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Bovine immunoglobulin A (IgA)-binding activities of the surface-expressed Mig protein of *Streptococcus dysgalactiae*

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The Mig protein of *Streptococcus dysgalactiae* is a type III immunoglobulin G (IgG)-binding protein, expressing IgG- and α_2-macroglobulin (α_2-M)-binding receptors. This study showed that the Mig protein also displays binding activities to bovine immunoglobulin A (B-IgA). Biotin-labelled bovine serum IgA bound immobilized recombinant Mig and α_2-M receptors derived from Mig, as well as the native Mig extracted from the surface of *S. dysgalactiae* strain SDG8 and the α_2-M receptor released from the isogenic *mig* mutant strain Mig8-Mt, as determined by Western blotting and ELISA. There was no B-IgA binding activity to the immobilized IgG receptor derived from Mig or the proteins in the culture supernatant from the *mig* mutant strain Mig7-Mt, in which expression of Mig or Mig-related peptides on the cell surface was completely abolished. In a reciprocal experiment, biotin-labelled Mig was found to bind immobilized bovine serum IgA but not human IgA (H-IgA). The binding of Mig to bovine serum IgA was competitively inhibited by unlabelled Mig, intact and truncated α_2-M receptors, and bovine serum IgA, but not by the Mig-IgG receptor, H-IgA or B-IgG. The binding of Mig and partially purified bovine secretory IgA (B-sIgA) was also characterized by Western blotting. Membrane-immobilized B-sIgA did not react with the biotin-labelled Mig, whereas soluble B-sIgA showed binding activity to the immobilized α_2-M receptor of Mig. It is therefore concluded that the 11 kDa N-terminal region of the α_2-M receptor of the *S. dysgalactiae* Mig protein specifically binds soluble and immobilized bovine serum IgA, as well as soluble B-sIgA. This is believed to be the first report of a B-IgA-binding protein in *S. dysgalactiae.*

Keywords: IgG, α_2-macroglobulin, receptor, bovine mastitis

INTRODUCTION

Streptococcus dysgalactiae, the Lancefield serological group C bacterium, is one of the most common environmental pathogens capable of causing bovine mastitis, a disease responsible for large economic losses in the dairy industry. Normal methods of hygiene and antibiotic therapy have little effect in the prevention of *S. dysgalactiae* infections. In spite of its high prevalence, little is known about factors that contribute to the virulence of *S. dysgalactiae* (Calvinho *et al.*, 1998). Therefore, looking for virulence factors expressed on the bacterial surface and using them as vaccine targets might be an effective way to prevent bovine mastitis associated with *S. dysgalactiae* infections.

A few surface-expressed proteins in *S. dysgalactiae* have been characterized. The list includes the α_2-macroglobulin (α_2-M)- and immunoglobulin G (IgG)-binding protein Mig (Jonsson & Müller, 1994), the α_2-M-, albumin- and IgG-binding protein Mag (Jonsson *et al.*, 1994), and fibrinogen- and IgG-binding M-like proteins (Vasi *et al.*, 2000). Some *S. dysgalactiae* surface receptors that bind to host-derived plasma and matrix proteins such as albumin, fibronectin, collagen, vitronectin and plasminogen have also been reported (Calvinho *et al.*, 1998). Recently, the role of Mig in the virulence of *S.*

Abbreviations: α_2-M, α_2-macroglobulin; AP, alkaline phosphatase; B-, bovine (immunoglobulins); H-, human (immunoglobulins); sIgA, secretory immunoglobulin A.

dysgalactiae has been investigated in our laboratory. Our research indicates that the Mig protein is involved in resisting phagocytosis by bovine neutrophils (PMNs) in the presence of bovine serum (Song *et al*., 2001). Thus, the Mig protein, an M-like protein, is considered a potential virulence factor of *S. dysgalactiae*. In bovine mastitis, the mucosal defence system in the mammary gland plays a major role in the prevention of infection. Since immunoglobulin A (IgA) is the predominant class of immunoglobulin present on mucosal surfaces, and constitutes an important defence mechanism against microbial infections, IgA-binding receptors expressed by bacteria might be involved in the development of mastitis by binding IgA and helping the micro-organism evade the immunological surveillance of the host. In group A and B human streptococcal pathogens, species-specific human-IgA (H-IgA)-binding receptors have been reported. With the exception of SibA (Fagan *et al*., 2001), most IgA-binding proteins of group A streptococcal strains are M or M-like proteins that may play a role in protection against phagocytosis. These include the proteins Arp4 (Johnsson *et al*., 1994; Lindahl & Åkerström, 1989), Sir22 (Stenberg *et al*., 1994) and ML2.2 (Bessen, 1994). IgA-binding receptors were also found in *Streptococcus agalactiae*, including Bac (Hedén *et al*., 1991) and *β*-antigen proteins (Cleat & Timmis, 1987; Russell-Jones *et al*., 1984). Although all of them show specific binding activities to H-IgA, the group A streptococcal receptors bind both serum and secretory IgA (sIgA), whereas the group B streptococcal receptor binds mainly serum IgA (Lindahl *et al*., 1990). Other H-IgA-binding receptors were also reported in *Streptococcus pneumoniae*, including a 52 kDa protein (Serhir *et al*., 1995) and a 59 kDa protein SpsA that specifically binds H-IgA secretory component (Hammerschmidt *et al*., 1997). One of the possible roles of the IgA-binding proteins appears to be protection against phagocytosis, since binding of H-IgA to receptors in *Streptococcus pyogenes* has been found to interfere with bacterial adhesion to the host cells (Fluckiger *et al*., 1998).

Since *S. dysgalactiae* is a major bovine mastitis-causing pathogen and the bovine mammary gland mucosal defence system plays an important role in protection against infections, we investigated the presence of bovine IgA-binding receptors in *S. dysgalactiae* strains isolated from cases of bovine mastitis. Here we report that the surface-expressed Mig protein, a well-characterized α_2-M- and IgG-binding protein of *S. dysgalactiae*, exhibits specific binding activities to serum and secretory bovine IgA (B-IgA and B-sIgA, respectively). We used a Mig-expressing wild-type strain, two isogenic *mig* mutant strains, purified recombinant Mig, and Mig-derived peptides, to test B-IgA-binding activities. We were able to identify the IgA-binding domain in Mig and map it to the 11 kDa N-terminal region of the α_2-M receptor portion of Mig. This finding consolidated our conclusion that the Mig protein possesses multiple binding activities, and is a potential virulence factor of *S. dysgalactiae* strains causing bovine mastitis.

