Molecular studies on streptococcal surface proteins

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The Streptococcal Blr and Slr Proteins Define a Family of Surface Proteins with Leucine-Rich Repeats: Camouflaging by Other Surface Structures

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Regions with tandemly arranged leucine-rich repeats (LRRs) have been found in many prokaryotic and eukaryotic proteins, in which they provide a remarkably versatile framework for the formation of ligand-binding sites. Bacterial LRR proteins include the recently described Slr protein of Streptococcus pyogenes, which is related to internalin A of Listeria monocytogenes. Here, we show that strains of the human pathogen Streptococcus agalactiae express a protein, designated Blr, which together with Slr defines a family of internalin A-related streptococcal LRR proteins. Analysis with specific antibodies demonstrated that Blr is largely inaccessible on S. agalactiae grown in vitro, but surface exposure was increased ~100-fold on mutants lacking polysaccharide capsule. In S. pyogenes, surface exposure of Slr was not affected in a mutant lacking hyaluronic acid capsule but was increased ~20-fold in mutants lacking M protein or protein F. Thus, both Blr and Slr are efficiently camouflaged by other surface structures on bacteria grown in vitro. When Blr and Slr exposed on the bacterial surface were compared, they exhibited only little immunological cross-reactivity, in spite of extensive residue identity, suggesting that their surface-exposed parts have been under evolutionary pressure to diverge functionally and/or antigenically. These data identify a family of immunologically diverse streptococcal LRR proteins that show unexpected complexity in their interactions with other bacterial surface components.

Recent work has identified an InlA-related LRR protein, designated Slr (Spy1361), in the gram-positive bacterium Streptococcus pyogenes (group A streptococcus), the cause of acute pharyngitis (“strep throat”), skin infections, streptococcal toxic shock syndrome, and several other diseases (45, 46). This surface-localized protein, which has an LRR region in the C-terminal part, has the characteristics of a lipoprotein, implying that it is attached to the bacterial cell membrane via a cysteine residue in the N-terminal region. In contrast, InlA is covalently attached to the cell wall via an LPXTG sequence in the C-terminal part (4), while the LRR region is located in the N-terminal half, implying that the LRR region is located most distally from the cell wall in both of these proteins (Fig. 1A).

Our work was focused on the human pathogen Streptococcus agalactiae (group B streptococcus), the most important cause of life-threatening diseases like pneumonia, sepsis, and meningitis in newborns (7). Although the two streptococcal species S. agalactiae and S. pyogenes cause different diseases and express many different virulence factors, some surface proteins expressed by these pathogens are closely related (29). This situation prompted us to analyze whether S. agalactiae expresses an LRR protein related to Slr. Here, we describe such a protein, designated Blr (for group B, leucine rich), and compare it with the Slr protein of S. pyogenes. These studies demonstrated that Blr and Slr define a family of streptococcal LRR proteins that exhibit extensive amino acid residue identity but nevertheless show only weak immunological cross-reactivity when exposed on the bacterial surface. Remarkably, access of antibodies to Blr or Slr was strongly reduced by other surface components on bacteria grown under laboratory conditions. Access to Blr was inhibited by the polysaccharide capsule of S. agalactiae, and access to Slr was inhibited by M protein and
protein F of \textit{S. pyogenes}. These interactions are likely to affect the in vivo function of the streptococcal proteins and are of general interest for studies of bacterial surface proteins.

\textbf{MATERIALS AND METHODS}

\textbf{Bacterial strains, plasmids, and media.} \textit{S. agalactiae} BM110 is a capsular serotype III strain of a putative high-virulence clone (29, 35, 52). The \textit{S. agalactiae} type III strain COH1 and its isogenic acapsular mutant, COH1-13 (47), were from C. Rubens (Children's Hospital, Seattle, Wash.), and the \textit{S. agalactiae} type II strain 1954/92 was from R. Facklam (Centers for Disease Control and Prevention, Atlanta, Ga.). Strains of \textit{S. agalactiae} representing the nine known capsular serotypes were available in our laboratory. The \textit{S. pyogenes} M6 wild-type strain JRS4 and its M-negative derivative, JRS145, were from J. R. Scott (Emory University, Atlanta, Ga.) (5). The protein-F-negative JRS4 mutant SAM1 and the double mutant SAM2, which lacks both M6 and protein F, were from E. Hanski (Hebrew University, Jerusalem, Israel) (16). The \textit{S. pyogenes} M5 Manfredo strain (34) and its M-negative mutant, /H9004 M5, have been described previously (20). The encapsulated \textit{S. pyogenes} M18 strain 87-282 and its capsule-negative mutant, TX72, were from M. R. Wessels (Children's Hospital, Boston, Mass.) (63). All \textit{S. agalactiae} strains were grown in Todd-Hewitt broth (TH) at 37°C without shaking; all \textit{S. pyogenes} strains were grown in TH supplemented with 0.2% yeast extract (THY) in 5% CO\textsubscript{2} at 37°C without shaking.

