Surface-Expressed Mig Protein Protects *Streptococcus dysgalactiae* against Phagocytosis by Bovine Neutrophils†

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The *mig* gene of *Streptococcus dysgalactiae*, a major bovine mastitis pathogen, encodes two plasma protein-binding receptors, α₂-macroglobulin (α₂-M) and immunoglobulin G (IgG). In this study, the *mig* gene from one *S. dysgalactiae* isolate was cloned and expressed in *Escherichia coli*. The IgG receptor region encoded by *mig* was conserved in 16 *S. dysgalactiae* strains. An isogenic *mig* mutant was constructed by allele replacement mutagenesis of the wild-type gene in *S. dysgalactiae*. The IgG-binding activity was lost in the *mig* mutant strain, whereas the α₂-M receptor activity was still expressed but was detected only in the culture supernatant. In flow cytometry phagocytosis and bacterial-colony-counting bactericidal assays, the wild-type strain was found to be significantly more resistant to phagocytosis and killing by bovine neutrophils (PMNs) than the *mig* mutant strain when bacteria were preincubated with bovine serum. We therefore speculate that the Mig protein of *S. dysgalactiae* plays a role in virulence of the bacteria by binding to the plasma protein α₂-M or IgG and thus preventing phagocytosis by bovine PMNs.

The Lancefield serological group C bacterium *Streptococcus dysgalactiae* is one of the most common pathogens of bovine mastitis and causes large economic losses in the dairy industry. It is capable of survival in the mouth, vagina, and skin of healthy animals as well as bedding and pastures (32). Because of its environmental location, normal hygiene methods and antibiotic therapy are less effective in preventing *S. dysgalactiae* infections than infections with other contagious pathogens. Therefore, an effective way to prevent *S. dysgalactiae* mastitis might be to identify conserved potential virulence factors expressed on the cell surface as targets for vaccines.

*S. dysgalactiae* expresses various receptors on its cell surface that bind to host-derived proteins such as immunoglobulin G (IgG), α₂-macroglobulin (α₂-M), albumin, fibronectin, fibrinogen, collagen, vitronectin, and plasminogen (5, 23, 30). These receptors mediate the interaction between the host and the bacterium, and therefore they might be involved in the adhesion or invasion to the host cells or in resistance to the host defense system. Two of these receptors, IgG and α₂-M, have been identified in a surface-expressed protein, designated Mig (12). The IgG receptor expressed by Mig belongs to the type III IgG-binding receptor family, and its sequence shares homology with other type III IgG receptors. However, in contrast to the two or three repeated domains in the extensively studied protein G in the human group C and G streptococci (2, 25, 27), the IgG-binding region of Mig contains five repeats and it binds goat IgG via both the Fc and Fab domains (31). Although the role of the IgG receptor of Mig in *S. dysgalactiae* virulence is unclear, the IgG receptor of the group A streptococcus (GAS) strains has been found to be involved in virulence in a mouse skin infection model (21).

The other receptor present in the Mig protein binds to the universal protease inhibitor α₂-M, but only to the complex form (fast form) of α₂-M, the α₂-M–trypsin complex (α₂-M–T). This is in contrast to the α₂-M receptor in GAS, which binds only to the native form (slow form) of α₂-M (1, 16). The DNA sequence encoding the α₂-M receptor portion of the *mig* gene is different from other streptococci surface-expressed α₂-M receptors, such as Mag in *S. dysgalactiae* (10), Zag in *Streptococcus zooepidemicus* (11), and protein G from human group G streptococci (17, 28). Recently, a novel α₂-M receptor, carried by the protein G-related α₂-M-binding protein (Grab) from human GAS strains was found to be more virulent than the Grab–mutant in a mouse infection model (24). Furthermore, the α₂-M bound to the bacterial surface via Grab was still capable of inhibiting the activity of proteases, thereby protecting important virulence factors from proteolytic degradation (24). Another role for the α₂-M receptor was found in *S. dysgalactiae*, where the binding of α₂-M–T to *S. dysgalactiae* cells interferes with phagocytosis by bovine neutrophils (PMNs), but the specific α₂-M receptor was not identified in that study (29).

In this study, the degree of conservation of DNA regions encoding the α₂-M- and IgG-binding regions of Mig was assessed by Southern blot analyses of genomic DNA from several *S. dysgalactiae* isolates. In addition, a *mig* mutant strain was constructed by allele replacement mutagenesis in *S. dysgalactiae*, and its ability to resist phagocytosis and killing by bovine PMNs was investigated in a parallel analysis with the wild-type strain. We report here that the IgG receptor region encoded by *mig* was conserved in 16 *S. dysgalactiae* strains, while the *mig* α₂-M region was present in 5 strains only. Furthermore, we found that the wild-type strain was more resistant to the phagocytosis and killing by bovine PMNs than the *mig* mutant strain in the presence of serum. This mechanism of resistance to phagocytosis is probably mediated by the binding of α₂-M–T to the α₂-M receptor and not to binding of IgG to the IgG receptor of Mig.