METHODS

Bacterial strains and media. The Lancefield group C streptococcal isolate *S. dysgalactiae* ATCC 43078, renamed SDG8 in this study, was obtained from the American Type Culture Collection. The *mig* mutant strain Mig8-Mt derived from *S. dysgalactiae* SDG8 was described previously (Song *et al*., 2001). The group A streptococcal *S. pyogenes* strain 'M5 Manfredo' was originally obtained from the Memphis VA Hospital Culture Collection, University of Tennessee, Memphis, TN, USA, and kindly provided by Professor M. A. Kehoe, Newcastle University, UK. The Lancefield group B streptococcus *S. agalactiae* strain SB35, expressing the H-IgA-binding protein Bac (Hedén *et al*., 1991), was kindly provided by Dr G. Lindahl, Lund University, Lund, Sweden. The *Escherichia coli* strains DH5α [*ϕ*80d*lacZ*ΔM15, *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 deoR*, Δ(*lacZYA–argF*) *U169*], BL21(DE3) [F^- *dcm ompT hsdS*(r_B^- m_B^- *gal* (DE3)] and the cloning vector pBluescript II SK were from laboratory collections. *E. coli* XL10-Gold Kan^r [Δ(*mcrA*)183 Δ(*mcrCB–hsdSMR–mrr*)173 *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte (F′ *proAB* $lacI^qZ$ΔM15 Tn*10* Tn5 Amy)] was obtained from Stratagene. The expression vector pET-15b, carrying an N-terminal 6 × His-Tag was obtained from Novagen. The temperature-sensitive shuttle vectors pEU904 and pG^+host 9 were generous gifts from Dr J. R. Scott, Emory University, Atlanta, GA, USA, and Dr E. Maguin, Institut National de la Recherche Agronomique (INRA), France, respectively. The *E. coli* strain TG1 (dev) [F′ *traD36* $lacI^q$ Δ(*lacZ*)M15 $proA^+B^+$/ *supE* Δ(*hsdM–mcrB*) *recA*::*tet thi* Δ(*lac–proAB*) *repA*:*km*] used as a host for pG^+host 9 was also kindly provided by Dr E. Maguin.

Streptococcal strains were grown in Todd–Hewitt culture medium (Oxoid) or agar supplemented with 0·5% yeast extract (THY) at 37 °C in a 5% CO_2 atmosphere. *E. coli* strains were cultured in Luria–Bertani (LB) medium. When required, antibiotics were added to the following concentrations: 50 μg ampicillin ml^{-1}; 50 μg carbenicillin ml^{-1}; 1 μg erythromycin ml^{-1}; 100 μg kanamycin ml^{-1}; 200 μg rifampicin ml^{-1}; and 200 μg spectinomycin ml^{-1}.

Immunoglobulins. Purified B-IgA and bovine IgM (B-IgM) were obtained from Inter-Cell Technologies and Sigma, respectively. Human IgA (H-IgA) was obtained from Cappel. Bovine IgG_1 (B-IgG_1) and IgG_2 (B-IgG_2) were from Jackson ImmunoResearch Laboratories. Rabbit anti-B-sIgA was obtained from Nordic Immunology. Alkaline phosphatase (AP)-conjugated goat anti-B-IgG (H + L) was obtained from Kirkegaad & Perry Laboratories. Polyclonal antibodies against Mig raised in rabbits and purification of specific Mig IgG antibodies have been described previously (Song *et al*., 2001).

DNA manipulations. Plasmid DNA was prepared with DNA preparation kits from either Qiagen or Sigma. *S. dysgalactiae* genomic DNA was extracted as described previously (Song *et al*., 2001). Either $CaCl_2$-treated (Sambrook *et al*., 1989) or PEG-treated (Kurien & Scofield, 1995) *E. coli* cells were used for transformation. Oligonucleotides other than those specified, used for amplification of the *mig* structural gene and the α_2-M- and IgG-binding regions have been described previously (Song *et al*., 2001). Restriction endonucleases, *Taq* DNA polymerase and dNTPs were obtained from Amersham Pharmacia Biotech. T4 DNA ligase was from USBiological. PCR amplification was run for 35 cycles of 45 s at 94 °C, 45 s at 55 °C and 1 min at 72 °C with an initial step of 3 min at 95 °C for denaturation, followed by a final extension of 5 min at 72 °C. The Geneclean Spin Kit (Bio 101) and UltraClean