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\caption{Analysis of the Blr protein of \textit{S. agalactiae}. (A) The Blr protein exhibits extensive residue identity to Slr of \textit{S. pyogenes} (46), and both of these predicted lipoproteins are related to \textit{L. monocytogenes} InlA, which is attached to the wall via an LPXTG motif. Amino acid residue identities of different regions are indicated in percent between the proteins. In all three proteins, the regions with LRRs are probably located distally to the bacterial surface. There are 12.5 LRRs in Blr, 10.5 LRRs in Slr, and 15 LRRs in InlA. The positions of histidine triad motifs (HXXHXXH) in the N-terminal part of Blr and Slr are indicated. R, repeat; P, partial repeat. (B) Amino acid sequence of the C-terminal part of the Blr protein, including the LRR domain. The conserved sequence of LRRs in \textit{L. monocytogenes} InlA is indicated in bold at the top, and the corresponding residues in the LRRs of Blr and Slr are highlighted in gray. In Blr, each LRR has a length of 22 residues, with the exception of the seventh repeat, which is one residue shorter. (C) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified recombinant Blr and Slr. Molecular weight markers (in thousands) are noted at the left of the gel.
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\end{figure*}
BumH and ligated to BumH-cleaved fik2α, generating plasmid pBR8⋅5. Finally, the whole insert in plasmid pH9262 was amplified by PCR, cleaved with SalI and XhoI (recognition sequences introduced through the primers), and ligated into SalI/XhoI-cleaved pBR8⋅5, generating plasmid pH9262. This construct was transformed into strain BM110, and the mutant (BM110 ΔAla-36) was recovered after homologous recombination (42). The structure of the mutant was verified by PCR. Analysis by reverse transcriptase-PCR demonstrated that the 36-bp mutation did not have a polar effect on transcription of the gene located downstream.

Construction of a Rib-negative S. pyogenes mutant. A Rib-negative mutant, designated Rn69, was derived from strain BM110. An insert harboring the rib gene and the flanking chromosomal regions upstream (−2.2 kb) and downstream (−2.0 kb) was recovered from a PC139 derivative (46, unpublished data) by cleavage with SalI and XhoI (recognition sequences introduced through the primers) and ligated into SalI/HindIII-cleaved pRS325, generating plasmid pTA06. Cleavage of plasmid pTA06 with BglII could be used to remove a large part of the central repeat region in rib because each repeat unit contains a BglII cleavage site (61). BumH-cleaved fik2α was therefore ligated into BglII-cleaved pTA06, generating plasmid pTA110. This plasmid was transformed into strain BM110, and a Rib-negative mutant was recovered after homologous recombination (42). The structure of the mutant was verified by PCR.

Construction of capsule-negative S. pyogenes mutants. Capsule-negative S. pyogenes mutants were derived from the wild-type strain BM110 and its Rib-negative mutant, Rn69. The construction of the mutants employed a pRS325 derivative, in which the chloramphenicol (Cat) resistance gene (cat) was surrounded by regions derived from the cpx operon of S. aureus scrtitoplast III (47). For construction of this plasmid, a region of the cpx operon (1,308 bp), including 233 bp of the 3′ end of ocfA, an intergenic region of 471 bp, and most of ocfD (104 bp), was amplified by PCR. The resulting PCR product was cleaved with SalI and XhoI (recognition sequences introduced through the primers) and ligated into SalI-cleaved pTA06. This construct was transformed into strain BM110, and a Rib-negative mutant was recovered after homologous recombination (42). The structure of the mutant was verified by PCR.

