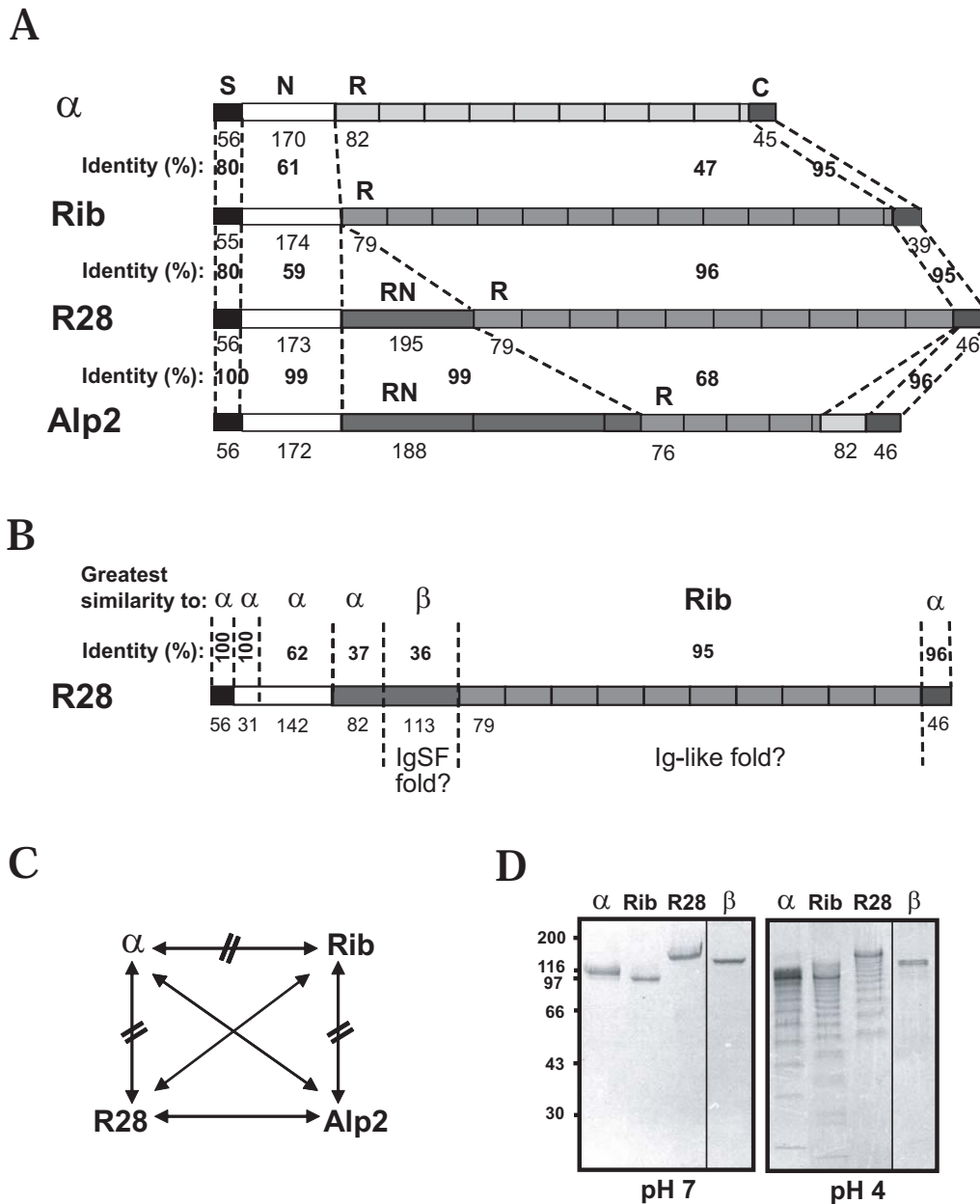
FIG. 2. The Alp family of proteins. (A) Comparison of the four known members of the Alp protein family: the α, Rib, R28, and Alp2 proteins. S, signal peptide; N, nonrepeated N-terminal region; R, repeat region; C, C-terminal region. The number of amino acid residues in each region and the percent residue identity (bold numbers) between corresponding regions are indicated. Note that the designation Alp3 has been used as an alternative name (130) for the R28 protein (235). Each of the three proteins α, Rib, and R28 has 9 to 12 repeats with a length of 79 or 82 residues, as indicated. These repeats are identical within one protein but not between proteins, and the number of repeats varies among clinical isolates. The nonrepeated region of R28 contains a 195-residue subregion, designated RN, which does not fit into the alignment with Rib. This region is 99% identical to a type of repeat found in the N-terminal half of the Alp2 protein. Thus, the Alp2 protein contains two types of tandem repeat (designated RN and R), unlike the other members of the family. This figure is based on published sequences for the four proteins (78, 130, 165, 235, 261). The data for Alp2 are based on the sequence reported by Glaser et al. (78). (B) Schematic representation of the R28 protein. As indicated, R28 can be viewed as a chimera, derived from the two Alp family proteins α and Rib and the unrelated β protein (235). Predictions about the three-dimensional structure are given below the protein. The region that shows similarity to β corresponds to a region in β predicted to have an IgSF fold (12), and our analysis of the R28 region suggests that it has a similar structure. The repeats in the C-terminal part of R28 have been proposed to have a fold related to the IgSF fold (35). Modified from reference 235 with permission of the publisher. (C) Immunological relationship among the four known members of the Alp protein family. Solid arrows indicate immunological cross-reactivity, and broken arrows indicate lack of cross-reactivity. For example, the α protein cross-reacts with Alp2 but not with Rib or R28. Most of the data summarized in the figure were obtained with rabbit antisera raised against purified proteins (132, 234–236), but the relationship between R28 and Alp2 was analyzed with an extract of strain D136C (NEM316), which expresses Alp2 (78, 234). (D) Laddering pattern in the α, Rib, and R28 proteins analyzed by SDS-PAGE. Purified preparations of the three proteins, and the control protein β (which is not a member of the Alp family), were subjected to SDS-PAGE under ordinary conditions (with boiling of samples at pH = 7) or after boiling of samples for 15 min at pH = 4. Under the latter conditions, the three Alp family proteins form a regularly laddered pattern due to hydrolysis of acid-sensitive Asp-Pro bonds in the repeats during sample preparation. Modified from reference 235 with permission of the publisher.

related protein that was designated "epsilon" (47) or Alp1 (202). However, it is not yet known whether these putative Alp-related proteins are expressed on the bacterial surface and the corresponding genes have been only partially sequenced. We first describe each of the four known family members and then focus on some properties common to these proteins.

The α protein. The α protein is unrelated to the β protein, the other component of the c antigen (92, 107), and is commonly found in strains of serotypes Ia, Ib, and II but almost never in type III strains and only rarely in type V strains (6, 16, 67, 112, 124, 163, 236, 243) (Table 1). The α protein is resistant to trypsin, a property that contributed to its identification and has been used to analyze strains for expression of α (67, 163, 268). Bevanger and Naess (19) reported that α purified from streptococci elicits antibodies that protect mice against infection, a result that was confirmed in experiments with recombinant protein (164).

The gene for α was cloned from strain A909, a commonly used reference strain of serotype Ia. Sequence analysis of the α-encoding gene, named *bca* (not to be confused with *bac*, the gene encoding the *S. agalactiae* β protein [92]) revealed that a large part (~75%) of the protein was composed of nine 82-residue tandem repeats, for which the sequence was also identical at the nucleotide level (165). The calculated molecular mass for the mature protein (i.e., the protein without the 56-residue signal sequence [236]) is 103 kDa. Of note, the repetitive structure of the *bca* gene made the sequencing difficult (165), a problem also encountered during sequencing of genes encoding other members of the Alp family (235, 261). Because these genes cloned in *E. coli* are unstable, sequencing of such cloned genes may underestimate the number of repeats (261). However, the true number of repeats may be estimated from ladder patterns in PCR or sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (see below).

The Rib protein. The Rib protein was identified as a unique high-molecular-weight protein in cell wall extracts of type III strains (236). Characterization of the purified protein indicated that it was related to α, although the two proteins did not cross-react immunologically. In particular, the N-terminal sequences of the two purified proteins were found to be related but not identical. Moreover, Rib was resistant to trypsin, varied in size among clinical isolates, and gave rise to a ladder pattern in Western blots, like the α protein. Most importantly, antibodies to Rib conferred protective immunity against lethal infection with Rib-expressing strains (236). The available evidence indicates that Rib is expressed by the large majority of serotype III strains, by many type II strains, and by a few type V strains (6, 139, 236, 252).

The sequence for Rib was determined in strain BM110, a member of the putative high-virulence type III clone (261). The processed form of Rib in this strain has a 174-residue N-terminal region and 12 identical 79-residue repeats and has a calculated molecular mass of 123 kDa (Fig. 2A). As previously reported for α, the repeats of Rib are identical even at the nucleotide level (165, 261). The amino acid residue identity to α is 61% in the N-terminal region and 47% in the repeat region. Thus, the sequencing of Rib showed that Rib and α are members of a family of streptococcal surface proteins with extremely repetitive sequence (261). Of note, the genome of the sequenced type V strain (252) has the gene (*rib*) encoding

Rib. The sequence deduced for Rib in that strain is completely identical to the sequence previously determined in a type III strain (261), except that the type V protein has 14 repeats. The paper on the type V sequence (252) reports that the Rib protein of that strain has repeats with a length of only 67 amino acid residues, but that conclusion is due to a mistake during analysis of the nucleotide sequence reported in the same paper.

The R28 protein (Alp3). The R28 protein was first identified in strains of *S. pyogenes* (group A streptococcus) (137), and molecular characterization demonstrated that it is a member of the same family as Rib and α (235). The nonrepeated N-terminal region in R28 is considerably longer than the corresponding regions in α and Rib, while the repeat region is closely related (95% identity) to that in Rib. This similarity between R28 and Rib may explain an immunological cross-reactivity observed in early studies (137). The R28 protein is expressed not only by some strains of *S. pyogenes* but also by many *S. agalactiae* strains of serotypes V and VIII (6, 130, 131), and these *S. agalactiae* strains express R28 proteins that are identical except for different number of repeats (124, 130). Lachenauer et al. (130) used the novel name Alp3 for the protein expressed by *S. agalactiae*, but because the proteins expressed by *S. pyogenes* and *S. agalactiae* are 98% identical, we prefer to retain the original name R28 for both proteins. Like other members of the Alp protein family, R28 elicits protective immunity (234, 235).