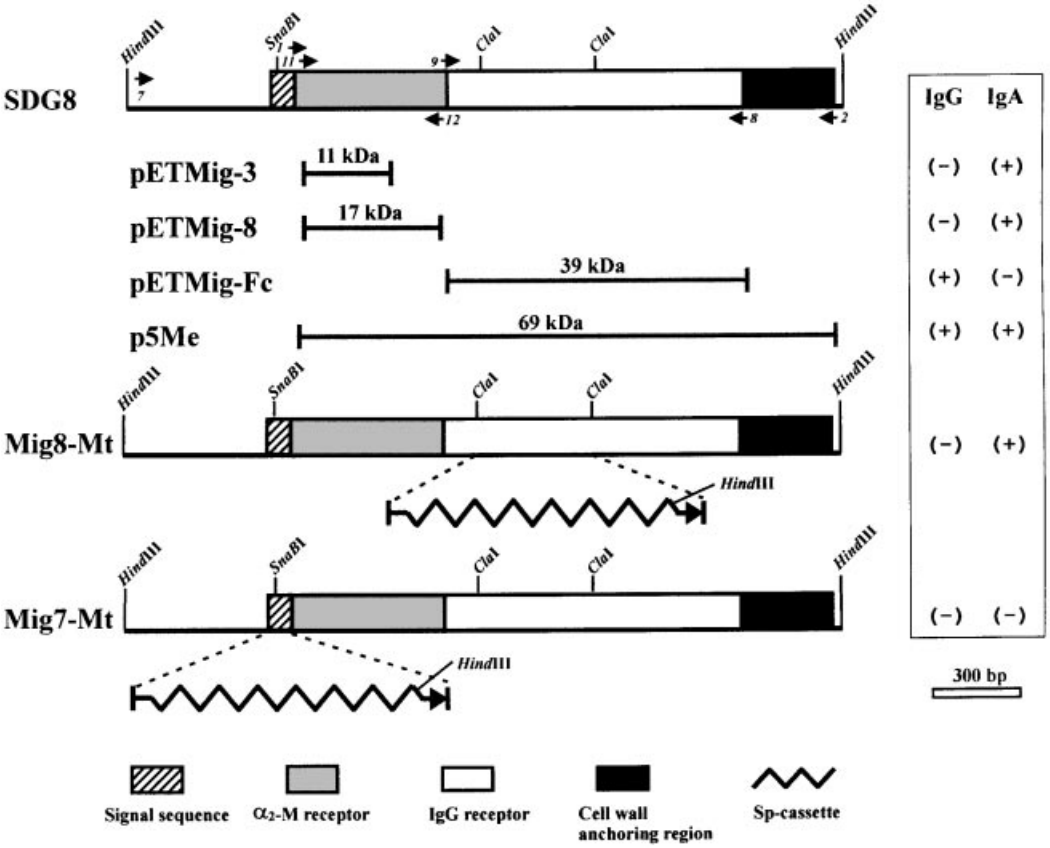

Fig. 1. Schematic maps of the *mig* gene in the wild-type strain SDG8, and isogenic *mig* mutant strains Mig8-Mt and Mig7-Mt. The construction and chromosomal map of the Mig8-Mt strain has already been described (Song *et al.*, 2001) and is presented here to allow comparison. The locations of the PCR primers used to construct plasmids and generate probes are indicated with arrows. The numbers on the arrows correspond to the Mig primers described previously (Song *et al.*, 2001). Restriction enzyme sites used for strain construction and Southern blots are marked. The names of the plasmids, and the coding location and mass of Mig-related protein products encoded, are represented below the map for the wild-type SDG8 strain. The block symbols indicate regions of the Mig protein, and the zig-zag line indicates the Sp-cassette used for mutagenesis. On the right side, the binding activities to AP-conjugated goat IgG (IgG) and biotin-labelled bovine serum IgA (IgA) of the purified and culture supernatant proteins of the *mig* mutant strains are indicated.

GelSpin DNA Purification Kit (Mo Bio Laboratories) were used to purify PCR products or plasmid DNA fragments from agarose gels when needed.

Construction of plasmids and strains. The plasmid p5Me expressing the mature Mig protein, and the isogenic *mig* mutant strain Mig8-Mt (Fig. 1) expressing α_2-M receptors only in the culture medium, have been described previously (Song *et al.*, 2001). To construct plasmids expressing intact and truncated α_2-M-binding receptors, a 0·5 kb fragment encoding the α_2-M-binding region of Mig was amplified with Mig-11 and Mig-12 primers (Fig. 1) from two templates. The entire α_2-M receptor region was amplified from *S. dysgalactiae* SDG8 genomic DNA, digested with *Nde*I and *Bam*HI, and cloned into the expression vector pET-15b to generate pETMig-8. The truncated version of the α_2-M receptor of Mig was amplified from pKSMig-3. This plasmid carries a point mutation in the α_2-M coding region, resulting in a premature stop codon (data not shown). The amplified fragment was cleaved with *Nde*I and *Bam*HI, and ligated to pET-15b to generate pETMig-3. Finally, the IgG-binding receptor region of Mig was amplified from SDG8 genomic DNA with the Mig-9 and Mig-8 primers (Fig. 1) and cloned into pET-15b. One recombinant plasmid, pETMig-Fc, carrying a 1·1 kb insert, was selected for expression of the IgG-binding receptor protein.

To construct a *S. dysgalactiae* strain deficient in expression of the Mig protein on the cell surface, an approximately 1 kb fragment carrying the region upstream of the *mig* start codon and the entire α_2-M-binding region was amplified from SDG8 genomic DNA with Mig-7 and Mig-12 primers (Fig. 1). This fragment was cloned into the *Bam*HI and *Xba*I sites of pBluescript II SK. The resulting plasmid was named pMigmut-1. After treatment with *Sna*BI, the linearized pMigmut-1 was ligated to the 1·2 kb *aad9* gene from pEU904 encoding resistance to spectinomycin, amplified with *aad9*-01 (5′-TCGATAGCTTGCATGCCTGCAG-3′) and *aad9*-02 (5′-GAGGTCGACGGTATCGATAAGC-3′) primers. The resulting plasmid, pMigmut-6, carries the *aad9* gene inserted immediately downstream of the Mig start codon. The insert in pMigmut-6 was cleaved with *Eco*RV and *Not*I, and cloned into pG$^+$host 9 to generate pMigmut-7. This plasmid was transformed into SDG8, and allele-replacement of the chromosomal *mig* gene was performed as described previously (Perez-Casal *et al.*, 1993). Colonies resistant to spectinomycin and sensitive to erythromycin were isolated at 37 °C and the strain was designated Mig7-Mt (Fig. 1).

Southern blots. To characterize the *mig* mutant strain Mig7-Mt, probes specific to the *mig* IgG-binding region and to the spectinomycin-resistance cassette (Sp-cassette) were labelled with DIG-dUTP (Roche) by PCR with the Mig-9/Mig-8 and *aad*9-01/*aad*9-02 primers, respectively. Conditions for labelling, hybridization and detection were similar to those previously described (Song *et al*., 2001).