The wells of microtiter plates (Maxisorp; Nunc) were coated with protein by incubation overnight with 50 μg/ml of protein in SPE8⋅2l at room temperature for 1 h with gentle shaking, the samples were centrifuged and the bacteria were washed twice with PBSAT. To detect bound antibodies, Alexa Fluor 488-conjugated goat anti-rabbit immunoglobulin G (Molecular Probes) was added and the incubation was continued for 20 min with gentle shaking. After washes with PBSAT, the samples were analyzed by flow-cytometry and immunofluorescence microscopy. Fading of the fluorochrome was reduced by the use of a SlowFade light antisoot kit (Molecular Probes).

Analysis of the immune response in infected mice. C3H/HeN mice were injected i.p. with a sublethal dose (109 CFU/ml) of S. agalactiae serotype III capsule, conjugated to tetanus toxoid, was the kind gift of D. L. Kasper (Boston, Mass.). Antiserum to S. pyogenes type III capsule, conjugated to tetanus toxoid, was the kind gift of D. L. Kasper (Boston, Mass.).
of the strain indicated. The mice were sacrificed after 1 month, and sera were collected and assayed for the presence of anti-Blr antibodies. For this purpose, the wells of microtiter plates (Falcon 9612, Becton Dickinson) were coated overnight with Blr (4 µg/ml in PBS), washed with PBSAT, and blocked with the same buffer for 1 h. Following washing, mouse serum (50 µl, diluted as indicated in PBSAT) was added to each well, and the plates were incubated for 2 h. After the wells were washed with PBSAT, 10 µl rabbit anti-mouse immunoglobulin (2025H, diluted 2,000-fold in PBSAT, DakoCytomation) was added to each well and the plates were incubated for 1 h. After further washing, bound rabbit antibodies were detected with 125I-labeled protein G (4–10,000 cpm in 50 µl PBSAT). All incubations were performed at room temperature with gentle shaking, except the coating step, which was performed at 4°C without shaking.

Nucleotide sequence accession number. The GenBank accession number for the nucleotide sequence of the blr gene of strain BM110 is DQ242614.

RESULTS

Sequence comparison of Blr and Slr and purification of recombinant proteins. Through searches of genomic databases, we found that a homolog of the S. pyogenes slr gene was present in the two published genomes of S. agalactiae (12, 58), as also noted by Sutcliffe and Harrington (54). Preliminary work showed that this homolog, designated blr, was also present in S. agalactiae strain BM110, which was used for most of the work described here because it is a member of a putative high-virulence clone of the clinically important serotype III (29, 35). Sequencing of blr in BM110 and comparison with the blr genes in the two published S. agalactiae genomes demonstrated that the three genes encode predicted Blr proteins with 98% amino acid residue identity. In each strain, the blr gene encodes an 877-residue polypeptide (including signal sequence), with a predicted molecular weight of 97,590 in strain BM110 (Fig. 1A). The N-terminal part of Blr contains a predicted lipobox (residues 19 to 23), which is required for covalent attachment of a lipoprotein to the cell membrane (54, 55), while the C-terminal part, which includes 12.5 LRRs, is predicted to be located distally to the surface. Each LRR contains 22 amino acid residues, with the exception of the seventh repeat, which is one residue shorter.

The N-terminal parts of Blr and Slr show 40% residue identity, and the LRR regions show 62% identity (Fig. 1A). While Blr contains 12.5 LRRs, there are 10.5 LRRs in the Slr protein (46). As expected from the work with Slr (46), Blr shows sequence similarity to InlA, with 32% residue identity between the LRR regions. Because InlA is attached to the bacterial cell wall via a C-terminal LPXTG motif (4), the LRR region is predicted to be located distally to the bacterial surface in all three proteins, a location that may favor interactions with ligands. Interestingly, the LRRs of InlA, Blr, and Slr have the same length, 22 residues, except one LRR with a length of 21 residues. In InlA, it is the sixth repeat that only contains 21 residues (49). The N-terminal part of Blr includes eight histidine triad motifs (HIXHIXH), while four such motifs were found in Slr (Fig. 1A). The same motif, for which no function has been identified, has been described for several surface proteins of Staphylococcus pneumoniae (1, 64). Together, the similarities between Blr and Slr demonstrate that these two proteins are members of a family of streptococcal proteins with LRRs.