The R28 protein may be viewed as a chimera derived from the three *S. agalactiae* proteins α, β, and Rib (Fig. 2B; also see below). This finding suggests that the gene encoding R28 may have arisen in *S. agalactiae*, followed by horizontal gene transfer to *S. pyogenes* (235).

Interestingly, there is some evidence that the R28 protein may have played a pathogenetic role in the epidemics of puerperal fever (childbed fever) that caused the death of numerous parturient women during earlier centuries and represents the classical example of a nosocomial infection (3, 235). These epidemics were due to infections with *S. pyogenes* (135) and should not be confused with neonatal infections caused by *S. agalactiae*, but in both cases the infection was most probably initiated by bacteria initially present in the vagina. Discussion of puerperal fever is beyond the scope of this review, but the putative association between R28 and nosocomial infections may be of relevance for studies of R28-related proteins such as Esp of the nosocomial pathogens *Enterococcus faecalis* and *E. faecium* (see below).

The Alp2 protein. The Alp2 protein was identified in a serotype V strain (132) and has a sequence virtually identical to that of R28 in the N-terminal two-thirds (130) (Fig. 2A). This protein is more rare than the other members of the Alp family and was found in only a few strains of serotypes Ia, III, and V (78, 124, 130). Unlike the other Alp family proteins, Alp2 has two types of tandem repeat. One type of repeat, here designated RN, corresponds to a nonrepeated part of R28 and includes the region in R28 that is related to the β protein (235) (Fig. 2B). The sequenced variants of Alp2 have one or two such repeats (78, 130). The second type of tandem repeat, located in the C-terminal half of Alp2 and here designated R, corresponds to the repeats identified in the other members of the Alp family. For Alp2, the number of such repeats varies between two and four in different *S. agalactiae* strains (78, 130).

The most C-terminal part of Alp2 includes a single region most similar to repeats in the α protein. Thus, the sequence organization in the Alp2 protein is more complex than in the other members of the Alp family. Of note, the genome of the sequenced type III strain (78) contains the gene encoding Alp2.

Proteins in the Alp family have chimeric but stable sequences. The known proteins in the Alp family appear to represent stable and distinct entities. For example, the Rib protein expressed by two *S. agalactiae* strains of serotypes III and V have identical sequences except for different number of repeats (252, 261) and the R28 protein sequenced in *S. pyogenes* is 98% identical to that sequenced in several type V and type VIII strains of *S. agalactiae* (130, 235). Moreover, partial sequencing of the four known Alp family members in many *S. agalactiae* strains indicated that these proteins have very stable sequences (124). Thus, different members of the Alp family vary only a little or not at all in sequence between strains, except for variation in repeat number.

In spite of their stability, members of the Alp family appear to have a chimeric structure, as first observed for the R28 protein, which can be viewed as a chimera derived from the three *S. agalactiae* proteins α, β, and Rib (235) (Fig. 2B). Similarly, the Alp2 protein can be viewed as a chimera derived from R28 and other members of the Alp family (130). The most surprising result of this analysis is that one region in R28 shows sequence homology to a region in the *S. agalactiae* β protein, which is not a member of the Alp family and has very different sequence organization (see below). Interestingly, the corresponding region in β is predicted to be evolutionarily related to eukaryotic proteins and to have the Ig superfamily (IgSF) fold (12). The β-like region in R28 may also have this structure (our unpublished data), suggesting that it corresponds to a mobile domain with the IgSF fold.

Although the chimeric structure of Alp proteins suggests that the corresponding genes can recombine with each other, and possibly also with other genes, the emergence of a new family member appears to be a rare event, because only four members of the family have been identified so far, and these proteins are genetically stable. The family members identified so far may have been selected because of their superior fitness. Nevertheless, it is conceivable that novel Alp family members will arise and become common in other epidemiological settings.

Immunological and Immunochemical Properties

Because Alp family members hold promise as vaccine components (140), it is of relevance to identify protective epitopes. Only limited information is available concerning the location of such epitopes, but the isolated N-terminal (nonrepeated) region of α is known to elicit protective immunity, as demonstrated in studies involving passive and active immunization (80, 122). Moreover, there is some evidence that the repeat region of α may be a target for protective immunity, as concluded from one experiment employing a monoclonal antibody (122, 156). However, it is not yet known whether immunization with the repeat region of an Alp family protein elicits protective immunity.

Variation in repeat number and its effect on immunological properties. For all members of the Alp protein family, the protein varies in size among different clinical isolates and the size

variability can readily be explained by variation in the number of repeats (130, 153, 154, 235, 236). For example, the size of the intact α protein varies between ~65 and ~165 kDa in different strains (153), corresponding to proteins with 8 to 19 repeat regions, and the size of the Rib protein varies between ~65 and ~125 kDa (236), corresponding to proteins with 8 to 15 repeat regions. Such size variation may arise through *recA*-independent slipped-strand mispairing during DNA replication (201) and is common in repetitive DNA sequences (150).

In spite of the extremely repetitive sequence, the size of proteins in the Alp family remains remarkably stable during growth in vitro, suggesting that the molecular events giving rise to size variability represent rare events (201, 236). However, rare size variants can be demonstrated to arise in vitro, as described in the α system (201). In contrast, considerable size variability has been found in vivo, when bacteria infect a host pretreated with protective anti-α serum (154). Interestingly, variants of α with fewer repeats are selected under these conditions, suggesting that reduction in repeat number allows bacteria to escape immunity. In agreement with this hypothesis, an *S. agalactiae* strain expressing an α protein with only one repeat was more virulent than a strain expressing the wild-type variant containing nine repeats when the virulence of the two strains was compared in mice pretreated with antibodies to the wild-type protein (82). However, no difference in virulence was observed in untreated mice, so these findings do not provide evidence that the repeats of α affect virulence under nonimmune conditions. Importantly, naturally occuring variants of α and other Alp proteins contain multiple repeats, suggesting that a long repeat region enhances virulence in humans. The appearance of variants with few repeats may therefore be interpreted as a side effect of host immunity, and it seems possible that it represents a dead end for the bacterium rather than an immune evasion mechanism. Nevertheless, this phenomenon may contribute to the size variation encountered among naturally occurring variants. Possibly, the average number of repeats in an Alp family protein represents the net result of a selective force (bacterial virulence) favoring proteins with many repeats and another selective force (host immunity) favoring variants with few repeats. While immune selection would result in rapid "contraction" of the repeat region, escape from such selection would allow expansion of the repeat region, which may be required for spread to a new host. In vivo, such dynamic selections might cause frequent size variability, resulting in the appearance of repeats that are identical even at the nucleotide level.

In addition to the possible role of the repeat region for immune evasion in an immune host, this region has been suggested to affect immune escape in another way, by reducing the immune response to the whole protein, and in particular to the N-terminal region, in a nonimmune host (80). This conclusion was based on the observation that the antibody response in mice to the N-terminal region of α was drastically reduced when the number of repeats was increased (80).

Immunological relationship between Alp family members. The immunological relationship among different Alp proteins has implications for the development of an *S. agalactiae* vaccine and for the use of antisera for serological typing of strains. Cross-reactivities observed among the four Alp family members are summarized in Fig. 2C (130, 132, 139, 234–236). Most

of these results were obtained with hyperimmune rabbit antisera raised against purified proteins, and they do not necessarily reflect the situation during a natural infection. In general, the cross-reactivities are surprisingly limited, given the extensive residue identity between different members of the Alp family. For example, the α protein showed no cross-reactivity with any of the Rib and R28 proteins, in spite of ~61% residue identity in the N-terminal regions and ~45% identity in the repeat regions (235, 261). Similarly, the Alp2 protein did not cross-react with Rib, in spite of the 60 to 67% residue identity in different regions (132). Even more surprisingly, the cross-reactivity between Rib and R28 was limited, in spite of the 59% residue identity in the most N-terminal region and the 95% identity in the repeat region (234). These data indicate that the sequence variability could represent antigenic variation, allowing bacteria expressing one protein in the family to evade protective immunity directed against some of the other proteins.

From a vaccine development point of view, it is of relevance that even a limited cross-reactivity may be sufficient to confer cross-protection, as shown by work with the Rib and R28 proteins (234). In that study, vaccination of mice with purified Rib from *S. agalactiae* protected against infection with an R28-expressing strain of *S. pyogenes* and vice versa; i.e., the cross-reacting Rib and R28 proteins even elicited cross-protection between strains of two different species (234). Moreover, a very weak cross-reactivity that is hardly detectable by immunochemical techniques may allow some cross-protection in vivo, as suggested by work with the Rib and α proteins in the mouse model (139). Of note, the cross-reactivities summarized in Fig. 2C indicate that a vaccine composed of the two proteins Rib and α might protect against *S. agalactiae* strains expressing any of the four Alp proteins so far identified (140).

Laddering pattern in gels. A remarkable property of proteins in the Alp family is their ability to form a regular ladder pattern in SDS-PAGE and Western blots, as first described for the α protein (153, 165) (Fig. 2D). Because of this property, the proteins in the family have often been referred to as laddering proteins. The distance between the steps in the ladder corresponds to the size of one repeat, and it was initially suggested that a novel mechanism may allow α-protein fragments of different sizes to be synthesized (165). However, immunochemical analysis subsequently indicated that the laddering phenomenon most probably is due to hydrolysis of acid-sensitive Asp-Pro bonds in the repeats during sample preparation (261). Indeed, purified preparations of the α and Rib proteins do not form a ladder in SDS-PAGE when samples are boiled under standard conditions at pH 7, while samples boiled at pH 4 give rise to the characteristic ladder pattern (Fig. 2D). Although these observations imply that the ladder pattern is an artifact, it is an interesting phenomenon that may aid in the identification of novel Alp family members. However, members of the Alp family may exist that lack acid-sensitive bonds in the repeats and therefore do not form ladders. Of note, the number of steps in a ladder observed on SDS-PAGE may be used to estimate the number of repeats in a protein (261). Laddering has also been observed during analysis of PCR products generated from genes encoding Alp proteins, and the distance between the steps in that ladder was estimated to correspond to one repeat (261). Such ladders, which may arise through slippage of the DNA polymerase during replication, can also be used to estimate the number of repeats in the gene.