Expression and purification of proteins in *E. coli*. The Mig protein was expressed from p5Me in *E. coli* and purified with a B-IgG affinity column (Song *et al*., 2001). To express the intact and truncated forms of α_2-M receptors of Mig, the plasmids pETMig-8 and pETMig-3 were transformed into *E. coli* BL21(DE3). Protein expression was induced by IPTG (Sigma) at a final concentration of 1 mM from exponential-phase cultures, in LB broth for 5 h at 37 °C. Cell pellets were harvested by centrifugation and suspended in a working buffer (50 mM NaH_2PO_4, pH 8·0, 300 mM NaCl) supplemented with 10 mM imidazole (Sigma). Cells were lysed by incubation with 1 mg lysozyme ml^{-1} (Sigma) on ice for 30 min, followed by sonication. Cellular debris was removed by centrifugation, and the supernatant was loaded onto a column packed with nickel-nitrilotriacetic acid (Ni-NTA) affinity agarose (Qiagen). After extensive washing with the above working buffer supplemented with 20 mM imidazole, the expressed proteins carrying a 6 × His-Tag at the N-terminus were eluted with the working buffer containing 250 mM imidazole, according to the supplier's recommendations (Qiagen).

To express the IgG-binding receptor, exponential-phase cultures of BL21(DE3) carrying pETMig-Fc were incubated at 30 °C in the presence of 10 mM glucose and carbenicillin, to maintain the resident plasmid. After 1 h incubation with 1 mM IPTG, rifampicin was added, and induction was continued for 2·5 h. Expressed protein was then purified as described for the α_2-M receptors (above).

Preparation of streptococcal surface proteins. The search for B-IgA-binding receptors in *S. dysgalactiae* was carried out using cell-surface proteins extracted by three different methods. The enzymic method involved treating bacteria with mutanolysin (Sigma) to isolate *S. dysgalactiae* cell-wall fractions, in the presence of a protease inhibitor cocktail (Roche) (Kling *et al*., 1999; Song *et al*., 2001). The buffer method involved incubation of the bacteria in 50 mM Tris buffer at different pH values (7·0, 9·0 and 11·0) overnight at 37 °C before removing cells by centrifugation (Lindahl *et al*., 1990). The chemical method used cyanogen bromide (CNBr) (Sigma) to remove proteins from the cell surface by cleavage of methionine residues (Faulmann *et al*., 1991). Briefly, bacterial cultures were washed once in 0·1 M phosphate-buffered saline (PBS) and cell pellets were suspended in PBS. An equal volume of CNBr solution (30 mg CNBr ml^{-1} in 0·2 M HCl) was added to bacterial suspensions to a final concentration of 15 mg CNBr ml^{-1}. After overnight incubation at room temperature with end-to-end rotation, the supernatant was collected by centrifugation and sequentially washed twice in Ultrafree-15 Biomax-5 K protein concentrators (Millipore) with the following solutions: 0·1 M HCl, 1 M Tris/HCl, pH 8·0, and PBS.

Purification of B-sIgA. Freshly collected bovine colostrum was centrifuged at 15000 ***g*** at 4 °C for 1·5 h to remove the top lipid layer. The clear middle layer was transferred to a clean centrifuge tube and centrifugation was repeated. The supernatant was treated with 2% glacial acetic acid to remove casein, and the solution was cleared by centrifugation at 15000 ***g*** at 4 °C for 1 h. The supernatant was transferred into a clean tube and the pH neutralized with NaOH. An equal volume of saturated $(NH_4)_2SO_4$ was added to the solution, and precipitated immunoglobulins were collected by centrifugation at 3000 ***g*** for 30 min at 4 °C. The pellet was suspended in PBS and dialysed in a large volume of PBS at 4 °C. To remove IgG from the B-sIgA preparation, the dialysed solution was passed three times through a Protein G-affinity column (MAbTrapGII, Pharmacia). The column flow-through portion was collected and washed twice in PBS with a Biomax-30 K protein concentrator (Millipore) prior to analysis.

Biotinylation of proteins. Prior to biotin coupling, 0·5–1 mg of the purified proteins were equilibrated in 0·1 M $NaHCO_3$ buffer at pH 8·0 by centrifugation in a protein concentrator (Millipore). Labelling was initiated by adding 50 µg biotin amidocaproate *N*-hydroxysuccinate ester (Sigma) in DMSO (Sigma) to the samples and incubation was carried out for 1 h at room temperature with end-to-end mixing. Excess labelling solution was removed in a protein concentrator and concentrated proteins were equilibrated in PBS by two rounds of centrifugation in protein concentrators.

SDS-PAGE and Western blots. SDS-PAGE was performed according to the standard method (Sambrook *et al*., 1989). Gels were either stained with Coomassie brilliant blue or transferred onto nitrocellulose membranes (Bio-Rad). For Western blots, membranes were first blocked with PBS supplemented with 0·05% Tween 20 (PBS-T). Membranes were incubated for 2 h with either biotin-labelled proteins at a dilution between 1:250 and 1:500 and followed with AP-conjugated streptavidin (1:2000 dilution) (Life Technologies), or with the B-sIgA preparation at a dilution of 1:250 followed by 1:2000 diluted biotin-labelled second antibody and AP-conjugated streptavidin. The membranes were washed three times with PBS-T for 10 min between incubations. Blots were developed in AP buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris/HCl, pH 9·5) supplemented with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma). Low-range and high-range protein standards (Bio-Rad) were used to estimate the molecular mass of protein bands. The concentration of protein samples was determined on a microtitre plate with a DC Protein Assay kit and the Microplate Manager Software (Bio-Rad), using bovine serum albumin or IgG (Pierce) standards.

ELISA. Binding and binding-inhibition assays were performed in high-binding Immulon-2 U-bottom microtitre plates (Dynex Technologies). Each well was coated with 100 µl of either purified Mig or B-IgA, at a concentration of 2 µg ml^{-1}, in 50 mM sodium carbonate buffer (pH 9·6) and incubated for 16 h at 4 °C. After four washes with TBST (0·1 M Tris/HCl, pH 7·5, 0·17 M NaCl, 0·05% Tween 20), the wells were coated with 200 µl TBSTg (TBST + 0·5% gelatin) for 16 h at 4 °C to block non-specific binding. After washing twice with TBST, 50 µl of non-labelled inhibitor proteins, diluted in TBSTg at various concentrations, were added to each well and followed with 50 µl of biotin-labelled B-IgA or Mig at a dilution of 1:2000. The reaction was performed at room temperature for 2 h and terminated by washing four times with TBSTg. Finally, plates were incubated with 100 µl per well of AP-conjugated streptavidin diluted at 1:3000 for 1 h and developed with *p*-nitrophenyl phosphate (Sigma) in DE buffer (0·1 M diethanolamine, 0·5 M $MgCl_2$, pH 9·8) for 0·5 h. The absorbance was measured at 405 nm with a reference wavelength of 490 nm in a Bio-Rad 3550 microplate reader. The percentage of binding was calculated from the ratio of the absorbance in the wells containing the inhibitor proteins to the absorbance in wells without inhibitor proteins (100% binding). The percentage of inhibition was calculated as 100 minus the percentage of binding.