The gene encoding Slr was present in all strains of S. pyogenes analyzed by Reid et al. (46). To analyze different S. agalactiae strains for the presence of the blr gene, genomic DNA from 17 isolates, including at least one strain for each of the nine known capsular serotypes, was analyzed by PCR. The blr gene was present in all 17 isolates tested (data not shown), implying that most, if not all, strains of S. agalactiae harbor the blr gene.

Interestingly, Blr and Slr show homology not only to internalin A of L. monocytogenes but also to a putative internalin-related surface protein in Bacillus anthracis (44). In contrast, searches of databases did not reveal any predicted LRR proteins closely related to Blr or Slr in the gram-positive cocci Staphylococcus aureus, Streptococcus pneumoniae, and Enterococcus faecalis.

For purification of the Blr and Slr proteins, DNA fragments of blr and slr corresponding to amino acids 24 to 877 and 24 to 792, respectively, were cloned into the expression vector pGEX-4P-2. In these DNA fragments, the first 69 nucleotides, corresponding to the first 23 amino acids, were deliberately omitted to avoid potential toxicity due to the signal sequence and the lipobox (13). Each protein was overexpressed in E. coli BL21 and purified to apparent homogeneity (Fig. 1C), and antisera was raised in rabbits.

The Blr protein is located on the S. agalactiae surface but is camouflaged by the capsule. The presence of a putative lipobox in Blr and the report that the related Slr protein is exposed on the surface of S. pyogenes (46) suggested that Blr would be exposed on the surface of S. agalactiae. However, antibodies to Blr reacted only weakly with washed whole S. agalactiae bacteria (see below). This result was not due to low titer of the antiserum or release of the protein into the culture supernatant (data not shown).

We hypothesized that the weak reactivity of S. agalactiae bacteria with anti-Blr was not due to low-level expression of the protein but to camouflaging by other surface components. The capsule was of particular interest in this context, because it has been suggested that it may reduce the exposure of S. agalactiae surface proteins (57). Surface exposure of Blr in a capsule-negative BM110 mutant was therefore analyzed. Because BM110 and most other S. agalactiae serotype III strains express the major surface protein Rib (29, 52, 61), we also analyzed whether lack of this protein affected the ability of antibodies to detect Blr. Moreover, surface exposure of Blr was analyzed in a double mutant lacking both capsule and Rib.

The BM110 mutants were constructed as described in Materials and Methods. Characterization of the mutants with specific antibodies demonstrated that they had the expected properties (Fig. 2). Thus, the capsule-negative mutant (BM110-22) reacted with anti-Rib but not with antcapsule antibodies (Fig. 2B), the Rib-negative mutant (RM69) reacted only with antcapsule antibodies (Fig. 2C), and the double mutant (RM69-16) lacked reactivity with both antisera (Fig. 2D).

Analysis of surface exposure of Blr on the capsule- and Rib-negative mutants yielded striking results (Fig. 3A). Anti-Blr antibodies reacted well with the capsule-negative mutant, but to obtain similar reactivity with the wild-type strain, −100-fold more antibodies were required, indicating that −100-fold less Blr was exposed on the surface of the latter strain. Compared to the capsule-negative mutant, an additional −4-fold increase in Blr exposure was observed for the double mutant lacking both capsule and Rib. However, exposure of Blr was not increased with the mutant lacking only Rib. The strongly
increased reactivity of anti-Blr with the two strains lacking capsule was not due to exposure of a cross-reacting protein, because the anti-Blr serum is highly specific and detects only Blr in extracts of \textit{S. agalactiae} (see below). These data indicate that the capsule camouflages surface-localized Blr and also suggest that Rib contributes to the masking, at least in the absence of capsule. This result was not unique to strain BM110, because strongly increased reactivity with anti-Blr was also observed with a capsule-negative mutant of another \textit{S. agalactiae} strain, COH1 (47) (Fig. 3B).