Biological Function

Conclusive evidence that proteins in the Alp family contribute to virulence is lacking, and their major biological function remains unclear. Indeed, the most important property as yet identified for these proteins is probably their ability to elicit protective immunity in an animal model. However, the finding that most, if not all, strains of *S. agalactiae* express an Alp family protein suggests that these proteins may have an important biological function. Interestingly, a recent report suggests that one function for these proteins may be to bind human glycosaminoglycans (11).

Virulence of bacterial mutants. Use of an α-negative mutant suggested that α might have some effect on mouse virulence in the early parts of an infection, but it was not excluded that this result was due to a polar effect (144). Moreover, experiments with bacterial mutants lacking Rib or R28 have not provided evidence that these proteins act as virulence factors in the mouse (235; T. Areschoug and G. Lindahl, unpublished data). Together, these data suggest that proteins in the Alp family have little or no effect on virulence in the mouse model of invasive infection. Nevertheless, each of the different Alp proteins is a target for protective antibodies in the mouse system (19, 132, 235, 236). These findings may appear to be paradoxical but can be readily explained by assuming that Alp proteins act only as targets for antibodies in the mouse, allowing complement deposition and/or Fc-mediated phagocytosis.

Interactions with human epithelial cells. The R28 protein, which was first identified in *S. pyogenes*, promotes binding of that pathogen to the human cervical epithelial cell line ME-180, indicating that R28 may act as an epithelial cell adhesin (235). However, attempts to demonstrate that other members of the Alp family also act as adhesins have been negative. Thus, a mutant of *S. agalactiae* lacking the α protein was not affected in binding to ME-180 cells (30), and similar results were obtained with a Rib-negative mutant (J. Waldemarsson, G. Lindahl, and T. Areschoug, unpublished data). However, the α protein may promote invasion of ME-180 cells by *S. agalactiae* (30), possibly by interaction with host cell glycosaminoglycans (11).

Related Proteins in Other Bacterial Species

Proteins with sequence similarity to the Alp proteins have been identified in several other bacterial species, a similarity that could reflect horizontal gene transfer, shared evolutionary origin, or convergent evolution (Fig. 3A). The first protein to be identified in this group was Esp of *E. faecalis* (225). Possibly, Esp and other Alp-related proteins represent a group of virulence factors with similar function in different species. The identification of these proteins enhances interest in the Alp family and vice versa. Indeed, studies of some of these proteins may provide information about the other proteins. Such comparisons are particularly relevant, because little is known concerning the biological function of the different proteins. Of note, two of the Alp-related proteins, Esp of *E. faecalis* and Bap of *Staphylococcus aureus*, have been implicated in biofilm

A

B

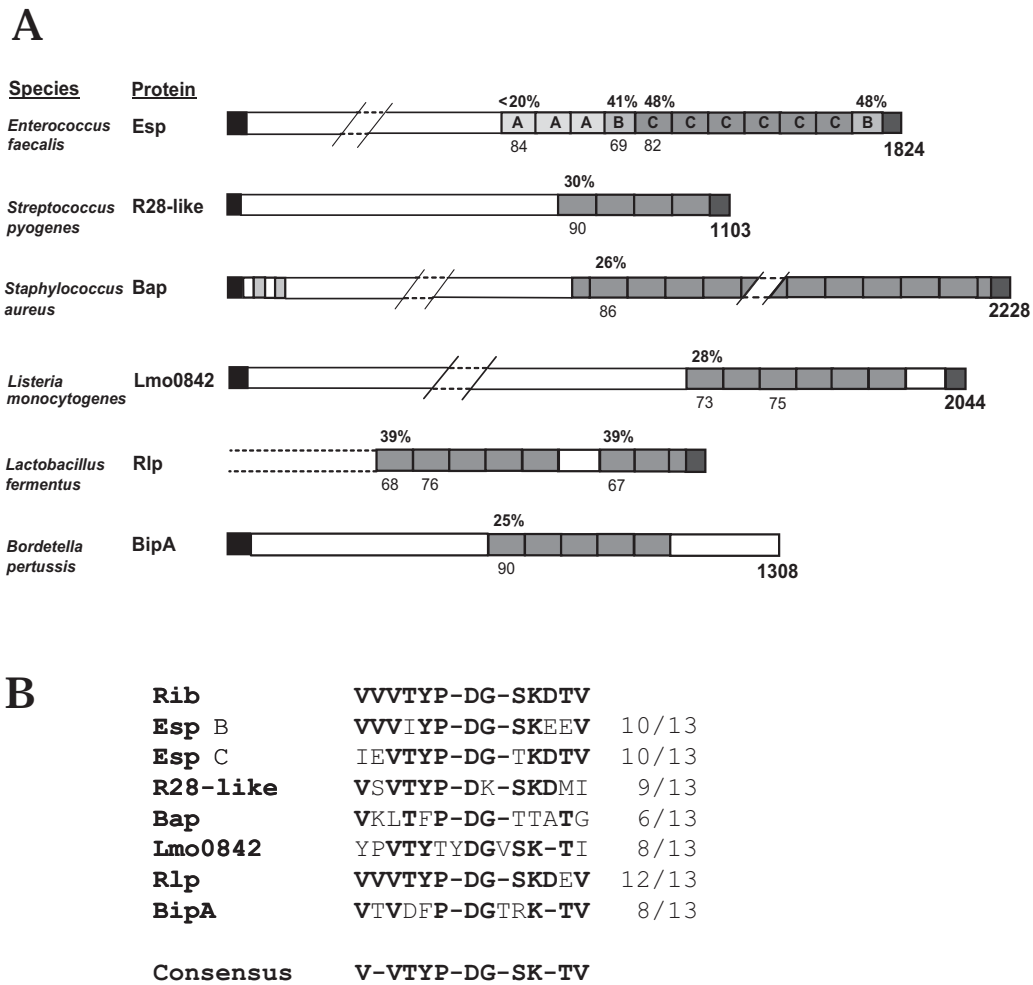|		
Rib	`VVVTYP-DG-SKDTV`	
Esp B	`VVVIYP-DG-SKEEV`	10/13
Esp C	`IEVTYP-DG-TKDTV`	10/13
R28-like	`VSVTYP-DK-SKDMI`	9/13
Bap	`VKLTFP-DG-TTATG`	6/13
Lmo0842	`YPVTYTYDGVSK-TI`	8/13
Rlp	`VVVTYP-DG-SKDEV`	12/13
BipA	`VTVDFP-DGTRK-TV`	8/13
Consensus	`V-VTYP-DG-SK-TV`	

FIG. 3. Proteins in other bacterial species related to members of the Alp protein family. (A) Schematic representations of six Alp-related bacterial proteins. The bacterial species and the name of the protein are indicated to the left. For all proteins, the C-terminal part includes a region with long tandem repeats showing sequence similarity to the C-terminal repeat region of Alp family members. The lengths of the different repeats are indicated below each protein. The percentages indicate residue identity to the repeats of the Rib protein, the Alp family member to which these proteins show the greatest similarity. Note that the Esp protein of *E. faecalis* has a more complex structure than the other proteins and has three types of repeats, the tandem A and C repeats and the nontandem B repeats, of which the B and C repeats show similarity to the repeats of Rib and other Alp proteins. The complete sequence is not available for the Rib-like protein (Rlp) of *L. fermentus*. With one exception (BipA), these different proteins have an LPXTG motif in the C-terminal region, which may allow covalent anchoring to the cell wall. The BipA protein of the gram-negative bacterium *B. pertussis* is probably bound to the bacterium via an N-terminal hydrophobic region. The black boxes to the left represent signal peptides. See the text for details and references. (B) Alignment of sequences in a conserved region present within the repeats. Because these sequences are most similar to the corresponding sequence in Rib among different Alp proteins, the corresponding sequence in Rib is also included. Residues identical to those in Rib are shown in bold type, and the number of identities is indicated to the right. The consensus sequence represents residues present in at least five of the eight sequences.

formation, suggesting that this could be a general property of Alp-related proteins.

Like the Alp family members, the related proteins in other bacterial species contain extended regions composed of long repeats (Fig. 3A). With one exception, these proteins have been identified in gram-positive bacteria and were predicted to have the characteristics of surface proteins with a C-terminal LPXTG motif. The sequence similarity to the Alp family proteins is most pronounced in one part of the repeats, where a consensus sequence can be identified (Fig. 3B) (59, 225). Although the residue identity to members of the Alp family is not always extensive, the identification of the consensus sequence, together with the similarity in overall sequence organization, suggests an evolutionary and functional relationship among the different proteins. This hypothesis is supported by the prediction that the Esp protein of *E. faecalis* has repeats with Ig-like fold, like Rib and other Alp proteins (35). Interestingly, the group of Alp-related proteins includes at least one member expressed by gram-negative bacteria, the BipA protein of *Bordetella* species.

Esp in *E. faecalis* and a closely related protein in *E. faecium*. The ~202-kDa Esp protein of *E. faecalis* was identified through the presence of localized sequence identity to the Rib and α proteins of *S. agalactiae* (225). Because the *esp* gene was found in many *E. faecalis* strains isolated from patients with invasive infections but only rarely in commen-

sal isolates, it was suggested that Esp contributes to the virulence of this important cause of nosocomial infections (225). Other investigators have reported that the *esp* gene may also be common among commensal isolates, a result that might reflect geographical differences (258). The function of Esp is not known, but it enhances the ability of *E. faecalis* to cause urinary tract infection in an animal model (224) and contributes to the formation of biofilms on abiotic surfaces (255), although other surface structures also are important for biofilm formation (127, 167, 255).

Esp differs from the Alp family proteins in having three different types of repeat (A, B, and C), as well as longer N- and C-terminal regions (Fig. 3A). As a result, Esp is about twice the size of the *S. agalactiae* proteins. The A and C repeats of Esp are tandemly arranged, while the two B repeats are located in different parts of the sequence. Within the A and C repeat regions, the sequences of different repeat units are almost identical, but the number of repeats varies among different clinical isolates. However, all *esp* genes include A and C repeats, suggesting that both regions are important for the function of the protein (225, 255). In contrast to the variability in repeat number between different clinical isolates, the number of repeats within one strain remained stable after passages on laboratory medium, as described also for the Rib and α proteins of *S. agalactiae* (201, 236). The 69-residue B repeats and the 82-residue C repeats are related and show extensive sequence similarity to members of the Alp protein family (225). Of note, these repeats do not contain the acid-labile Asp-Pro bonds that give rise to a ladder pattern in Alp family proteins analyzed by SDS-PAGE (261).