Determination of DNA sequences. The nucleotide sequences of constructed plasmids were determined on an ABI 373 DNA automatic sequencer (Applied Biosystems) at the Plant Biotechnology Institute (National Research Council, Saskatoon, SK, Canada). The sequence data were analysed with applications in the GCG software package (Wisconsin Package Version 10.1; Genetics Computer Group) and DNA-fold software (Michael Zuker, Washington University, St Louis, MO, USA) provided by the Canadian Bioinformatics Resources.

Determination of N-terminal protein sequences. Prior to analysis, proteins expressed with the His-Tag system were pre-treated with thrombin protease (Novagen) to remove N-terminal amino acid residues derived from the vector. After gel purification, the sample bands were transferred onto PVDF membranes (Millipore) by electro-blotting in a transfer buffer containing 10 mM CAPS (pH 11) and 20 % methanol. Samples were analysed either at the Victoria Protein Microchemistry Centre, University of Victoria, Victoria, BC, Canada, or at VIDO with a 490 Procise Protein Sequencer (Applied Biosystems).

RESULTS

Expression of Mig and Mig-derived peptides

The plasmids and bacterial strains constructed for this study as well as their Mig-related protein products are summarized in Fig. 1. The plasmid pETMig-3 expressed an 11 kDa protein (α_2-M-TR), corresponding to a truncated form of the α_2-M receptor contained within Mig. The complete α_2-M receptor region of Mig (α_2-M-R) is expressed by pETMig-8 as a 17 kDa product, while the IgG-binding domain is expressed by pETMig-Fc as a 39 kDa protein. The plasmid p5Me expressed the mature form of Mig (Song *et al.*, 2001). The bacterial strain Mig8-Mt expressed the α_2-M-R receptor, and since it lacks the Mig C-terminal anchor region this receptor was only found in the culture supernatants (Song *et al.*, 2001). The strain Mig7-Mt is deficient in the expression of Mig on the cell surface (see below). The protein products expressed by the pET system contain 40 aa derived from the vector, in addition to the Mig-related peptides, resulting in protein products of approximately 16 kDa for α_2-M-TR and approximately 22 kDa for α_2-M-R. As previously observed (Song *et al.*, 2001), the relative mass of these protein products migrating in SDS-PAGE gels was larger than expected.

The purified products encoded by the plasmids pETMig-Fc, pETMig-8 and pETMig-3 were characterized by Western blotting. As expected, the α_2-M-R and α_2-M-TR expressed by pETMig-8 and pETMig-3, respectively, only reacted with anti-Mig polyclonal antibodies but not with the AP-conjugated IgG, whereas the IgG-R expressed by pETMig-Fc reacted with both antibodies (data not shown).

Construction and characterization of the *mig* mutant strain

To construct an isogenic *mig* mutant strain of *S. dysgalactiae* that did not express any part of Mig on the cell surface, a Sp-cassette was inserted immediately downstream of the Mig start codon. The chromosomal map of the Mig7-Mt strain was analysed by Southern blots using probes specific to the *mig* IgG-binding region and to the Sp-cassette coding sequence. When the *mig* probe was used, a 2·5 kb and a 2·0 kb *Hin*dIII fragment were detected in the SDG8 and Mig7-Mt genomic DNA, respectively (data not shown). The smaller size in the *mig* mutant strain was due to the introduction of a new *Hin*dIII site in the Sp-cassette (Fig. 1). With the Sp-cassette probe, no signal was found in the genomic DNA of SDG8 whereas a 1·7 kb *Hin*dIII fragment was present in the mutant strain (data not shown), indicating that the Sp-cassette has been inserted into the *mig* at the desired location.

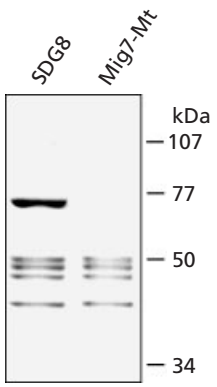

Fig. 2. CNBr preparations of the wild-type strain SDG8 and the *mig* mutant Mig7-Mt strain. Protein extracts were separated on an 8 % SDS-PAGE gel, and stained with Coomassie blue.

The loss of Mig on the cell surface and in the culture supernatant of the Mig7-Mt strain was tested by Western blots. No signals were detected from cell wall and concentrated culture supernatant preparations by using either AP-conjugated goat anti-B-IgG or rabbit anti-Mig polyclonal antibodies followed by AP-conjugated goat anti-rabbit IgG (data not shown). We also examined a protein preparation of Mig7-Mt obtained by CNBr extraction. Coomassie-blue staining of the SDS-PAGE gel revealed that as with the previously constructed *mig* mutant strain Mig8-Mt (Song *et al.*, 2001), only four protein bands were present in the CNBr extract of Mig7-Mt compared to the five bands present in the wild-type SDG8 strain (Fig. 2). These results indicated that the expression of Mig on the surface of the Mig7-Mt strain was abolished. We used CNBr to remove the IgG-binding proteins from the cell surface because it has been observed that these proteins generally lack internal methionine residues that are the specific cleavage site of CNBr (Faulmann & Boyle, 1991). The extracted proteins therefore kept functional activities. N-terminal sequencing analyses were performed on all of the surface proteins extracted with CNBr from the wild-type SDG8 strain (Fig. 2). The sequence obtained of the N-terminal region of the approximately 75 kDa protein was ETIPAAVIVPVGL-DTTE, matching residues 1–17 of the expected mature

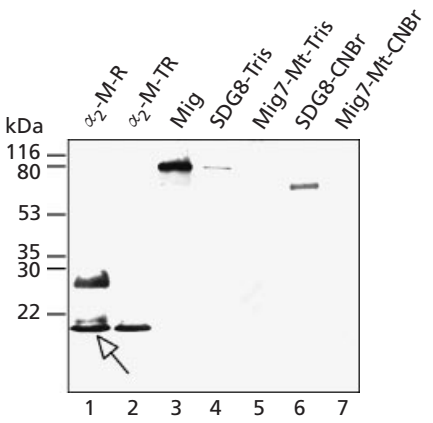

Fig. 3. Binding analyses of purified proteins and surface-protein preparations. The binding to biotin-labelled bovine serum IgA was analysed by Western blotting. Tris and CNBr indicate the method used to extract surface proteins from whole cells of the wild-type SDG8 and *mig* mutant Mig7-Mt strains. The purified α_2-M-R, α_2-M-TR and Mig are expressed by pETMig-8, pETMig-3 and p5Me, respectively. The band indicated by an arrow was further characterized by N-terminal protein sequencing (see text for details).