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Bacterial strains and media. The Lancelot field C S. dysgalactiae isolates ATCC 43078, renamed SDG8 in this study, and ATCC 27957 were obtained from the American Type Culture Collection. Other S. dysgalactiae strains isolated from milk of cows with mastitis were kindly provided by M. Chirino-Trejo, University of Saskatchewan, and by Agriculture Development and Marketing, Winnipeg, Manitoba, Canada. The clinical isolates were identified by the API 20 Strep diagnostic kit (BioMeierux, Quebec, Canada) and analyzed by amplified DNA Plus software provided by the same supplier. Escherichia coli strain DH5α [888locZam15 recA1 endA1 gyrA96 thi-1 hsdR17 (rK-mac) zymA arf(U169)] and cloning vectors pBluescript II KS, pSP-CR Script, and pAA505 were from laboratory collections. The temperature-sensitive shuttle vector pEU904 was a generous gift from June R. Scott, Emory University, Atlanta, Ga.

The S. dysgalactiae strains were grown either in Todd-Hewitt culture medium (Oxoid Ltd., Basingthoke, Hampshire, England) supplemented with 0.5% yeast extract (THY) or on sheep blood agar at 37°C with 5% CO2. The E. coli strains were cultured in Luria-Bertani medium. Antibiotics were added to the following concentrations when required: ampicillin, 50 μg/ml; and erythromycin (EM), 1 μg/ml; and spectinomycin (SP), 200 μg/ml; and erythromycin (EM), 1 μg/ml.

DNA preparations. Plasmid DNA was prepared with the Qiagen plasmid kit (Qiagen GmbH, Hilden, Germany). S. dysgalactiae genomic DNA was prepared by a modification of the method provided by Qiagen (Qiagen genomic DNA handbook). Briefly, bacteria grown in 50 ml of THY were harvested by centrifugation and then washed once in 0.1 M phosphate-buffered saline buffer (PBS), pH 7.2. The bacterial pellets were suspended in 11 ml of buffer B1 (50 mM Tris pH 7.2, 0.1% SDS at 37°C, and 1 min at 72°C with an initial denaturation step of 3 min at 95°C and a final extension of 5 min at 72°C). SDS-PAGE and Western blots. Proteins were analyzed by SDSPAGE as described previously (15). The purified IgG samples were analyzed on gels with β-mercaptoethanol excluded from the gel-loading buffer. Gels were either stained with Coomassie brilliant blue or transferred onto nitrocellulose membranes (Bio-Rad). After blocking with PBS-T buffer (PBS-0.05% Tween 20), the membranes were either incubated with rabbit anti-IgG polyclonal antibodies at a dilution of 1:1,000 and followed with AP-

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequences (5’ to 3’)</th>
<th>Restriction site</th>
<th>Position relative to the mig start codon</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mig-1</td>
<td>gcGCGccatgTTAGAAATAACTATAACTG</td>
<td>NcoI</td>
<td>81 to 108</td>
<td>Stop codon region</td>
</tr>
<tr>
<td>Mig-2</td>
<td>acggcgggTATGGTTCTTCTTATGTTT</td>
<td>Smal</td>
<td>2018 to 1992</td>
<td>Membrane-spanning region</td>
</tr>
<tr>
<td>Mig-3</td>
<td>GATTGCCCTCTAACAGAATATCTGA</td>
<td>EcoRV</td>
<td>147 to 173</td>
<td>α-M-binding region</td>
</tr>
<tr>
<td>Mig-4</td>
<td>AAAAGCACCggGCCGACGTACTGCTT</td>
<td>Smal</td>
<td>1978 to 1954</td>
<td>Membrane-spanning region</td>
</tr>
<tr>
<td>Mig-7</td>
<td>GGGGAGGAGTcgaGAAACTAAGGCAGAAAAC</td>
<td>XbaI</td>
<td>–446 to –417</td>
<td>mig upstream region</td>
</tr>
<tr>
<td>Mig-8</td>
<td>AGCATCCAGGGCACTGAGTAACTCTTTC</td>
<td>BamHI</td>
<td>1699 to 1671</td>
<td>Cell wall-spanning region</td>
</tr>
<tr>
<td>Mig-9</td>
<td>CAGGCCGCTTGGCTACGTTGCTAGTT</td>
<td>Ndel</td>
<td>606 to 634</td>
<td>IgG-binding region</td>
</tr>
<tr>
<td>Mig-12</td>
<td>GCCTGAAGTGGATCCCTCAGTACTGCTG</td>
<td>BamHI</td>
<td>620 to 583</td>
<td>α-M-binding region</td>
</tr>
<tr>
<td>Mig-13</td>
<td>TCTGGCTgaaTCTCTGCAACTTCAATTGT</td>
<td>BamHI</td>
<td>1740 to 1767</td>
<td>Cell wall-spanning region</td>
</tr>
<tr>
<td>Mig-14</td>
<td>taaGTCAAAGAACgtTGAACATTGCTCTT</td>
<td>HindIII</td>
<td>2029 to 2002</td>
<td>mig downstream region</td>
</tr>
</tbody>
</table>