To verify the results obtained in binding assays, in which radiolabeled protein A was used to detect bound antibodies, the surface exposure of Blr was also analyzed by immunofluorescence (Fig. 3C). The wild-type strain BM110 showed no reactivity with anti-Blr antibodies, while the acapsular mutant, BM110-22, reacted strongly with these antibodies. This reactivity was not specific, because the acapsular mutant did not react with preimmune serum. These data confirm that Blr is a surface protein, which is poorly accessible in the presence of the capsule.

The increased surface exposure of Blr with the capsule-negative mutants could be explained by camouflaging, but it could not be excluded that it reflected upregulation of Blr synthesis through an unknown mechanism. To analyze this possibility, we compared the total amount of Blr protein present in extracts of the wild-type strain BM110 and in the double mutant lacking capsule and Rib. Analysis of the extracts by Western blotting with anti-Blr as the probe indicated that the two extracts contained similar amounts of Blr (Fig. 3D). The protein detected in this blot was Blr and not a cross-reacting protein, because no signal was obtained for an extract prepared from a Blr-negative bacterial mutant (Fig. 3D, third lane). (See Materials and Methods for the construction of the Blr-negative mutant). These data indicate that the surface-localized Blr protein is indeed camouflaged by other cell wall components and in particular by the capsule.

To analyze whether the capsule causes camouflaging of surface proteins different from Blr, we used anti-Rib to compare the surface accessibilities of Rib in strain BM110 and its capsule-negative mutant (Fig. 3E). In contrast to Blr, exposure of Rib was not affected by the absence of capsule. This result does not exclude that the capsule camouflages other proteins but shows that it selectively camouflages Blr compared to Rib. Because Blr and Rib were detected by identical techniques, using rabbit antibodies, these data also show that the effect of the capsule on the binding of anti-Blr was specific.

The Slr protein of \textit{S. pyogenes} is also camouflaged: roles of M protein and protein F. The camouflaging of Blr in \textit{S. agalactiae} suggested that a similar situation might prevail for Slr in \textit{S. pyogenes}. Indeed, preliminary tests indicated that anti-Slr an-

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**FIG. 2.** Characterization of mutants of \textit{S. agalactiae} strain BM110 that lack polysaccharide capsule and/or protein Rib. (A) Binding of anticapsular and anti-Rib antibodies to the BM110 wild type (wt). A series of identical bacterial samples were mixed with antisera and diluted as indicated, and bound antibodies were detected by the addition of a standard amount of radiolabeled protein A, as described in Materials and Methods. Binding is presented as percentage of protein A added, explaining why binding may reach a maximal level at lower antibody dilutions and decreases at higher dilutions. (B, C, and D) Binding of anticapsular and anti-Rib antibodies to the BM110 mutants indicated. The capsule-negative mutant (Cap\(^{-}\)) is strain BM110-22, the Rib-negative mutant is strain Rm69, and the double mutant is strain Rm69-16. All experiments were performed three times with triplicate samples, and each panel shows representative data from one experiment. Binding observed with preimmune serum (<8%) has been subtracted.

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tibodies reacted only weakly with *S. pyogenes* wild-type bacteria. Because of the dramatic effect of the capsule in *S. agalactiae*, it seemed possible that the hyaluronic acid capsule that is expressed by at least some strains of *S. pyogenes* (62) might affect surface exposure of Slr. This hypothesis was analyzed with a capsule-producing (mucoid) strain of the clinically important serotype M18 and a capsule-negative mutant of that strain (63). Interestingly, absence of the hyaluronic acid capsule had no effect on surface exposure of Slr, as judged by the ability of antibodies to detect Slr (data not shown). This result suggests that the capsules of *S. agalactiae* and *S. pyogenes* differ in ability to mask the Blr and Slr proteins, respectively. Of note, the capsules of these two bacterial species are biochemically unrelated.