Mig protein, and indicating that the approximately 75 kDa product obtained by CNBr extraction was indeed Mig. We did not find sequence homology to Mig in any of the remaining four bands from the SDG8 strain, indicating that these four proteins were not degradation products of Mig. However, some of them exhibited homology to M1 and extracellular matrix-binding proteins of *S. pyogenes* (data not shown).

Binding activities of Mig and B-IgA analysed by Western blots

To examine and characterize B-IgA-binding receptors in *S. dysgalactiae*, commercial B-IgA was used as a probe in Western blots. Since many IgA-binding receptors in *S. pyogenes* also exhibit IgG-binding activities (Stenberg *et al.*, 1994) that may cause non-specific reactions with the second conjugated antibodies, we employed a direct testing approach by labelling B-IgA with biotin and used it to test B-IgA-binding receptors followed by AP-conjugated streptavidin and AP substrates as detection systems. The reaction background with the AP-conjugated streptavidin was found to be negligible in all cases.

We tested surface protein preparations extracted by each of three different methods (see Methods). Proteins extracted from whole SDG8 cells by enzymic (data not shown) or buffer treatments resulted in an approximately 80 kDa band that co-migrated with the purified recombinant Mig; both of these bound B-IgA (Fig. 3, lanes 4 and 3, respectively). The approximately 75 kDa Mig-derived protein obtained by CNBr extraction (Fig. 2) was also able to bind B-IgA (Fig. 3, lane 6). The difference in molecular mass of Mig extracted by the CNBr method and the buffer method was probably due to the nature of CNBr cleavage, which occurs at the C-terminal side of methionine residues. Upon inspection of the deduced protein sequence of Mig, we found a methionine residue at position 615 of the mature Mig protein (Song *et al.*, 2001). CNBr cleavage at this position would result in a mature peptide of 614 residues, with an estimated molecular mass of approximately 67 kDa. Taking into consideration the aberrant migration of these proteins on SDS-PAGE gels, the approximately 75 kDa protein observed on the SDG8 CNBr extract is consistent with the expected size of the CNBr-derived peptide of Mig.

To confirm the specific binding of Mig and labelled B-IgA, the recombinant Mig protein and the constructed isogenic *mig* mutant strains were tested. As expected, the recombinant Mig protein bound labelled B-IgA (Fig. 3, lane 3) whereas no binding was found in the buffer- and CNBr-preparations of the *mig* mutant strains Mig8-Mt (data not shown) and Mig7-Mt (Fig. 3, lanes 5 and 7, respectively). Surface protein preparations from *S. pyogenes* M5 Manfredo and *S. agalactiae* strain SB35, both of which express H-IgA-binding receptors, were included as controls. Neither preparation contained proteins able to bind B-IgA (data not shown). These results also suggested that the B-IgA-binding receptor present in *S. dysgalactiae* is different from the extensively studied H-IgA-binding receptor of other streptococci.

The binding of Mig to B-IgA was also characterized by using biotin-labelled Mig to detect immobilized B-IgA by Western blots. High-molecular-mass protein bands binding to labelled Mig were detected when proteins were separated in a 6 % polyacrylamide gel under non-reducing conditions (data not shown). In contrast, immobilized H-IgA and B-IgM did not exhibit any binding activities to the labelled Mig (data not shown).

We extended our study to analyse binding of Mig to the secretory form of IgA (B-sIgA) obtained from bovine colostrum. Prior to analysis, the partially purified B-sIgA preparation was characterized with antibodies against B-sIgA and B-IgG in Western blots. Under non-reducing conditions, high-molecular-mass bands were found in the B-sIgA preparation reacting with the rabbit anti-B-sIgA followed by AP-conjugated goat anti-rabbit IgG (data not shown). However, there was no reaction with the conjugated goat anti-B-IgG (data not shown). The results suggested that the B-sIgA preparation contains detectable amounts of B-sIgA and that it is free of IgG. However, when the membrane-immobilized B-sIgA was tested with biotin-labelled Mig, we did not detect binding (data not shown). In a reciprocal analysis, the ability of soluble B-sIgA to bind immobilized Mig and Mig-derived peptides was analysed using biotin-labelled rabbit anti-B-sIgA as the second antibody. As expected, the recombinant Mig protein showed binding. This was probably due in part to the IgG receptor binding to the secondary antibody, and in part to the α_2-M receptors of Mig binding to the B-sIgA. In the same experiment, the immobilized recombinant α_2-M receptor also showed binding to B-sIgA (data not shown). This indicated that soluble B-sIgA binds the immobilized α_2-M-binding receptor of Mig.