**a** Upper-strand and lower-strand primers are indicated with odd and even numbers, respectively.

**b** Lowercase letters indicate nucleotides substituted for creation of restriction sites or as a codon.

**c** A minus sign indicates a position upstream of the mig gene.

**MATERIALS AND METHODS**

**TABLE 1. Oligonucleotides used in this study**

**Primers**

- **Mig-1**: cGCAGCTTGCAGGCTTATCGATCTG
- **Mig-2**: acggcgggTATGGTTCTTCTTATGTTT
- **Mig-3**: GATTGCCCTCTAACAGAATATCTGA
- **Mig-4**: AAAAGCACCggGCCGACGTACTGCTT
- **Mig-7**: GGGGAGGAGTcgaGAAACTAAGGCAGAAAAC
- **Mig-8**: AGCATCCAGGGCACTGAGTAACTCTTTC
- **Mig-9**: CAGGCCGCTTGGCTACGTTGCTAGTT
- **Mig-12**: GCCTGAAGTGGATCCCTCAGTACTGCTG
- **Mig-13**: TCTGGCTgaaTCTCTGCAACTTCAATTGT
- **Mig-14**: taaGTCAAAGAACgtTGAACATTGCTCTT

**Description**

- **NcoI**: 81 to 108 Signal sequence cleavage region
- **Smal**: 2018 to 1992 Stop codon region
- **EcoRV**: 147 to 173 α-M-binding region
- **XbaI**: –446 to –417 mig upstream region
- **BamHI**: 1699 to 1671 Cell wall-spanning region
- **Ndel**: 606 to 634 IgG-binding region
- **BamHI**: 620 to 583 α-M-binding region
- **BamHI**: 1740 to 1767 Cell wall-spanning region
- **HindIII**: 2029 to 2002 mig downstream region
prepared with a HiTrap Protein G column (Pharmacia Biotech) from 1.6 ml of bovine serum samples according to the column supplier recommendations. Bovine IgG samples were examined, an AP-conjugated goat anti-bovine IgG (heavy plus light chains; Kirkegaard & Perry Laboratories, Gaithersburg, Md.) diluted at 1:500 was used. The membranes were developed in AP buffer (100 mM NaCl; 5 mM MgCl₂; 100 mM Tris · HCL, pH 9.5) supplemented with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyolphosphate. The concentration of the protein samples was determined on a microtiter plate with a DC protein assay kit (Bio-Rad). Erythrocytes were lysed with a lysis solution (168 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM tetrasodium EDTA), and the PMNs were washed twice in PBS-T or incubated with same amount of Mig with incomplete Freund’s adjuvant. Three weeks later, a second injection with same amount of Mig with incomplete Freund’s adjuvant was administered. The rabbits were humanely euthanized at 14 days after the boost injection, and serum samples were collected.

Determination of DNA sequences. The nucleotide sequences of both strands of the mig gene in S. dysgalactiae strain SDG8 were determined on an ABI 373 DNA automatic sequencer (Applied Biosystems) at the Plant Biotechnology Institute (National Research Council, Saskatoon, Canada) by using multiple primers (Table 1). The sequence data were analyzed with the Genetics Computer Group software provided by The Canadian Bioinformatics Resource.

Preparation of bovine PMNs. Whole blood from clinically normal 5- to 7-year-old dairy cows was collected in EDTA tubes. PMNs were prepared according to the method provided by Becton Dickinson Immunocytometry Systems (Mountain View, Calif.). Erythrocytes were lysed by a lysis solution (168 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM tetrasodium EDTA), and the PMNs were washed twice with 1× Hanks’ balanced salt solution before being suspended in 1× minimum essential medium without antibiotics. Prior to the assay, the viability and the number of PMNs were determined in a hemocytometer under a light microscope by using trypan blue dye (Gibco BRL, Life Technologies, Grand Island, N.Y.) exclusion test.