Although the hyaluronic acid capsule did not affect surface exposure of Slr, it seemed possible that Slr might be camouflaged by other surface components. Analysis of this problem was focused on two *S. pyogenes* surface proteins, the antiphagocytic M protein and the fibronectin-binding protein F (also known as Sfb1) (9, 16, 56). These two proteins were chosen for study because they are encoded in complex loci that play important parts in virulence, the *emm* and FCT loci, respectively, and because M protein, in particular, is a major component of the *S. pyogenes* cell envelope (3, 9, 36). The roles of M protein and protein F for surface exposure of Slr were studied with the M6 system, in which single mutants, lacking either protein, and a double mutant were available (5, 16). Surface exposure of Slr was increased more than 20-fold on all three strains and was even higher on the protein-F-negative mutant than on the other two mutant strains (Fig. 4A). Thus, absence of both proteins did not have a cumulative effect on the surface exposure of Slr. The detection of increased amounts of Slr on the mutants was not due to increased synthesis of the protein, because extracts of wild-type bacteria and of the mutants contained similar amounts of Slr, as demonstrated by Western blot analysis (data not shown). These results indicate that both M protein and protein F are required for camouflaging of Slr in the M6 strain of *S. pyogenes*. This result was not unique to the M6 system, because strongly increased exposure of Slr was also observed for an M-negative mutant derived from an M5 strain (Fig. 4B).

**FIG. 3.** The capsule of *S. agalactiae* inhibits binding of antibodies to Blr but not to protein Rib. (A) Binding of anti-Blr antibodies to *S. agalactiae* strain BM110 wild type (wt) and mutants of this strain. For each bacterial strain, a series of identical samples were mixed with antiserum and diluted as indicated, and bound antibodies were detected as described in Materials and Methods. The mutants used were those described in the legend to Fig. 2. (B) Binding of anti-Blr antibodies to *S. agalactiae* strain COH1 and its acapsular mutant, COH1-13 (Cap−). (C) Binding of anti-Blr antibodies to *S. agalactiae* wt strain BM110 and its acapsular mutant, BM110-22 (Cap−), analyzed by immunofluorescence. A control with preimmune (preimm) serum is shown for BM110-22. Bound antibodies were detected as described in Materials and Methods, and representative images from one experiment are presented. For each analysis, the same field is shown in the phase-contrast panel (Phase) and in the immunofluorescence panel (IF). (D) Western blot analysis of bacterial extracts, using anti-Blr as the probe. The extracts were prepared from *S. agalactiae* BM110 wild type, its Rib− and capsule-negative double mutant (Rm69-16), and the Blr-negative BM110 mutant, Δblr-36. Molecular weight markers (in thousands) are noted at the left of blots. (E) Lack of capsule does not affect surface exposure of protein Rib. Anti-Rib antibodies, diluted as indicated, were incubated with BM110 wild type and its acapsular mutant, BM110-22 (Cap−). This panel was derived from data presented in Fig. 2A and B. The binding experiments shown in panels A, B, and E were performed three times with triplicate samples, and each panel represents data from one experiment. Binding observed with preimmune serum (<8%) has been subtracted.
Immunological cross-reactivty between Blr and Slr. Because Blr and Slr exhibit extensive residue identity, it was of interest to analyze possible immunological cross-reactivity. It was not obvious that these two proteins would cross-react, as shown by analysis of the two S. agalactiae surface proteins Rib and α, which exhibit ~50% residue identity but show little or no immunological cross-reactivity (52, 81).

For examination of the immunological relationship between pure Blr and Slr, the proteins were immobilized in microtiter wells and analyzed for reactivity with anti-Blr and anti-Slr, using antisera that had the same titer against the homologous antigen (Fig. 5A and B). Both antisera reacted well not only with the homologous antigen but also with the heterologous protein, although the titer was highest against the homologous antigen. The greatest difference was observed for anti-Blr, but the reactivity of this antiserum was only ~4-fold lower against the heterologous antigen. Thus, Blr was not a virulence factor in this model. However, Blr was immunogenic during the course of an infection, as also reported for Slr (28, 46). This conclusion is based on analysis of antisera from mice infected with sublethal doses of different S. agalactiae strains. Mice infected with the type II strain BM110 and the type I strain 1954/92 produced antibodies to Blr (Fig. 6B, left and middle panels), while no antibodies were found in control mice infected with the Blr-negative mutant of BM110 (Fig. 6B, right panel).