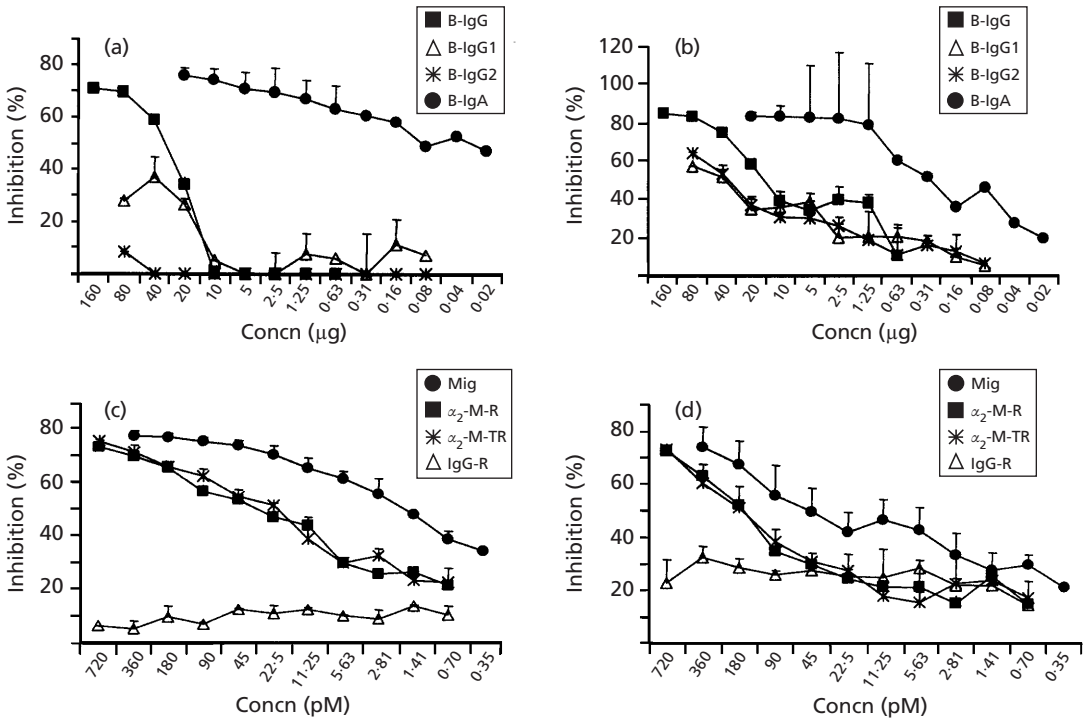

Fig. 4. Binding-inhibition assays. (a, c) Bovine serum IgA-coated microtitre plates incubated with biotin-labelled Mig in the presence of different concentrations of (a) B-IgG, B-IgG_1, B-IgG_2 and bovine serum IgA, or (c) Mig and Mig-derived α-$_2$M-R, α_2-M-TR and IgG-R proteins. (b, d) Mig-coated microtitre plates incubated with biotin-labelled B-IgA in the presence of different concentrations of (b) B-IgG, B-IgG_1, B-IgG_2 and B-IgA, or (d) Mig and Mig-derived α_2-M-R, α_2-M-TR and IgG-R proteins. Each point represents the mean $\pm$ SD of values obtained from four experiments.

Localization of B-IgA-binding-domains in Mig

To identify the B-IgA-binding domains of Mig, purified α_2-M and IgG receptors were tested with labelled B-IgA in Western blots. The α_2-M-R receptor expressed by pETMig-8 contained two protein products that bound B-IgA (Fig. 3, lane 1) but not IgG (data not shown), which were the expected approximately 22 kDa α_2-M-R receptor and a band migrating at approximately 16 kDa. To characterize this additional band in the α_2-M-R preparation, N-terminal protein sequencing was performed on the gel-purified protein pre-treated with thrombin protease to remove the N-terminal amino acid residues encoded by vector DNA. The sequence of the first 10 aa of the insert was determined to be PVGLDT-TELQ, matching the predicted N-terminal sequence of α_2-M-R expressed by pETMig-8. This indicated that the smaller peptide in the α_2-M-R preparation is the result of a truncated α_2-M receptor, probably arising from premature termination of transcription due to a large branched hairpin structure within the α_2-M coding region, with a free energy of $-48{\cdot}6$ kcal mol^{-1} ($-203{\cdot}3$ kJ mol^{-1}) as predicted by the DNA-fold software (data not shown).

The α_2-M-TR protein expressed by pETMig-3 (Fig. 1) is a truncated α_2-M receptor carrying the N-terminal 11 kDa region of Mig. This protein migrated at the same position as the smaller peptide expressed by pETMig-8, and bound labelled B-IgA but not IgG (Fig. 3, lane 2). This observation suggested that the B-IgA-binding receptor was located in the N-terminal 11 kDa region of Mig. This hypothesis was further strengthened by the finding that a 29 aa sequence, postulated to be a conserved binding domain to H-IgA in *S. pyogenes* (Johnsson *et al*., 1994), revealed 54% similarity and 46% identity to a region in the α_2-M-TR receptor (data not shown). Although these two proteins bind different species of IgA, a sequence or structure homology may exist among these IgA-binding receptors. The Mig Fc receptor encoded by pETMig-Fc bound only conjugated IgG but not B-IgA (Fig. 1), confirming that the B-IgA-binding receptor is located in the α_2-M-binding region.

Binding inhibition analysis by ELISA

Either B-IgA or Mig was used to coat microtitre plates, and binding assays were performed with either biotin-labelled Mig or biotin-labelled B-IgA. Unlabelled immunoglobulins, Mig and Mig-derived proteins were tested as inhibitors. On the B-IgA coated plates, the binding of Mig to B-IgA was inhibited by B-IgA, Mig, α_2-M-R and α_2-M-TR receptors, but not by B-IgG_1, B-IgG_2 or the Mig-IgG receptor expressed by pETMig-Fc (Fig. 4a, c). A similar result was obtained when B-IgA was tested on the Mig-coated plates (Fig. 4b, d).

A noteworthy phenomenon was that the binding-inhibition effects of the intact and truncated α_2-M receptors were comparable to each other, whereas both were lower than that of the complete Mig protein (Fig. 4c, d). This suggests that although the α_2-M-TR 11 kDa N-terminal fragment of Mig contains the B-IgA-binding receptor, a whole Mig protein might be required for a full binding activity, which is probably due to differences in the secondary structures between these proteins.