The *esp* gene is located on a large PAI, supporting the notion that the Esp protein contributes to virulence (223). Within this PAI, a well-defined short deletion occurs at considerable frequency during in vitro growth, causing the elimination of several genes in the PAI, including the entire *esp* gene. The biological relevance of this irreversible deletion is unknown, but it is reminiscent of the appearance under certain conditions of α-protein variants with few repeat regions (154). In vivo, such deletions might represent a dead end for the bacterium, because the deleted fragment would be irreversibly lost. It has been suggested that loss of Esp may cause detachment of bacteria from a biofilm, thereby allowing dissemination, but the problem with irreversible gene loss remains (257).

The gene for a protein closely related to Esp of *E. faecalis* is found in many strains of *E. faecium*, an increasingly important cause of nosocomial infections (59, 269), and epidemiological analysis indicates that this *esp*-related gene is associated with virulence (142, 269). As described for the Esp protein of *E. faecalis*, the number of A and C repeats varies among different clinical isolates of *E. faecium*, and some isolates may even lack A repeats (59, 142). Interestingly, the *esp* gene of *E. faecium* may be located on a pathogenicity island, like the *esp* gene of *E. faecalis*, but, except for *esp* and an *araC*-like gene, these two putative islands contain different genes (142).

Other related proteins. A predicted "R28-like" protein was recently identified in an erythromycin-resistant *S. pyogenes* M6 clone (10). Interestingly, this R28-like protein is encoded by a gene located on a mobile genetic element (in this case a prophage), like the genes encoding Esp in *E. faecalis* and in *E. faecium* (142, 223). The ~149-kDa R28-like protein was predicted to have identical 90-residue repeats, which have the conserved motif (Fig. 3B). The function of this putative protein is not yet known.

Another Alp-related protein is the ~239-kDa Bap protein, which promotes biofilm formation and is expressed by some *S. aureus* strains causing bovine mastitis but not by human *S. aureus* isolates (48). This biofilm-associated protein apparently interferes with the ability of *S. aureus* to interact with connective tissue components (49), a property that was suggested to counteract the ability of the bacteria to cause inflammation, thereby favoring colonization and persistence in the bovine mammary gland (50). Bap includes a region with nearly identical 86-residue repeats, which vary in number among different clinical isolates (50), and it is more closely related to the Esp protein of *E. faecalis* than to the Alp proteins of *S. agalactiae* (48, 255). Like the *esp* genes of *E. faecalis* and *E. faecium* (142, 223), the *bap* gene is localized on a PAI and may be localized on a transposon-like element within that PAI (257). The PAI carrying the *bap* gene is related to several other *S. aureus* PAIs, but the other PAIs carry toxin genes instead of the *bap* gene. According to an interesting proposal, the presence of these alternative genes represents two different bacterial strategies: while expression of Bap inhibits inflammation and promotes bacterial persistence, the expression of toxin promotes inflammation and more acute disease (50).

The predicted *Listeria monocytogenes* protein Lmo0842 is related to Alp proteins. This protein, which was identified by analysis of the genome of strain EGDe (34, 77), contains a region with six long repeats related to those of Rib and R28. The corresponding gene was found in almost all *L. monocytogenes* strains and in some strains of other *Listeria* species (58).

A putative surface protein designated Rlp (for "Rib-like protein") was identified in *Lactobacillus fermentum* (256). This protein is related to Rib and other members of the Alp family and also to the Esp protein of *E. faecalis* and the Bap protein of *S. aureus*. The gene encoding Rlp was only partially sequenced but is predicted to contain at least eight imperfect repeats. The function of this protein is not known.

The BipA protein is a surface protein expressed by many *Bordetella* strains (74, 238). It was identified and characterized in *Bordetella bronchiseptica* but is also expressed by most, if not all, strains of the major human pathogen *B. pertussis* and by some strains of *B. parapertussis* (74). Unlike the Alp proteins, BipA is most probably anchored to the cell wall via the N-terminal hydrophobic region while the C-terminal part is surface exposed (238). The central part of BipA is composed of 90-amino-acid repeats exhibiting limited homology to the repeats of Alp proteins (Fig. 3B). Although the homology is limited, it was readily identified in our search of the databases. Interestingly, the N-terminal nonrepeated region of BipA shows sequence similarity to the membrane localization domains of two extensively studied proteins implicated in interactions with human cells, intimin from enteropathogenic and enterohemorrhagic *E. coli* and invasin from *Yersinia* species (86, 152). However, there is no evidence that the C-terminal surface-exposed part of BipA promotes adhesion to human cells, like the extensively studied intimin and invasin proteins.
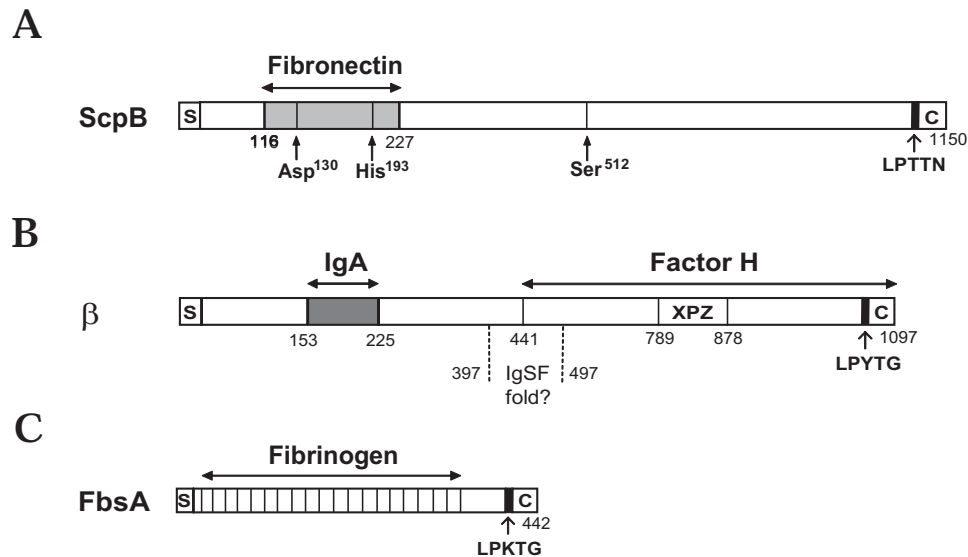
FIG. 4. Schematic representation of three *S. agalactiae* surface proteins with enzymatic and/or ligand-binding activity. (A) The ScpB protein, which was first identified as a C5a peptidase, has a Fn-binding region (13) that overlaps with the Ser-Asp-His catalytic triad required for enzyme activity (37). S, signal sequence. The C-terminal region (C) includes the putative wall-anchoring motif LPTTN. Modified from reference 13 with permission. (B) The β protein has separate binding sites for human IgA-Fc (92, 107, 108, 214) and FH (5), as indicated. The XPZ region is a proline-rich and surface-exposed region with a highly periodic sequence (4). This region, which adopts the PPII helix structure (4), is not required for binding of FH (5). A region in the central part of the β protein is predicted to have an IgSF fold (12). The C-terminal region includes the putative wall-anchoring motif LPYTG. Modified from reference 5 with permission of the publisher. (C) The FbsA protein is composed almost entirely of 16-residue repeats, which are responsible for the ability of the protein to bind Fg (219). The number of repeats varies among different bacterial strains. The C-terminal region includes the putative wall-anchoring motif LPKTG. Modified from reference 219 with permission of the publisher.

THE C5a PEPTIDASE

General Properties

Streptococcal C5a peptidase (Fig. 4A) is a surface-localized serine protease that inactivates human C5a, a neutrophil chemoattractant produced during complement activation (264). This streptococcal enzyme is thought to contribute to virulence by interfering with neutrophil recruitment and may contribute to the poor inflammatory response observed in infected tissues (94, 211). Moreover, recent data show that the C5a peptidase binds fibronectin (Fn) and may promote bacterial invasion of epithelial cells (13, 40). This peptidase was first identified in *S. pyogenes* (264, 265), and a virtually identical enzyme was subsequently found in *S. agalactiae* (24, 42, 95, 248). These two proteins have been designated SCPA and SCPB (40), or ScpA and ScpB (13); in this review, we use the latter designations.

Both ScpA and ScpB have been reported to cleave the human 74-amino-acid C5a molecule between residues His-67 and Lys-68, causing the release of a short C-terminal C5a fragment (24, 45). Possibly, ScpA also cleaves C5a at a second site between Ala-58 and Ser-59 (2). Interestingly, the C-terminal end of C5a is thought to contain amino acids contributing to the biological activity of this chemoattractant (168), providing a possible molecular explanation for the ability of Scp to inactivate human C5a.

Both ScpA and ScpB have been studied in detail and are described here. Of note, a C5a peptidase is also found in group G streptococci isolated from cases of human disease (44).

Molecular biology of *S. pyogenes* C5a peptidase (ScpA). All strains of *S. pyogenes* produce the ~125-kDa surface protein ScpA, which is a highly specific serine protease with a con-served Ser-Asp-His catalytic triad (2, 37, 45, 110, 233). At least in some strains, a fraction of ScpA is found in culture supernatants, a property that could have functional significance (109, 184).

The *scpA* gene was first sequenced in *S. pyogenes* strains of serotypes M12 and M49 (37, 196), and gene sequences are now available also in the published genomes of M1, M3, and M18 strains (14, 66, 175, 230), which encode proteins that are 96% identical to that originally sequenced in an M12 strain (37). The C-terminal region of ScpA contains the sequence LPTTN, which is similar to the classical sorting motif LPXTG, suggesting that surface-localized ScpA may be covalently attached to bacterial peptidoglycan (37, 177). There are no long repeats in ScpA, but the region immediately upstream of the putative sorting motif contains 17-residue proline-rich repeats, which vary in number between three and five in different *S. pyogenes* strains and were suggested to span the bacterial cell wall (37, 125).

The gene (*scpA*) encoding ScpA is closely linked to the gene (*emm*) encoding M protein, the classical antiphagocytic virulence factor of *S. pyogenes* (68), and both genes are part of a regulon positively regulated by the Mga protein (51, 120). This coregulation provides circumstantial evidence that ScpA affects virulence, but ScpA does not exhibit the extensive sequence variability and antigenic variation that allows M protein to evade host immunity (68, 170, 177).