DISCUSSION

Proteins containing LRRs are common among both prokaryotes and eukaryotes. Their functions are diverse, ranging from RNase inhibition to lipopolysaccharide binding, but the common theme is believed to be involvement in different ligand recognitions (23, 25). The crystal structure of an LRR protein was first determined for porcine RNase inhibitor (22), which has a horseshoe-shaped structure in which the concave side is responsible for binding of the ligand, RNase A (24). A number of subsequent structural studies of other LRR proteins have shown great overall similarity to that of the RNase inhibitor. The remarkable ability of LRR proteins to specifically bind different ligands is underlined by the recent identification, in jawless vertebrates, of variable lymphocyte receptors composed of highly diverse LRRs (38, 39).

Here, we have characterized and compared two LRR proteins expressed by important human pathogens, the Blr protein of S. pyogenes and the previously described Slr protein of S. agalactiae (46). Together, these proteins define a family of streptococcal proteins with LRRs. Importantly, the genes for Blr and Slr are present in most, if not all, strains of S. pyogenes and S. agalactiae, respectively, making them of general interest for analysis of streptococcal pathogenesis. The Blr and Slr proteins are predicted to be lipoproteins and to be present on both bacterial cell surfaces and in the extracellular milieu.

FIG. 4. Two surface proteins of S. pyogenes, M protein and protein F, inhibit binding of antibodies to Slr. (A) Mutants of S. pyogenes strain JR54 (of serotype M3) were tested for binding of anti-Slr. M refers to the M-negative strain JR54. F refers to the protein F-negative strain JSAM1, and M' F' refers to the double mutant SAM2. (B) Binding of anti-Slr to S. pyogenes strain M5 Manfredo and its M-negative mutant, M5 (M'). Each experiment was performed three times with triplicate samples, and representative data from one experiment are shown. Binding observed with preimmune serum (<9%) has been subtracted. wt, wild type.
by other components of the cell envelope, as judged by the poor binding of anti-Blr and anti-Slr antibodies to wild-type bacteria. In *S. agalactiae*, this effect was mediated by the capsule and, to some extent, the major surface protein Rib, while M protein and protein F caused camouflaging of Slr in *S. pyogenes*. The demonstration that these surface components mask Blr or Slr does not exclude that other wall components not studied here may also be important for the camouflaging.

The LRR regions of Blr and Slr show considerable residue identity with the LRR region of *L. monocytogenes* InlA, suggesting that these three surface proteins may have related but not necessarily identical functions. Some support for this hypothesis comes from the fact that the repeats of the three proteins not only exhibit sequence similarity but have similar overall arrangements. In particular, the LRRs have the same length, 22 residues, in each of the three proteins, except for

FIG. 5. Analysis of immunological cross-reactivity between Blr and Slr. (A and B) Cross-reactivity of purified proteins. Microtiter wells were coated with Blr (coat Blr) or Slr (coat Slr), and binding of anti-Blr and anti-Slr, diluted as indicated, was analyzed by ELISA. The two antisera were adjusted to have the same titer against the homologous antigen. Premimmune serum was used as a control. (C and D) Cross-reactivity between Blr and Slr expressed on the surface of bacteria. The analysis in panel C employed the *S. agalactiae* strain Rm69-16, which lacks both capsule and Rib, and the analysis in panel D employed *S. pyogenes* strain SAM2, which lacks both M protein and protein F. For each bacterial strain, a series of identical samples were mixed with antisera and diluted as indicated, and bound antibodies were detected as described in Materials and Methods. Premimmune serum was used as a control. Each experiment was performed three times with triplicate samples, and each panel shows representative data from one experiment.

FIG. 6. Blr does not affect virulence in an i.p. infection model but is immunogenic in vivo. (A) Mice in one group were infected i.p. with a 90% lethal dose of *S. agalactiae* strain BM110, and mice in another group were infected with the same number of bacteria of the nonpolar Blr-negative (Blr<sup>−</sup>) BM110 mutant, Δblr<sup>−</sup>. Each group comprised 15 mice. Deaths were recorded regularly for 96 h. (B) Immune response to Blr in mice infected with a sublethal dose of *S. agalactiae*. An immune response was detected in mice infected with the Blr-expressing strains BM110 (serotype III) and 1954/92 (serotype II) but not in control mice infected with the Blr-negative BM110 mutant, Δblr<sup>−</sup>.
when antibodies are used to analyze surface expression on surface structures may cause that protein to go undetected exposure of Slr may be enhanced during certain stages of an environmental control (5, 31, 59), suggesting that surface extracellular proteins in S. pyogenes complicated in adhesion (17). Interestingly, expression of M protein may reduce the accessibility of a surface protein in E. coli.