Fluorescence labeling and opsonization of bacteria. PKH2 fluorescent dye (Sigma) was used to label S. dysgalactiae strains for the phagocytosis assays, modified from a previous report (8). Briefly, 6 ml of logarithmic-phase bacterial culture was washed once in PBS and suspended in 0.5 ml of labeling buffer (Sigma) in a polypropylene centrifuge tube. An aliquot of this suspension (0.2 ml) was diluted in 1 ml of labeling buffer and mixed with 1 ml of the same buffer containing 10 μl of the PKH2 dye. The reaction mixture (total volume, 2.210 ml) was incubated for 10 min at room temperature protected from the light. After two washes with PBS-0.5% bovine serum albumin (BSA) (fraction V; Boehr-
inger Mannheim, Mannheim, Germany), the labeled bacteria were suspended in 0.15 ml of PBS-0.5% BSA.

The bacterial osmoporation or serum treatment was performed by incubating mixtures of 100 μl of labeled bacteria and either 50 μl of a pool of heat-inactivated bovine sera (obtained from cows that had recovered from S. dysgalactiae mastitis) or 50 μl of purified IgG from the same bovine serum pool at various concentrations for 15 min at 37°C. The bacteria were then washed twice with 10 ml of PBS and suspended in 0.45 ml of Ca2+- and Mg2+-free Dulbecco’s PBS containing 5 mM glucose and 0.1% gelatin (PBSg). The viability of bacteria in each labeling samples was determined by plating 10-fold dilutions on THY.

Flow cytometry (FC)-based phagocytosis. Equal volumes (100 μl) of serum-treated or nontreated bacteria were mixed with bovine PMNs in a 96-well U-bottom microtiter plate (Nunclon surface; Nunc, Roskilde, Denmark) and incubated at 37°C for 45 min with gentle shaking in the dark. The reaction was stopped by the addition of 20 μl of 0.3 M EDTA. After two washes with 150 μl of PBSg containing 10 μg of gentamicin per ml (Gibco BRL), the PMNs were suspended in the same solution and incubated for 30 min at 37°C. Finally, the PMNs were washed twice with 150 μl of PBSg and suspended in 100 μl of ice-cold PBSg containing 2% formalin before the analysis. A FC assay was performed on a FACScan flow cytometer (Becton Dickinson, Mississauga, Ontario, Canada) with a 15-MW argon laser light source. Five thousand PMNs were counted for each sample, and cell populations were selected by gating according to their granularity and florescence.

Bactericidal assay. Killing of bacteria by bovine PMNs was measured by a viability assay modified from a previously report (20). Exponential-phase bacteria were washed once in PBS, suspended in Hanks’ balanced salt solution, and incubated in the presence or absence of bovine serum. Equal volumes (100 μl) of bacteria and bovine PMNs were mixed in an Eppendorf tube, and the mixtures were incubated at 37°C with end-to-end mixing. At the required incubation time points (0, 1, and 4 h), 50 μl of the reaction mixtures was transferred to a 96-well microtiter plate well containing 25 μl of 2% saponin (Sigma) in PBS. After incubation at room temperature for 10 min, the samples were diluted up to 1,000-fold in PBS, and three serial dilutions (50 μl of each sample) were plated on THY plates in duplicate. Prior to the counting of CFU, the agar plates were incubated for 16 h at 37°C with 5% CO2. The CFU count at time zero was used to calculate the initial ratio of bacteria to PMNs. The killing of bacteria by PMNs was calculated as the bacterial survival rate, measured as the CFU at 1 and 4 h relative to the CFU at time zero.

Statistical analysis. The P values of the phagocytosis and bactericidal analyses were obtained from paired t-test analysis (two tailed) with Excel (Microsoft) and Prism (GraphPad Software) software.

Nucleotide sequence accession number. The nucleotide sequence of the mig gene in S. dysgalactiae strain SDG8 has been deposited in GenBank under the accession number AF354651.

RESULTS

Molecular cloning and sequencing of the mig gene. The mig coding sequence was obtained from the plasmid p5Me, from which a mature Mig protein was expressed. The upstream sequence was determined from the plasmids pKSMig-3 and pPMig2-8, both carrying the same PCR product but in different vectors, while the 3’-end sequence was obtained from pMC-5e, carrying a DNA region spanning the mig stop codon (Fig. 1).

Assembled, the sequence revealed an open reading frame of 2,007 bp and 669 deduced amino acids with a molecular mass of 72,681 Da and a pl of 4.49. Except for a 15-bp extra sequence at the cell wall-spanning region, the sequence of the mig coding region of SDG8 was highly homologous to the mig gene of S. dysgalactiae SC1 (12), sharing 99 and 98% identity at the nucleotide and amino acid levels, respectively (data not shown).