Molecular biology of *S. agalactiae* C5a peptidase (ScpB). The *S. agalactiae* C5a peptidase (ScpB) was first sequenced in a strain of serotype II (42), and the sequence is now available for several additional strains (26, 78) for which the amino acid sequences are at least 96% identical to the first sequence (42).

ScpB is also virtually identical to ScpA of *S. pyogenes* (~95% residue identity) (26). This similarity between ScpA and ScpB indicates that horizontal gene transfer has occurred. The specific activity of purified ScpB is very similar to that of purified ScpA, in agreement with the almost identical sequences (233).

The gene encoding ScpB was found in all human isolates of *S. agalactiae* but only in a minority of bovine isolates (55, 73). As described below, the *scpB* gene is located immediately upstream of the *lmb* gene, which encodes another surface protein, the putative adhesin Lmb (73, 79), and a similar gene arrangement is found in *S. pyogenes* (see "The Lmb protein" and Fig. 5, below).

Analysis of the two known complete *S. agalactiae* genome sequences showed that the *scpB* gene is intact in the type III strain (78) but is frameshifted near the 3′ end in the type V strain (252). These genomes also contain the genes for two additional putative serine proteases related to ScpB (78, 252). Both of these proteins have the LPXTG motif (or the closely related LPXTS motif) in the C-terminal region, indicating that a family of surface-exposed ScpB-related proteases may be expressed by human *S. agalactiae* isolates. One of these proteases is the CspA enzyme, which was independently identified in another *S. agalactiae* strain (87) (see below). Of note, ScpB-related cell envelope proteases are also found in lactic acid bacteria, and all of these proteases were predicted to have a multidomain structure (65, 229).

Role of enzymatic activity. For both *S. pyogenes* and *S. agalactiae*, the function of the C5a peptidase is unclear and the species specificity of the enzyme is controversial. The latter point is important, because the mouse model is commonly used to analyze streptococcal pathogenesis.

In the *S. pyogenes* system, work with ScpA-negative mutants in a mouse model indicated that ScpA decreases recruitment of inflammatory cells, possibly because ScpA inhibits the function of mouse C5a (110, 183). In agreement with this result, ScpA was reported to have at least some ability to degrade mouse C5a in vitro (110).

In the *S. agalactiae* system, the ScpB enzyme was found to inactivate human C5a, but not C5a from several other species, including the mouse (23). In agreement with this finding, ScpB inhibited recruitment of neutrophils promoted by human C5a but not by mouse C5a (23, 28). To reconcile these findings with those made in the *S. pyogenes* system, it was suggested that degradation of mouse C5a occurs with markedly reduced efficiency and may not be physiologically relevant. Because of the similarity between ScpA and ScpB, it is unlikely that the results reported in the two systems reflect differences between the two enzymes. Thus, it is uncertain whether the streptococcal C5a peptidases have physiologically significant activity on mouse C5a.

Although an important function of ScpB may be to inactivate human C5a, evidence is accumulating that this protein also has other functions. This was first surmised from the finding that many *S. agalactiae* strains of the III-3 clone, a putative high-virulence clone, have little or no C5a peptidase activity in vitro, although the gene (*scpB*) is present in all *S. agalactiae* strains (26, 246). Interestingly, these enzyme-negative strains express an ScpB protein that lacks enzyme activity due to a deletion corresponding to four amino acids affecting the active site (26). Thus, enzymatic activity is not an essential feature of ScpB expressed by clinical isolates of *S. agalactiae*. Because the en-

zyme-negative strains have a high content of capsular sialic acid, it was suggested that C5a peptidase activity may not be needed in these strains because the capsule efficiently inhibits complement activation and production of C5a (246).

Role in fibronectin binding and cellular invasion. Two recent studies (13, 40) demonstrate that the ScpB protein not only is a C5a peptidase but also binds Fn. These studies were based on the previous finding that *S. agalactiae* bacteria bind to immobilized Fn (249). Binding between ScpB and Fn was demonstrated with pure components, and pure ScpB was shown to bind to human epithelial cells. However, bacterial mutants lacking ScpB were only partially affected in their ability to bind Fn, indicating that other Fn-binding proteins may exist in *S. agalactiae* (13, 40). The analysis by Beckmann et al. (13) located Fn binding to a 112-residue region in the N-terminal part of ScpB, a region that includes two of the amino acid residues in the catalytic triad of the enzyme (Fig. 4A). The C5a peptidase of *S. pyogenes*, ScpA, was included in some tests and, like ScpB, was found to bind to Fn and to human epithelial cells (40).

Studies with one ScpB-negative mutant (derived from an unencapsulated parental strain) indicated that it had a reduced ability to invade human epithelial cells in vitro, suggesting that ScpB promotes cellular invasion (40). This observation may be of relevance to the ability of *S. agalactiae* to invade human epithelial and endothelial cells in vitro and in vivo (76, 211, 212).

Role in virulence. As described above, the available data indicate that the Scp proteins of *S. pyogenes* and *S. agalactiae* have at least two functions: binding of Fn (which may promote adherence and/or invasion) and degradation of C5a. However, conclusive evidence is not yet available that ScpB contributes to virulence, although there is evidence for both ScpA and ScpB that the ability to inactivate C5a may delay infiltration of phagocytes (23, 110, 183, 248).

Evidence that ScpA contributes to virulence in *S. pyogenes* comes from the finding that ScpA-negative bacterial mutants were eliminated more rapidly than wild type bacteria from infected mouse tissue or from the nasopharynx of colonized mice (109–111). However, ScpA-negative mutants were not affected in their ability to cause lethal infection (110, 183) and ScpA had little effect on the ability of *S. pyogenes* to resist phagocytosis in human blood (109). Of note, a mutation in the *scpA* gene might have a polar effect on the *fba* gene located downstream in many *S. pyogenes* strains (197, 250), complicating studies of such mutants (see below and Fig. 5).

Evidence that ScpB plays a role in virulence in *S. agalactiae* comes from a study employing C5-deficient mice (28). When such mice were infected with wild-type bacteria or with an ScpB-negative mutant, similar recruitment of neutrophils was observed, but when human C5 was administered together with the bacteria, neutrophil recruitment was higher in mice infected with the mutant bacteria, implying that ScpB produced by the wild-type bacteria inhibits neutrophil recruitment in vivo by degrading C5a formed after cleavage of C5. In another study, an ScpB-negative bacterial mutant (derived from an uncapsulated parental strain) showed somewhat increased sensitivity to phagocytosis by human cells and was cleared more efficiently than the parental strain from the lungs of infected mice, suggesting that ScpB contributes to virulence (39).

Concerning the role of ScpB in virulence, it is of interest that some *S. agalactiae* strains, predicted to belong to a high-virulence clone, express an ScpB protein that lacks enzyme activity (26). This finding suggests that the ability to bind Fn and promote invasion may be more important than the enzymatic activity, at least in some strains. The lack of enzymatic activity in these high-virulence strains even suggests that C5a peptidase activity may attenuate virulence, as proposed for some other bacterial surface structures (75), including Fn-binding proteins of *S. aureus* (161) and *S. pyogenes* (176, 182).

Immunization studies. The conserved sequence and surface localization of Scp make it an interesting vaccine candidate. Due to the sequence similarity between ScpB and ScpA, it is even conceivable that a vaccine containing one protein might confer protection against both *S. agalactiae* and *S. pyogenes* (109). These arguments raise the important question whether C5a peptidase elicits protective immunity.

Concerning *S. pyogenes*, intranasal immunization of mice with recombinant ScpA induced salivary IgA and serum IgG antibody responses (109). Moreover, such immunization reduced the potential of *S. pyogenes* strains of different M type to colonize the nasopharynx of mice, indicating that ScpA is indeed of interest for the development of a vaccine against *S. pyogenes* disease. Immunization of mice with recombinant ScpB, which is almost identical to ScpA, also reduced the ability of *S. pyogenes* to colonize mouse tissues (43). Of note, ScpA is immunogenic in humans, as shown by the appearance of anti-ScpA antibodies during many (but not all) natural *S. pyogenes* infections (185, 228). However, anti-ScpA antibodies do not appear to be opsonic (228), possibly because the *S. pyogenes* strains also express the antiphagocytic M protein, but they might protect against infection by inhibiting adhesion to (or invasion of) epithelial cells.

Concerning *S. agalactiae*, Bohnsack et al. (29) made the interesting observation that normal human serum contains IgG antibodies that inhibit the enzymatic activity of soluble ScpB, but not of ScpB expressed on the surface of *S. agalactiae*. However, the surface-located enzyme of a nonencapsulated bacterial mutant was inhibited by such antibodies. Thus, the polysaccharide capsule may allow surface-localized ScpB to evade neutralizing antibodies, although the protein retains the ability to cleave C5a. Nevertheless, hyperimmune rabbit anti-ScpB serum was reported to have some opsonic activity in studies with mouse macrophages or whole human blood (38), and immunization of mice with pure ScpB enhanced the clearance of bacteria from the lungs of mice that had been inoculated intranasally with *S. agalactiae* (39). Although the latter tests employed only a single *S. agalactiae* strain of the uncommon serotype VI (presumably expressing ScpB), the results suggest that ScpB may be of interest for vaccine development, even if the enzymatic activity of the surface-localized protein is not inhibited by antibodies. Conceivably, anti-ScpB antibodies may protect against infection by interfering with Fn binding. It is not yet known whether anti-ScpB antibodies appear in connection with a clinical *S. agalactiae* infection, but the identification of antibodies recognizing ScpB in normal human serum (29) suggests that the protein may be immunogenic in humans.

THE β PROTEIN

General Properties and Sequence

The trypsin-sensitive β protein was initially identified as one of the two components of the *S. agalactiae* c antigen (18, 268), but is unrelated to the other component of that antigen, the α protein of the Alp family. The β protein interacts with two components of the human immune system, IgA-Fc and factor H (FH), suggesting that it plays a role in immune evasion, and it elicits protective immunity. This protein is found in almost all strains of serotype Ib and in a minority of strains of serotypes Ia, II, and V but almost never in strains of serotype III (6, 112, 124, 163, 236, 243). For unknown reasons, strains that express β usually also express the unrelated α protein, while the α protein is often expressed alone, in particular in strains of serotype Ia. The genes for these two proteins are located in the same part of the *S. agalactiae* chromosome (54), but they are not closely linked (130). It is not known whether β is a virulence factor, although its ability to bind different components of the human immune system suggests that it may contribute to virulence. Indeed, recent observations indicate that high level expression of β is associated with virulence (173).