S. pyogenes capsules in regulating the exposure of surface proteins, the studies with different bacterial systems focus interest on bacterial membrane, and not LPXTG proteins like Rib, which are attached to the bacterial cell wall, lipoproteins, which are attached to the bacterial cell wall via an LPXTG motif. Possibly, camouflaging by the capsule selectively affects lipoproteins, which are attached to the bacterial cell membrane, and not LPXTG proteins like Rib, which are attached to the peptidoglycan layer.

While our studies with S. agalactiae and the published studies with different bacterial systems focus interest on bacterial capsules in regulating the exposure of surface proteins, the ability of other surface proteins to camouflage Slr in S. pyogenes was more surprising. The mechanisms involved remain unclear, but there is precedence from E. coli that protein fimbriae may reduce the accessibility of a surface protein implicated in adhesion (17). Interestingly, expression of M protein and protein F in S. pyogenes is known to be subject to environmental control (5, 31, 59), suggesting that surface exposure of Slr may be enhanced during certain stages of an infection.

Importantly, the camouflage of a surface protein by other surface structures may cause that protein to go undetected when antibodies are used to analyze surface expression on wild-type bacteria grown in vitro. Indeed, analysis of an S. agalactiae type V strain with antibodies to a large number of putative surface proteins led to the conclusion that many of these proteins, including Blr, were not surface exposed (58). However, it now seems possible that not only Blr but also other proteins were camouflaged by the capsule of that strain. Thus, it will be of interest to include a capsule-negative mutant in future studies of putative S. agalactiae surface proteins. Similarly, it will be of interest to study surface exposure of S. pyogenes proteins not only with wild-type bacteria but also with mutants lacking M protein and/or protein F. Camouflaging may also be of relevance to the recent report that two surface proteins of S. pneumoniae, PsaA and PypA, were poorly accessible to antibodies (14).

A high degree of cross-reactivity was seen when the purified Blr and Slr proteins were analyzed by ELISA, but the cross-reactivity was unexpectedly found to be very limited when the two proteins were expressed on the surface of bacteria, i.e., under more physiological conditions. Thus, the epitopes that exhibited cross-reactivity in ELISA, when the proteins were exposed on the surface of microtiter wells, were largely inaccessible on the bacterial surface. The low cross-reactivity of the surface-exposed parts (which most likely include the LRR regions) may reflect structural differences that have been selected for during evolution, either because the surface-exposed parts have different functions or because they have evolved to escape cross-reacting antibodies. This result stresses the importance of analyzing cross-reactivity under physiological conditions.

A recent study described the S. agalactiae protein LrrG, which was reported to have LRR-like repeats and was predicted to be attached to the bacterial cell wall via an LPXTG motif (50). This protein is not related to the Blr and Slr proteins studied here, and the repeats in LrrG lack the classical LRR consensus sequence LXXLXXNXL. However, LrrG is related to a group of proteins with repeats designated TpLRR, which have been identified for several bacterial pathogens (21, 51). Of note, the LrrG protein is one of the proteins which Tettelin et al. (58) concluded is not exposed on the surface of S. agalactiae, but LrrG was nevertheless reported to be a target for antibodies that protect against S. agalactiae infection (50). Possibly, LrrG is exposed on the bacterial surface during certain stages of an infection but camouflaged by the capsule on bacteria grown in vitro.

In summary, our data show that the Blr protein of S. agalactiae and the previously described Str protein of S. pyogenes (46) are members of a family of streptococcal LRR proteins that exhibit extensive residue identity but are immunologically diverse. These proteins are associated with the bacterial surface, but on bacteria grown under standard conditions, Blr and Slr are largely camouflaged by other surface components. These interactions provide evidence for a heretofore-unexpected complexity in the relationship between different streptococcal surface structures. It will be of interest to analyze to what extent Blr and Slr are exposed during infections, to evaluate them as possible vaccine components, and to analyze whether they act as adhesins, like the InlA protein of L. monocytogenes.
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