A BLAST search revealed four proteins sharing overall sequence homology to the SDG8 Mig protein. They were protein G from human group G streptococcus (61% identity [27]), Mag from S. dysgalactiae (54% identity [10]), Zag from S. zooepidemicus (48% identity [11]), and Grab from Streptococcus pyogenes (31% identity [24]). Except for Grab, which has only one α2-M receptor related to protein G, all the other proteins express multiple receptors binding to α2-M, IgG, or albumin. When analyzed by regions, the homology between Mig and the other proteins was higher in the IgG-binding region (sequence identity with Mag, protein G, and Zag, 99, 83, and 71%, respectively). In contrast, the sequence encoding the α2-M-binding domain of Mig was less conserved, with identities between 25 and 30%.

Construction and characterization of the mig-mutant strain. Recent work suggests a role in virulence for the α2-M-binding region of the GAS and group C streptococcus surface proteins (see Discussion). We attempted the construction of an isogenic mutant lacking only the Mig α2-M-binding region with no success. However, we were able to obtain a mutant in which an antibiotic-resistant cassette replaced sequences downstream of the α2-M region. Briefly, the mig-internal 420-bp ClaI fragment present in p5Me was replaced with a blunted EcoRI-ClaI fragment containing an Sp cassette to generate p5Me-Sp (Fig. 1). The mig-Sp insert was cloned into a temperature-sensitive suicide vector, and this construct was named pMig-1 (Fig. 1). For allele replacement mutagenesis, pMig-1 was transformed into S. dysgalactiae and selected for single crossover in the presence of EM at 30°C. The strain carrying the plasmid was incubated at 37°C and plated on SP. Bacteria in which the double crossover between homologous plasmid and chromosomal sequences had occurred were selected from colonies resistant to SP but sensitive to EM. One such isolate, Mig8-Mt, was selected for further analysis.

To identify and characterize the constructed mig mutant strain, PCR amplifications were carried out using the primers Mig3 and Mig-4, which anneal to the sequences flanking the Sp cassette insertion site (Fig. 1). PCR products of 1.8 and 2.5 kb were obtained from respective genomic DNAs of strains SDG8 and Mig8-Mt (data not shown). The 0.7-kb difference in size between the two strains results from the insertion of the 1.2-kb Sp cassette, minus the 420 bp of the ClaI fragment within the mig coding region deleted during the mig mutant construction (Fig. 1). In Southern blot analysis, genomic DNAs of the two strains were cleaved with HindIII and probed with a total of four specific probes (see Materials and Methods). As expected, genomic DNA of SDG8 did not hybridize to the Sp probe, whereas strain Mig8-Mt showed a 2.4-kb fragment homologous to the Sp probe (Fig. 2B), suggesting that the Sp cassette has been inserted into the wild-type strain. Neither genomic DNA showed homology to the Em probe (data not shown), indicating that in the case of the Mig8-Mt strain a double cross between homologous sequences present on the plasmid pMig-1 and on the SDG8 chromosome had occurred. When the α2-M-1 probe was used, 2.5- and 2.4-kb HindIII fragments were detected in the SDG8 and Mig8-Mt genomic DNAs, respectively (Fig. 2A). The smaller fragment in the mutant results from the introduction of an extra HindIII site close to the 3’ end of the Sp cassette (Fig. 1). The HindIII bands of 2.5 kb in SDG8 and 2.4 kb in Mig8-Mt were also present when the IgG probe was employed (Fig. 2C). As expected from the restriction map of the mutant strain, an extra 1.2-kb HindIII band was also detected in Mig8-Mt, since the IgG probe spanned the ClaI site used to construct the mutant (Fig. 1). These results indicate that the Mig8-Mt strain carries a mutation on the mig gene. The restriction map of the mutant...
DNA probes containing the mig gene alone, since the export signal and the /H9251S. dysgalactiae/ of 16 bacterial cell wall or membrane turnover or release of the culture supernatant of the wild-type strain could be due to either bacterial cell wall or membrane turnover or release of the Mig protein in the concentrated supernatant of Mig8-Mt but not in the cell wall preparations (Fig. 3B), indicating that Mig8-Mt still expressed the upstream regions (Fig. 1). When detected with the antibodies against Mig, a band at about 28 kDa was found in concentrated culture supernatants of Mig8-Mt but not in the cell wall preparations (Fig. 3B), indicating that Mig8-Mt still expressed the α2-M receptor but it was lost into the medium. Concentrated culture supernatants and whole-cell extracts of the wild-type strain exhibited the ca. 80-kDa bands reacting to the goat IgG (Fig. 3B). The presence of the Mig protein in the concentrated culture supernatant of the wild-type strain could be due to either bacterial cell wall or membrane turnover or release of the Mig protein from the cell wall by a cytolytic protease, as is the case for the M protein of GAS (24).