Sequencing of the β protein (92, 107) identified a 37-residue signal peptide and a typical C-terminal wall-anchoring region with an LPXTG motif (Fig. 4B). Unlike many other surface proteins of gram-positive cocci, the β protein lacks long tandem repeats, but the C-terminal half includes a proline-rich region with unique periodic sequence, the so-called XPZ region, which varies in length among different bacterial strains, a property that may be used for epidemiological purposes (4, 15, 124). The sequence for β was found to be identical in two different *S. agalactiae* strains (92, 107), except for a single amino acid residue and a size difference in the periodic XPZ region. Of note, β is released in almost pure form from washed bacteria incubated at elevated pH, implying that this LPXTG protein may not be covalently linked to the cell wall (4, 146). This property provides a simple way to purify the protein (146).

The proline-rich XPZ region in the C-terminal half of β adopts the polyproline II (PPII) helix structure (4), an extended type of structure important for protein-protein interactions (104, 270). The significance of this type of structure is underlined by its presence in collagen and by the finding that class II major histocompatibility complex molecules bind peptides in a PPII-like conformation (104, 270). However, the function of the XPZ region in β is not known, but it is present in all variants of β and is exposed on the cell surface (4), suggesting that it may participate in interactions with host proteins.

A noteworthy feature of the β protein is the presence of a region that most probably has the IgSF fold (Fig. 4B). This region was identified by Bateman et al. (12), who searched databases for prokaryotic protein sequences showing homology to eukaryotic proteins known to have the IgSF fold. One of only two prokaryotic sequences identified corresponds to the central part of the β protein. This region may represent a prokaryotic progenitor of molecules of the eukaryotic IgSF family, but it may also represent a case of horizontal gene transfer from eukaryotes to bacteria (12). As described above, the R28 protein of the Alp family includes a region showing

homology to the β protein, and we have noted that this region in R28 corresponds to the region in β predicted to have an IgSF fold. Together, these findings suggest that the region with the predicted IgSF fold may correspond to a mobile domain.

Binding of human IgA-Fc and factor H. The β protein binds with high affinity to the Fc part of human serum IgA of both subclasses, but, unexpectedly, it binds poorly to secretory IgA, the molecular form of IgA found on mucosal surfaces, where *S. agalactiae* initiates infections (146, 214). The IgA-Fc-binding site is localized within a 73-residue region in the N-terminal part of the protein (92, 108) and it was suggested that a 6-residue sequence within this region is essential for binding (108). A second IgA-Fc-binding site was proposed to reside in the central part of the β protein (107), but this observation may have been due to antibody activity in the polyclonal serum IgA used for the analysis. Indeed, normal serum is known to often contain antibodies to β (19, 146, 254). The binding site for β in IgA-Fc is localized in the Cα2-Cα3 interdomain region (194), at a site also used by IgA-binding M proteins of *S. pyogenes* and by the human IgA receptor CD89 (FcαRI) (194, 195). Consequently, the streptococcal IgA-binding proteins inhibit binding of IgA to CD89, thereby inhibiting IgA effector function, a property that may contribute to immune evasion (194).

A few *S. agalactiae* strains express alternative forms of the β protein that are surface localized but do not bind IgA (32, 173). The reason for the inability of these variants to bind IgA is not known. In addition, some strains may lack surface expression of the β protein, while they secrete a low-molecular-weight variant of the protein (32).

The β protein also binds human FH, a single-chain ∼150-kDa plasma protein that inhibits the activation of complement via the alternative pathway (5). The major FH-binding site in β is localized in the C-terminal half of the protein and does not overlap with the IgA-binding site (5) (Fig. 4B). Thus, β has separate binding sites for two important components of the human immune system, IgA and FH. Like many other complement regulators, FH is a member of the regulators of complement activation family and is composed of modules designated SCR or CCP, of which there are 20 in FH (149, 171). Of note, FH binds to several bacterial pathogens, which may exploit this human complement inhibitor for protection against complement attack and phagocytosis (147, 205). In β-expressing *S. agalactiae*, surface-bound FH retains its ability to act as a complement regulator, suggesting that such *S. agalactiae* strains may utilize bound FH to inhibit complement attack (5), but it is not yet known whether the β protein has antiphagocytic properties. Indeed, bacterially bound FH might also have other functions, e.g., to promote adhesion to human cells (5). The major binding site for β in FH may be localized in the SCR 8-11 region (105).

Immunization studies. Several experimental studies have shown that the β protein elicits protective antibodies, a property that may be of interest for vaccine development. In passive-immunization experiments, protection was observed with antiserum raised against β protein purified directly from streptococci (19) or raised against recombinant protein (164). In active immunizations, maternal immunization of mice with β protein protected pups against infection with a β-expressing strain (155) and maternal immunization with a conjugate composed of β and the type III capsular polysaccharide protected

pups against strains expressing either component (157), implying that protective antibodies are transferred transplacentally. In the human system, there is evidence that many women have anti-β IgG antibodies that can be transferred across the placenta (72). These observations suggested that the level of naturally acquired maternal β-specific IgG antibodies might be correlated with protection of human neonates from disease, but such a correlation was not found (129).

Related proteins in other bacterial species. The β protein is distantly related to several other surface proteins expressed by important gram-positive pathogens. These β-related proteins include members of the *S. pneumoniae* PspC protein family (101), the *S. pyogenes* Fn-binding protein Fba (250), and the *S. pyogenes* M1 protein (our unpublished data). Interestingly, these different surface proteins all share the ability to bind human FH (52, 103, 121, 189), suggesting that they all play related roles in pathogenesis. These bacterial proteins may all have similar FH-binding sites, as suggested by analysis of two of the proteins (105). In addition to these similarities between β and other FH-binding bacterial proteins, the region in β predicted to have an IgSF fold is homologous to a region in the R28 protein, as described above.

THE Lmb PROTEIN

The Lmb Proteins of *S. agalactiae* and *S. pyogenes*

The Lmb protein of *S. agalactiae* is a surface-exposed lipoprotein (73, 232) that shows homology to members of the Lra1 family of proteins, which have been implicated in adhesion and metal transport in gram-positive bacteria (106). This protein, which is expressed by most, if not all, *S. agalactiae* strains, was first identified as a laminin-binding surface protein and was suggested to play a role in colonization and/or invasion of damaged epithelium (232). It has also been identified in *S. pyogenes*, and the sequence is virtually identical in different strains both within and between the two species (61, 232, 251).

The nomenclature in the field is somewhat confusing. The name Lmb was introduced for the *S. agalactiae* protein (232), but the virtually identical protein in *S. pyogenes* has been referred to as Lsp or Lbp (61, 251). In this review we use the name Lmb, whether the protein is expressed in *S. pyogenes* or in *S. agalactiae*.

In spite of its name, the ability of the Lmb protein to bind laminin and promote binding to tissues is controversial. Pure laminin was reported to bind pure recombinant Lmb protein in the *S. pyogenes* system (251), but in another study binding of laminin to Lmb was not detected in similar assays (61). Binding of laminin to Lmb has also been analyzed with bacterial mutants affected in the *lmb* gene, but analysis of such mutants is problematic because a mutation in the *lmb* gene may affect the expression of multiple genes for unknown reasons (61). Moreover, a mutation in the *lmb* gene may have a polar effect on the gene located downstream (see below). Nevertheless, comparison of different bacterial mutants did not provide evidence that Lmb exposed on the surface of *S. pyogenes* binds laminin (61). Moreover, binding of laminin to whole *S. agalactiae* bacteria was not observed in another analysis employing whole bacteria (249). Thus, it is unclear whether Lmb binds laminin. However, there is some evidence that the Lmb protein of *S. pyo-*
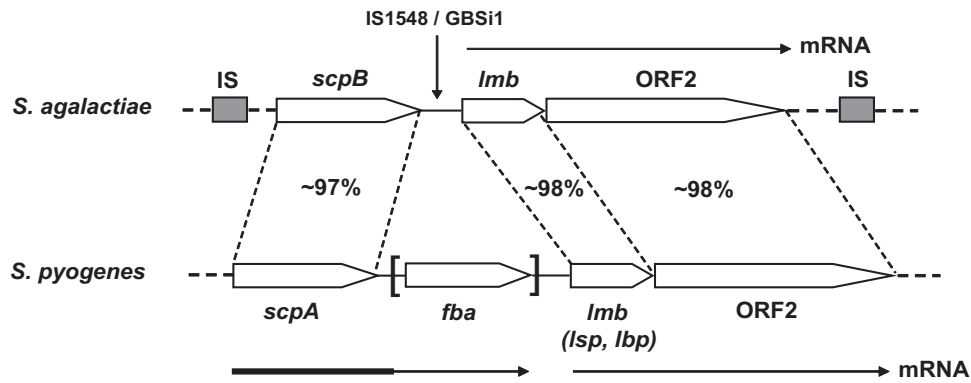
FIG. 5. Genetic structure of a chromosomal region, conserved between *S. agalactiae* and *S. pyogenes*, that includes genes for the surface proteins Scp and Lmb. The figure summarizes data from several studies (61, 73, 79, 197, 232, 251). The *scp and lmb* genes encode the C5a peptidase and the Lmb protein, respectively, while the function of ORF2 is unknown. These three genes are virtually identical in the two genomes, as indicated, while the surrounding chromosomal regions are unrelated (61, 73). In *S. agalactiae*, the three genes are located within a putative composite transposon that also includes several other genes and is bordered by IS elements, which are indicated in the figure but not drawn to scale (73). Many strains of *S. agalactiae* have the IS element IS*1548* or the intron GBSi1 inserted between *scpB* and *lmb*. In *S. pyogenes*, the genes do not appear to be located within a transposon. In many (but not all) strains of *S. pyogenes*, the *fba* gene, which encodes the Fn-binding surface protein Fba, is located between *scpA* and *lmb* (61, 197, 250). In both species, the *lmb* and ORF2 genes are cotranscribed (61). In *S. pyogenes* the *scpA* gene is cotranscribed with *fba*, and both genes are controlled by the Mga protein, but a strong transcriptional attenuator is probably located between the two genes, as indicated (197).

genes affects (directly or indirectly) adhesion to and/or invasion of human epithelial cells, but possible polar effects of *lmb* mutations complicate analysis of the data (61, 251). Thus, it is also unclear whether Lmb contributes to adhesion, like typical members of the Lra1 family. Evidence is also lacking that Lmb participates in metal transport, like Lra1 proteins (61). Although the function of Lmb is unclear, the fact remains that this protein may be exposed on the surface of most *S. agalactiae* strains, making it of interest for analysis of pathogenesis and for vaccine development. Interestingly, an *S. agalactiae* mutant with reduced virulence, obtained by STM, is affected in the *lmb* gene or a related gene (113, 242). However, it is not yet known whether Lmb elicits protective immunity.