**Distribution of the mig gene in S. dysgalactiae strains.** A total of 16 S. dysgalactiae isolates, including two strains from the American Type Culture Collection, were examined for the presence of sequences homologous to the mig gene by using DNA probes containing the mig-specific α2-M and IgG receptor-coding regions. Southern blot analysis of HindIII digested-genomic DNA revealed that five strains possessed sequences homologous to the α2-M-2 probe (Fig. 1), but the sizes of those bands varied between 2.1 and 2.7 kb (Fig. 4A). The same five strains were also positive in PCR amplifications of the mig α2-M coding region with the Mig-11 and Mig-12 primers (Fig. 1), but they exhibited bands of the same size at 0.5 kb (data not shown). Further PCR analysis of these strains with primers Mig-9 and Mig-8 (Fig. 1), amplifying the IgG receptor-encoding regions, indicated that the 0.6-kb size difference found with the α2-M-2 probe was located on this region (data not shown). In the mig gene, one IgG-binding repeat is encoded by a ca. 200-bp DNA fragment. Taking into account the size differences of the IgG receptor-coding regions, the five α2-M positive strains therefore might carry three to six IgG-binding repeats instead of only five repeats, as are present in the mig genes of the SDG8 and SC1 strains. In contrast to the α2-M-2 probe, the IgG probe (Fig. 1) detected homologous sequences in all the tested strains with a total of seven different hybridization patterns (Fig. 4B). As expected, in the five isolates possessing the specific mig α2-M sequences, the IgG probe hybridized to HindIII fragments of the same size (numbered bands in Fig. 4), suggesting that both regions were part of the same genetic unit.

**Phagocytosis.** To determine the role of the Mig protein in resistance to phagocytosis, SDG8 and Mig8-Mt were labeled with the fluorescent dye PKH2, and the percentage of intracellular microorganisms was measured by FC after ingestion of the bacteria by bovine PMNs. No deleterious effects on the bacterial cell viability were observed after labeling with PKH2 (data not shown). Optimal conditions for phagocytosis were obtained with a ratio of bacteria to PMNs of about 10:1. The results from four individual experiments indicated that the wild-type strain SDG8 (66%) and the mutant Mig8-Mt (66%) were phagocytosed at the same rate (P > 0.05) in the absence of PMNs.

**Fig. 2.** Southern blot analysis of S. dysgalactiae SDG8 and Mig8-Mt digested with HindIII. Blots A and B were probed with γ2P-labeled α2-M-1 and Sp probes, respectively; blot C was probed with the DIG-labeled IgG probe.

**Fig. 3.** Western blot analysis of the proteins of concentrated culture supernatants (S) and cell wall (CW) preparations extracted from SDG8, Mig8-Mt, and Mig8-2-M. Samples were separated by SDS-PAGE, transferred to nitrocellulose membranes, and assayed for binding to IgG (A) by using AP-conjugated goat anti-rabbit IgG and to the Mig protein (B) by using rabbit polyclonal antibodies against Mig and AP-conjugated goat anti-rabbit IgG.
of bovine serum (Fig. 5A). When bovine serum was included in the assay, SDG8 was more resistant to phagocytosis than Mig8-Mt (34 versus 69% ingested bacteria, respectively; \( P < 0.05 \)) (Fig. 5B). This result indicated that the Mig protein in SDG8 is capable of protecting the bacterium from phagocytosis in the presence of antiserum.

To investigate if the IgG receptor of the SDG8 Mig protein was involved in the resistance to phagocytosis, affinity-purified IgG was used in the phagocytosis assays. When a ratio between 5 and 13 bacteria per PMN was used, and from four individual experiments, we observed similar bacterial ingestion rates when the wild-type strain SDG8 was preincubated with the purified IgG at 1-, 2-, and 4-mg/ml concentrations (data not shown). This result suggests that the Mig-mediated higher resistance to phagocytosis of SDG8 (Fig. 5B) described above was probably due to the binding of \( \alpha_2 \)-M in the serum to the IgG receptor but not to the binding of \( \alpha \)-M to the Mig receptor.