Genetics of the region encoding Lmb and ScpB: linked genes for surface proteins. In both *S. agalactiae* and *S. pyogenes*, the *lmb* gene is located in a short chromosomal region that has similar structures in the two bacterial species and includes the virtually identical *scp* genes (Fig. 5). However, adjacent sequences in the two genomes show no homology, indicating that the region has been horizontally transferred (61, 73). Because it includes genes for surface proteins, the structure of this region is of relevance here.

In *S. agalactiae*, the *lmb* gene is located just downstream of *scpB* and is followed by an open reading frame, ORF2, that is found also in *S. pyogenes* and has unknown function. Thus, the region of homology between the two bacterial species includes three genes in the genome of *S. agalactiae*, and two of these genes encode known surface proteins, ScpB and Lmb. Of note, the *lmb* gene and ORF2 are cotranscribed (61). The three *S. agalactiae* genes are located within a putative composite transposon that also comprises several other genes and is bordered by IS elements (Fig. 5). The complexity of this region is further underlined by the finding that many strains of *S. agalactiae* have a mobile genetic element, the intron GBSi1 or the IS element IS*1548*, inserted between the *scpB* and *lmb* genes (79). It is not known whether these genetic elements affect

transcription of the surrounding *scpB* and *lmb* genes. Interestingly, the presence of the intron may be a marker for the putative high-virulence serotype III clone described in "Genomics" (above) (79, 247).

The location of the *scpB-lmb* region on a composite transposon may allow horizontal gene transfer, a hypothesis supported by the finding that most *S. agalactiae* isolates of bovine origin lack the *scpB* and *lmb* genes, suggesting that they are acquired or lost together (55, 73, 272). In contrast, all human isolates were found to have both genes (73, 272). Similarly, human isolates of group C and G streptococci harbor close homologues of the genes, while animal isolates of these bacterial species lack the genes (73). Together, these findings suggest that Lmb and ScpB may play a particularly important role for the ability of human *S. agalactiae* isolates to colonize and/or to cause disease.

There is no evidence that the conserved genes in *S. pyogenes* are located within a composite transposon (73), and in many strains the *scpA* and *lmb* genes are separated by another gene, *fba*, which encodes Fba, a proline-rich surface protein that binds Fn (61, 197, 250). Thus, the *scp-lmb* region region encodes three surface proteins in *S. pyogenes* strains that have the *fba* gene.

OTHER SURFACE PROTEINS OF *S. AGALACTIAE*

Some *S. agalactiae* surface proteins have been less extensively studied than those described above. As described below, these proteins include some LPXTG proteins and the Sip protein, which elicits protective immunity.

The Fibrinogen-Binding FbsA Protein

The FbsA protein promotes the binding of human fibrinogen (Fg) to the surface of *S. agalactiae* (219). Like many other surface proteins in gram-positive bacteria, FbsA has a putative

signal peptide, a repeat region, and a C-terminal wall-anchoring region with an LPXTG motif (Fig. 4C). The gene for FbsA was detected in 25 of 27 *S. agalactiae* strains, and it was not excluded that the remaining strains have *fbsA*-related genes (219). Thus, this protein may be of general interest for analysis of *S. agalactiae* pathogenesis and for vaccine development.

The processed form of FbsA is almost entirely composed of 16-amino-acid repeats, which are responsible for the Fg-binding property of the protein. The number of repeats was found to vary between 3 and 30 among different *S. agalactiae* strains, as predicted from gene sequences, but a single repeat binds Fg, as demonstrated with synthetic peptides (219). Interestingly, a mutation in the *fbsA* gene caused a reduction in the ability of *S. agalactiae* to grow in whole human blood, suggesting that FbsA contributes to phagocytosis resistance. It is not yet known whether FbsA affects virulence in mice, elicits protective immunity, and/or is a target for protective antibodies.

A second Fg-binding *S. agalactiae* protein, unrelated to FbsA, was recently identified in two independent studies (85, 102). This protein, which was designated FbsB (85) or Fgag (102), has a typical signal peptide but lacks the LPXTG motif or other wall-anchoring signatures, suggesting that it is not a surface protein but is secreted into the extracellular medium. Conclusive evidence is not yet available that FbsB/Fgag is expressed in *S. agalactiae*, but preliminary evidence suggests that it is indeed present in culture supernatants (85, 102). This protein has also been reported to be present on the bacterial surface (252), but the situation is unclear because a bacterial mutant lacking the Fg-binding surface protein FbsA was completely unable to bind human Fg, implying that this strain does not express FbsB/Fgag on the surface (85, 219). Interestingly, FbsB/Fgag occurs in at least three allelic variants which have similar C-terminal regions but virtually unrelated N-terminal regions, indicating that this protein identifies a novel family of secreted Fg-binding proteins (102). The function of FbsB/Fgag is not known, but it may contribute to bacterial invasion of human epithelial cells (85). Of note, the protein found in bovine *S. agalactiae* strains may bind bovine Fg but not human Fg (102).

The Sip Protein

The ~45-kDa Sip protein was identified after immunological screening of a genomic library (33). Amino-terminal sequencing of the purified protein identified a 25-residue signal peptide, and Sip is exposed on the bacterial surface (208). However, a wall-anchoring motif has not been identified, and the mechanism of surface attachment is unknown. Importantly, immunization of mice with purified recombinant Sip elicited protective immunity against lethal infection with *S. agalactiae* strains of several serotypes (33, 159). Moreover, Sip was identified in *S. agalactiae* strains representing all nine serotypes and is highly conserved (33, 208). Together, these data indicate that Sip is an interesting candidate for the development of a protein-based vaccine against *S. agalactiae* disease.

The function of Sip is not known, nor is it known whether Sip contributes to virulence. Interestingly, genes encoding putative Sip homologues have been identified in the genomes of *S. pyogenes* and *S. pneumoniae* (33).

CspA and Other Surface-Localized Enzymes

In addition to the C5a peptidase (ScpB), several other surface-associated *S. agalactiae* enzymes have been described, and some of them have been reported to elicit protective immunity.

The ~153-kDa CspA protein is a surface-associated protease with an LPXTG wall-anchoring motif (87). The gene (*cspA*) encoding this protein is widely distributed among clinical isolates of *S. agalactiae* (87) and is also present in each of the two sequenced genomes (78, 252). As mentioned above, the CspA protease is related to the C5a peptidase (ScpB), but CspA does not cleave C5a (87). Of note, CspA was reported to cleave the α-chain of human Fg and to promote evasion of phagocytosis (87), but the latter property is probably not due to the ability to cleave Fg, because it was identified in a system where bacteria were incubated with neutrophils and serum, i.e., in the absence of Fg. Interestingly, absence of CspA reduces virulence in a neonatal-rat sepsis model, but it is not yet known whether this protein elicits protective immunity.

Several enzymes proposed to be surface localized were identified in a proteomic analysis aimed at characterizing surface proteins of a serotype III strain (98). This analysis identified the Rib protein, but none of several other previously described surface proteins, such as Lmb, FbsA, Sip, or ScpB. Remarkably, the proteomic analysis mainly identified enzymes commonly thought to be intracellular, including superoxide dismutase, glyceraldehyde-3-phosphate dehydrogenase, enolase, an arginine deiminase (acid glycoprotein), ornithine carbamoyltransferase, and phosphoglycerate kinase. Other reports also indicate that some of these enzymes may be surface localized in *S. agalactiae* and other gram-positive bacteria (41, 187, 188, 222), but the significance of these findings remain unknown. Nevertheless, rabbit antibodies to the ornithine carbamoyltransferase and phosphoglycerate kinase enzymes of *S. agalactiae* conferred some protection against lethal infection in a neonatal-mouse model, supporting the hypothesis that the enzymes are surface exposed also in vivo (98). However, it is not yet known how these enzymes, which lack signal sequence and a known cell wall-sorting motif, are retained at the cell surface.

A ~52-kDa glutamine synthetase that may be surface exposed was identified by Suvorov et al. (244). This protein, which was found in all *S. agalactiae* strains analyzed, shows sequence similarity to known bacterial surface proteins, but it is not yet known whether it indeed is exposed on the bacterial surface.

The Spb1 Protein

The predicted 53-kDa Spb1 protein was identified by subtractive hybridization of DNA from a serotype III strain of the putative hypervirulent clone ET1/III-3 (1). Interestingly, the gene encoding Spb1 was found only in type III strains belonging to this clone and not in the sequenced type III and type V strains (78, 252). The gene sequence indicates that Spb1 has the characteristics of a surface protein, including a signal sequence and an LPXTG motif, and it is predicted to show segmental homology to some adhesins identified in other bacteria, both gram-positive and gram-negative. A Spb1-negative mutant was only slightly affected in the ability to adhere to epithelial cells but was significantly affected in the ability to invade such cells, suggesting that the Spb1 protein promotes

internalization (1). Of note, two other *S. agalactiae* surface proteins, the Alp family α protein and ScpB, have also been reported to promote invasion of epithelial cells (30, 40). It is not yet known whether the Spb1 protein affects mouse virulence or elicits protective immunity.

Additional Surface Proteins, Including R and X Antigens

Using antisera raised against whole bacteria, Wilkinson (267) concluded that four R antigens (designated R1 to R4) occur in various combinations in strains of *S. pyogenes* and *S. agalactiae*, and three of them were identified in *S. agalactiae* (71, 128, 267). Little is known about the molecular properties of these different antigens, and it is not known whether they have more than one component, like the c antigen, which is composed of two distinct proteins (18). However, some anti-R sera probably include antibodies elicited by members of the Alp protein family (31). For example, antiserum raised against the R reference strain D136 may recognize the Alp2 protein of that strain (78) and anti-R4 antisera may recognize the Rib protein (17). Immunological methods of identifying surface proteins may be misleading because of cross-reactivities and should be used with caution (130, 235). Of note, an early study by Lindén (148) indicated that antibodies against an R antigen from *S. agalactiae* serotype III protected mice against infection with R-positive serotype II strains, but, unexpectedly, these antibodies were not protective against R-positive serotype III strains.

An "R-like" surface protein, designated R5 or BPS, was recently identified in a minor fraction of *S. agalactiae* strains and was reported to elicit protective immunity (62). The gene for this protein was also found in the genome of the sequenced type V strain (252). Except for the C-terminal region, which includes the LPXTG motif, this protein shows no sequence homology to other surface proteins of *S. agalactiae*.

An antigen designated X was identified by Pattison et al. (193), who analyzed a subpopulation of bovine *S. agalactiae* isolates that apparently lacked capsular polysaccharide. Such strains were reported to express either of two protein antigens, one related to the R28 protein of *S. pyogenes* and one (X) with different serological properties. Little is yet known about the X antigen, but it may elicit opsonizing antibodies and may be of high molecular weight (141, 203, 204).

A 60-kDa protein designated Bsp (for "group B secreted protein") was identified as a minor component in culture supernatants of a type III strain (206). Sequence analysis showed that Bsp has a typical signal peptide and a C-terminal region with an LPXTG motif, suggesting that at least some Bsp molecules may be located on the bacterial surface, although this protein was identified in supernatants (206). However, Bsp was not found in cell wall extracts, and the most C-terminal region does not have the amino acid composition typical of LPXTG-anchored protein. Thus, it is unclear whether Bsp is anchored to the cell wall and/or exposed on the surface. Nevertheless, this protein is of interest for analysis of *S. agalactiae* pathogenesis, because it was found in supernatants from all 31 *S. agalactiae strains* tested, including strains from all of the major serotypes (206). Moreover, the two sequenced *S. agalactiae* genomes (78, 252) encode putative Bsp proteins that are virtually identical to that first identified. Sequence similarities between Bsp and proteins identified in other bacteria suggest that Bsp

may be a peptidoglycan-cleaving endopeptidase, and there is some evidence that it affects cell shape (206). However, a Bsp-deficient mutant showed normal growth in culture media, was not affected in autolysis, and showed normal sensitivity to antibiotics acting on the cell wall. It is not yet known whether Bsp affects virulence or elicits protective immunity.

A surface protein designated Fbs was identified in *S. agalactiae* strains of serotype V (6), which has recently emerged as a clinically important serotype (22). This protein, which has not yet been sequenced, should not be confused with the Fg-binding FbsA protein (219). Analysis with antiserum raised against purified Fbs indicated that it is expressed by about one-third of all human type V strains but rarely by strains of serotypes Ia, Ib, II, and III. Antibodies to Fbs conferred protective immunity, making it of potential interest as a vaccine component (6).

Finally, two putative *S. agalactiae* surface proteins were identified through the use of alkaline phosphatase fusions, and passive immunization with antisera against these proteins was reported to provide partial protection against infection (99). Little is yet known about these proteins, which lack typical signal peptides and wall-anchoring motifs.

REGULATION OF SURFACE PROTEIN EXPRESSION

The subject of gene regulation is beyond the scope of this review, but findings concerning the regulation of surface protein expression are briefly summarized. Generally, little is known about gene regulation in *S. agalactiae*, and only a few studies have addressed the regulatory mechanisms that control genes encoding surface proteins.

Sequence analysis of the *S. agalactiae* genome (78, 252) revealed a large number of possible transcriptional regulators, some of which may be similar to systems that affect the expression of surface proteins in *S. pyogenes*, including CovS/CovR, Rgg (RopB), and the RALP family response regulators RofA and Nra (126). Thus, *S. agalactiae* and *S. pyogenes* may to some degree have similar systems controlling the expression of surface proteins.

Several studies have analyzed regulatory systems that influence the ability of *S. agalactiae* to bind Fg. Spellerberg et al. (231) described a putative two-component regulatory system, designated *rgf* (for "regulator of fibrinogen binding"), that affects the ability of *S. agalactiae* to adhere to immobilized Fg, suggesting that it may influence the expression of the FbsA protein. This regulatory system may also repress the expression of ScpB.

The *S. agalactiae* RogB protein is a novel member of the RALP family, exhibiting ~50% sequence identity to the RofA and Nra proteins of *S. pyogenes* (84). RogB positively regulates the ability of *S. agalactiae* to bind immobilized Fg and Fn and affects the ability of the bacteria to adhere to a human epithelial cell line. Moreover, RogB was demonstrated to activate expression of the Fg-binding FbsA protein, but it did not affect the expression of the Fn-binding ScpB protein (84).

Another bacterial factor affecting expression of FbsA was reported by Samen et al. (215), who described an oligopeptide permease possibly involved in quorum-sensing-dependent regulation of *fbsA* expression.

Little is yet known about regulation of genes encoding members of the Alp family, but a recent study reported that expres-

sion of the α protein undergoes limited phase variation in vitro, due to changes in the number of pentanucleotide repeats located upstream of the structural gene (202). Of note, the two known genome sequences show that the allelic genes encoding the Alp family members Rib and Alp2 are located downstream of a gene encoding a putative AraC type of regulator (78, 252). Similarly, the Alp-related Esp proteins of *E. faecalis* and *E. faecium* are located downstream or upstream of an *araC*-like gene (142, 223). Possibly, these *araC*-like genes control the expression of the adjacent gene encoding a surface protein.

S. AGALACTIAE SURFACE PROTEINS AS VACCINE COMPONENTS

For most bacterial pathogens, the ideal vaccine would probably include a single surface protein that elicits protective immunity and is expressed by all clinical isolates. It remains to be seen whether such a vaccine can be developed for *S. agalactiae* or any other gram-positive pathogen. Indeed, any surface antigen that is a target for protective immunity in natural infections may exhibit antigenic variation, necessitating the development of multivalent vaccines (97). However, vaccine-induced immunity may be different from natural immunity (36), and the interesting possibility remains that a vaccine can be developed on the basis of one or a few surface proteins, even if other surface proteins exhibit antigenic variation. For *S. agalactiae*, such a protein may be included among those described in this review or may be identified from genome sequences (78, 252). Because protective antibodies are transferred transplacentally, a vaccine intended for protection against neonatal disease may be administered to pregnant women or teenage girls (7, 155, 190).

Protein Vaccines and Conjugate Vaccines

The *S. agalactiae* proteins that have so far been most extensively studied as potential vaccine components are the members of the Alp family. Importantly, all four known members of the Alp family elicit protective immunity in a mouse model (19, 132, 235, 236), and it seems possible that a vaccine composed of two of these proteins (Rib and α) would protect against the majority of *S. agalactiae* infections (139, 140). Of note, the Rib and α proteins elicit protective immunity even without adjuvant, a considerable advantage with regard to the development of a human vaccine (140). Moreover, inclusion of the nontoxic adjuvant cholera toxin B subunit may allow the development of a mucosal vaccine (138).

Among other *S. agalactiae* surface proteins that elicit protective immunity, the Sip protein is of particular interest because it is highly conserved and elicits antibodies that protect against infection with strains of several different serotypes (33, 159). Thus, Sip may have the properties desired for an ideal vaccine component. Several additional surface proteins, including the C5a peptidase (ScpB), other surface-localized enzymes, and the β protein, also hold promise as components of a future protein-based vaccine against *S. agalactiae* infections. Among these proteins, the ScpB protein is of particular interest because it is conserved and expressed by all strains of *S. agalactiae*, but it is not yet known whether immunization with this protein protects against lethal infection in an animal model.

Because the polysaccharide capsule is an important target for protective antibodies, work is in progress to develop a multivalent *S. agalactiae* conjugate vaccine based on capsular polysaccharides of several serotypes conjugated to tetanus toxoid or another carrier (7, 9, 190–192, 226, 227). An interesting option is to use an *S. agalactiae* protein as the carrier, because the carrier itself may then elicit immunity (190). Indeed, a conjugate composed of the type III polysaccharide and β protein elicited protective immunity against infection with *S. agalactiae* strains expressing either component (157), and similar results were obtained with a conjugate composed of type III polysaccharide and the α protein (81). Moreover, immunization of mice with a conjugate composed of type III polysaccharide and the ScpB protein elicited antibodies to both components, but protection against infection was not analyzed (38).

Finally, it seems possible that conjugate vaccines can be developed, in which a protective *S. agalactiae* surface protein is used as the carrier for capsular polysaccharide isolated from another important pathogen, e.g., *Haemophilus influenzae* or *Neisseria meningitidis*. Ideally, such a conjugate vaccine would elicit protective immunity against each of the two pathogens.

CONCLUDING REMARKS AND PERSPECTIVES

Evidence is accumulating that a variety of *S. agalactiae* surface proteins contribute to virulence and/or are of relevance for vaccine development. This review has focused on the proteins that have so far been most extensively studied, but the list of interesting proteins may soon expand, due to the wealth of information that is becoming available from genome sequences. The information about known proteins reviewed here provides the framework for future studies of different proteins.

For all of the proteins described in this review, their exact function remains unclear. In this regard, *S. agalactiae* is similar to most other bacterial pathogens, for which much less is known about the biological function of surface proteins than about the biochemical and immunochemical properties of such proteins. However, the available evidence indicates that the Alp family proteins and the ScpB, Lmb, FbsA, and Spb1 proteins may participate in the early stages of an *S. agalactiae* infection by virtue of their ability to promote binding to extracellular matrix components and/or their ability to promote interactions with human epithelial cells. In later stages of the infection, evasion of host innate immunity is essential, and the ability of ScpB to degrade C5a may affect virulence. Moreover, both of the FbsA and CspA proteins may contribute to phagocytosis resistance, and in strains that express the β protein the ability of this protein to bind human IgA and factor H may contribute to immune evasion. Nothing is known about the possible role of surface proteins in the pathogenesis of meningitis, but it is of interest that most cases of meningitis may be caused by high-virulence serotype III strains that express the Rib and Spb1 proteins.

In summary, *S. agalactiae* surface proteins hold promise as vaccine candidates and are likely to play key roles during various stages of an infection. Moreover, the finding that related proteins are found in several other important pathogens suggests that pathogenetic mechanisms used by *S. agalactiae* may be shared by several bacterial species. Further characterization of these proteins therefore promises to generate information

that is of general interest for the understanding of bacterial pathogenesis and for vaccine development.

ACKNOWLEDGMENTS

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