The role of complement receptors was excluded by analyzing the phagocytosis of strain SDG8 incubated with either a heat-inactivated or an unheated bovine serum pool. Similar bacterial internalization rates were observed in two groups (data not shown). To rule out the influence of other serum proteins, we incubated SDG8 with purified IgG prepared from the same serum pool prior to the phagocytosis assay. Similar ingestion rates of the wild-type strain were observed for the control and with 0.2 to 0.8 mg of IgG (\( P \) values between 0.7919 and 0.9319, \( n = 4 \)), suggesting that IgG does not influence phagocytosis by PMNs of \( S. \) dysgalactiae. To confirm this observation, we performed several complementary experiments on SDG8. First, we did not observe any differences in phagocytosis of control and SDG8 cells preincubated with a different serum pool obtained from four cows challenged with SDG8 (\( P = 0.566, n = 2 \)). Second, another \( S. \) dysgalactiae strain, ATCC 27957, was analyzed in the same way by using a serum pool containing specific antibodies against this strain. As in the case with SDG8, we did not see a significant enhancement of phagocytosis (\( P = 0.5896, n = 2 \)). Third, an \( S. \) agalactiae strain was incubated with bovine serum containing antibodies against \( S. \) agalactiae, and its resistance to phagocytosis was analyzed by the same method. A significantly higher ingestion rate was observed with the opsonized sample than the nonopsonized one (\( P < 0.0001, n = 4 \)), suggesting that the methodology used for \( S. \) dysgalactiae was appropriate.

**Bactericidal assay.** To investigate the roles of serum proteins in the intracellular bacterial survival rate, bacteria were incubated with bovine PMNs in the absence or presence of bovine serum for different time points. The internalized bacteria were released by lysis of the cells with saponin, and viable counts were determined by plating on THY. The lysis of PMNs was confirmed by microscopic examination, and no deleterious influence of the detergent on the bacterial viability was observed (data not shown). The optimal ratio of bacteria and PMNs in this assay was between 1:1 and 6:1. From six
individual experiments, we found that the serum-free SDG8 and Mig8-Mt strains survived at similar rates after incubation with PMNs for 1 h (27% for both) and 4 h (46% for SDG8 and 40% for Mig8-Mt; \( P > 0.05 \)). When the bacteria were incubated with bovine serum, a significant difference in the survival rate was observed between the two strains after incubation with PMNs for 4 h (93% for SDG8 and 35% for Mig8-Mt; \( P < 0.01 \)) but not for 1 h (25% for SDG8 and 27% for Mig8-Mt; \( P > 0.05 \)). These data suggest that in the presence of serum proteins, the wild-type strain is more resistant to the killing by PMNs after being phagocytosed than the mig mutant strain.

**DISCUSSION**

The mig gene of the *S. dysgalactiae* strain SC1 contains \( \alpha_2\)-M- and IgG-binding regions with five repeat units (12). We sequenced the mig gene from another *S. dysgalactiae* strain, SDG8, and its sequence exhibited high homology to the mig gene of SC1. However, among the five mig-positive strains in our collection, the size of the DNA encoding the IgG-binding region varied, with a difference of up to 0.6 kb. Based on the restriction map of the mig gene and the size of the IgG-binding repeat units (ca. 0.2 kb) in SDG8, we speculate that three to six IgG-binding repeats might exist in the Mig proteins of these other strains. The number of IgG-binding repeats correlates with the capacities for binding to IgG, since protein G (two or three IgG-binding repeats) binds to the IgG-Fc part (9, 27) and the Mig protein (five IgG-binding repeats) simultaneously binds to both IgG Fc and Fab regions. An 11-residue peptide derived from a single protein G repeat was shown to inhibit the binding of protein G to human IgG Fc fragments. Despite the amino acid differences (4 out of 11), a similar peptide from the first repeat of the Mig protein inhibited the binding of protein G to human IgG Fc (31). This suggests that the remaining conserved amino acids or the secondary structure of the peptide might play a role in the binding to the Fc portion of IgG.

The distribution of the mig gene in a total of 16 *S. dysgalactiae* isolates was investigated in this study. All of them possessed DNA fragments that hybridized to the IgG probe (Fig. 4B), suggesting that the IgG-binding sequence of mig is highly conserved in these strains. Only five strains (31%) were found to carry the mig \( \alpha_2\)-M-homologous sequences linked to the IgG-binding domains (Fig. 4A). This suggests that genes other than mig encode the IgG receptors in the rest of the *S. dysgalactiae* strains. The mag gene of *S. dysgalactiae* encodes a surface protein capable of binding to IgG, albumin, and \( \alpha_2\)-M (10). While the IgG-binding domains of mig and mag are highly related (99% identity [this work]), the \( \alpha_2\)-M receptors are not (25% identity). Only three strains (19%) were found to carry sequences homologous to the mag \( \alpha_2\)-M-binding region (data not shown) and none of them were the previously identified five mig-positive strains. Among the total 16 strains, the percentage of isolates carrying \( \alpha_2\)-M receptors was only 50% (31% mig and 19% mag), which was much lower than the 73% found in a direct binding assay using labeled \( \alpha_2\)-M–T (23). This suggests that other types of \( \alpha_2\)-M receptors with unique sequences might exist in *S. dysgalactiae*, especially in the mig- and mag-negative strains.

Besides Mig and Mag in *S. dysgalactiae*, \( \alpha_2\)-M receptors were also identified in several other proteins in streptococci, such as Zag in *S. zooepidemicus* (11), protein G in human group G streptococcus strain 148 (17, 28), and the protein G-related \( \alpha_2\)-M receptor Grab in human group A *S. pyogenes* (24). As the binding of \( \alpha_2\)-M to the bacterial \( \alpha_2\)-M receptors is highly dependent upon conformation, the sequences encoding \( \alpha_2\)-M-binding receptors are unique among these proteins. Furthermore, the pattern of binding of streptococcal cells to the \( \alpha_2\)-M protein of the infected hosts was divergent. The \( \alpha_2\)-M receptors from human group A and G streptococci bind only to the native form of \( \alpha_2\)-M, whereas the \( \alpha_2\)-M receptors from bovine and equine group C streptococci bind only to \( \alpha_2\)-M–T (16, 17). The effects on phagocytosis of these two kinds of binding are also different. The binding of native \( \alpha_2\)-M to *S. pyogenes* enhanced phagocytosis by PMNs (29). It is possible that in GAS, binding of \( \alpha_2\)-M provides protection against virulence factor degradation by interfering with intracellular host cell proteases following phagocytosis of the bacterium. Recent findings support this hypothesis. The \( \alpha_2\)-M receptor expressed by the protein Grab of human group A *S. pyogenes* strains has been shown to be involved in virulence in a mouse infection model via binding to \( \alpha_2\)-M, thereby inhibiting activities of both bacterial and host proteases and thus protecting important virulence determinants from proteolytic degradation (24). The binding of \( \alpha_2\)-M–T to *S. dysgalactiae* inhibited phagocytosis (29), perhaps by protecting other virulence factors against host protease degradation.

In bovine mastitis, the PMN-mediated phagocytosis is the most important host defense system in the mammary gland (3). The concentration of immunoglobulins (3) and other types of IgG-binding domains was shown to be involved in virulence in a mouse infection model via binding to \( \alpha_2\)-M, thereby inhibiting activities of both bacterial and host proteases and thus protecting important virulence determinants from proteolytic degradation (24). The binding of \( \alpha_2\)-M–T to *S. dysgalactiae* enhanced phagocytosis by PMNs (29), perhaps by protecting other virulence factors against host protease degradation.
ceptors that mediate phagocytosis, thereby inhibiting bacterial ingestion. In our phagocytic killing study, a very significant survival rate of the serum-incubated wild-type strain compared to the mig mutant strain correlates with a previous observation that the binding of α2-M–T to S. dysgalactiae whole cells inhibited phagocytic killing (29) and thus played a role in virulence of S. dysgalactiae. The α2-M protein bound to the bacterial surface via the Grab protein of S. pyogenes inhibits the activities of bacterial and host proteases, thereby preventing bacteria or some other virulence factors from proteolytic degradation (24). In the case of S. dysgalactiae, the mechanism of resistance to phagocytosis mediated by the α2-M receptor in Mig remains undetermined, since Mig binds only to the trypsin complex form of α2-M. It is unclear if α2-M–T bound to the bacterial surface via the α2-M receptor still traps and inhibits the activities of proteases, since the enzymatic activity of α2-M–T against low-molecular-mass substrates was unimpaired (22).

In human group A S. pyogenes strains, the M protein has been shown to protect the bacteria against phagocytosis by PMNs (6). Recently, an M-like protein was also isolated from a strain of S. dysgalactiae (30). A comparison of the amino acid sequence of this protein to that of Mig indicated a low degree of homology (data not shown). Although Mig and the M proteins do not share extensive amino acid homology, Mig possesses structural features similar to the M family of proteins, namely, an alpha-coiled-coil structure, repeated amino acid sequences, a carboxy-terminal region embedded in the cell wall, and the conserved sequence LPTEEG essential for anchoring to the cell membrane. A functional classification of the M proteins is their ability to confer resistance to phagocytosis (6). The mechanism by which the M protein protects the bacteria appears to be binding to the serum protein factor H, which regulates the activity of complement deposited on the cell surface (6). Although some of the proteins that bind Mig and M are different, it is tempting to include Mig as a member of the M-protein family, since they exert the same biological function, i.e., protection of the bacterium against the immunological surveillance of the host.

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