DISCUSSION

Most pathogenic bacteria and viruses use mucosal pathways such as the oral, intestinal and mammary gland as their port of entry for invasion into their host. In the case of the bovine mammary gland, immunoglobulins are one of several active defence mechanisms for protection of the gland from bacterial infections. The concentration of the immunoglobulin classes varies with the stage of lactation and health of the mammary gland. In colostrum, and in secretions obtained from dry animals, the concentration of IgA increases considerably with respect to the IgA levels in milk (International Dairy Federation, 2000; Sordillo *et al*., 1997), while in inflamed udders, there is also an increase in the levels of IgA, suggesting passive transfer from the serum to the mammary secretions (Guidry *et al*., 1980). These higher levels of IgA in mammary secretions could serve as a first line of protection against a pathogen. Several pathogenic Gram-positive bacteria encode surface proteins capable of binding to immunoglobulins, and it is postulated that this binding might help the bacterium to evade the immunological surveillance of the host. Surface proteins encoding IgA-binding receptors have been reported in *S. pneumoniae*, and streptococcal strains of groups A and B, but these proteins specifically bind only H-IgA (Bessen, 1994; Cleat & Timmis, 1987; Fagan *et al*., 2001; Hammerschmidt *et al*., 1997; Hedén *et al*., 1991; Johnsson *et al*., 1994; Russell-Jones *et al*., 1984; Serhir *et al*., 1995; Stenberg *et al*., 1994). Since IgA-binding receptors might play a role in bacterial pathogenesis, we were interested in examining the existence of B-IgA-binding receptors in *S. dysgalactiae*, an environmental pathogen that can infect and cause subsequent inflammation of the bovine mammary gland.

Among surface protein preparations, an approximately 80 kDa band extracted by the enzyme and buffer methods, and an approximately 75 kDa band extracted by the CNBr method from the wild-type SDG8 strain, were found to bind labelled B-IgA. In contrast, these bands were absent in the same preparations of the isogenic *mig* mutant strains Mig8-Mt (data not shown) and Mig7-Mt (Fig. 3), suggesting that both the 80 kDa and the 75 kDa B-IgA-binding proteins were Mig or a Mig-derived protein. The 80 kDa protein bands migrated at the same size in the gel and displayed similar binding activities to the purified recombinant Mig protein in reactions with IgG and anti-Mig antibodies, indicating that the approximately 80 kDa B-IgA-binding protein extracted by the enzyme and buffer methods is the mature Mig protein. The use of the CNBr extraction method was based on the previous observations that type II (Otten *et al*., 1992; Raeder *et al*., 1992) and type III (Hedén *et al*., 1991) IgG-binding receptors of streptococci were efficiently solubilized with CNBr. Because of a shortage of internal methionine residues in these IgG-binding receptors, the extracted proteins still exhibit biological functions. We therefore applied this method to extract Mig, a type III IgG-binding receptor (Jonsson & Müller, 1994), and other surface proteins from *S. dysgalactiae* strains. The approximately 75 kDa B-IgA-binding protein in the CNBr extract of the SDG8 strain displayed similar antibody-binding activities (data not shown) to purified Mig, although the size was a little smaller than the native protein. We postulate that the smaller size of the CNBr-extracted Mig (approx. 75 kDa) is due to the cleavage of a methionine residue by CNBr in the C-terminal region of Mig, because the N-terminal sequence of the 75 kDa protein was identical to the N-terminal sequence of the mature Mig protein determined in our protein sequence analysis.

To identify the B-IgA-binding domains in Mig, the ability of Mig-derived recombinant α_2-M and IgG receptors to bind labelled B-IgA was tested. Both intact and truncated α_2-M receptors, expressed from pETMig-8 and pETMig-3, respectively, bound B-IgA (Fig. 3, lanes 1 and 2), whereas no binding to B-IgA was observed with the IgG receptor expressed by pETMig-Fc (data not shown). Both forms of the α_2-M receptors also exhibited specific inhibition of the binding of labelled B-IgA and Mig, and vice versa (Fig. 4c, d), indicating that the B-IgA-binding region is located within the truncated form of α_2-M receptor corresponding to the N-terminal 11 kDa region of Mig. Furthermore, we also analysed B-IgA-binding activities in concentrated cell culture supernatants of two *mig* mutant strains. Due to difference in the Sp-cassette insertion site, the Mig8-Mt expressed a 28 kDa peptide containing the α_2-M receptor into the culture supernatant (Song *et al*., 2001), whereas the Mig7-Mt constructed in this study entirely lost the expression of Mig. As expected, the 28 kDa peptide expressed by Mig8-Mt bound B-IgA but no binding proteins were found in the Mig7-Mt preparations (data not shown). This observation further confirmed our finding that the B-IgA-binding domain of Mig was located at the N-terminal α_2-M-binding region.

Potential conserved H-IgA receptor sequences have been described and tested in a few studies in streptococcal species. These include the 29 and 50 aa peptides derived from the *S. pyogenes* Arp4 (Johnsson *et al*., 1994) and Sir22 proteins (Johnsson *et al*., 1999), respectively. Also tested were the protein motifs ALXGENXDLR from the *S. pyogenes* Arp4 and ML2.2 proteins (Bessen, 1994), MLKKIE derived from the β-antigen of the c protein complex of group B *S. agalactiae* (Jerlström *et al*., 1996), and YRNYPT from *S. pneumoniae* (Hammerschmidt *et al*., 2000). However, only the 50 aa peptide derived from the *S. pyogenes* Sir22 protein exhibited functional activities in its isolated form (Johnsson *et al*., 1999). These proteins are species-specific H-IgA-binding recep-

tors. However, the 29 conserved residues that were derived from the *S. pyogenes* Arp4 protein (Johnsson *et al.*, 1994) shared about 50 % homology with the deduced Mig protein sequence (data not shown). This homologous region was located within the 11 kDa α_2-M-TR region, which is in agreement with the observation that the 11 kDa α_2-M-TR peptide blocked the binding activities between Mig and B-IgA in our binding assays (Fig. 4). Although the specific B-IgA-binding sequences have yet to be identified in Mig, the sequence similarity between H-IgA- and B-IgA-binding receptors suggests that there might be some common features shared among them. Currently, we are investigating some other B-IgA-binding proteins with divergent plasma-protein-binding profiles in different *S. dysgalactiae* strains. The sequence data of these studies will enable us to identify conserved B-IgA-binding peptide sequences. However, the sequences or regions responsible for the full B-IgA-binding activities might depend on the conformation of a larger region or whole molecule. This speculation is based on our binding inhibition assay result that the whole Mig protein had a higher inhibitory effect than the α_2-M receptors at the same concentration (Fig. 4c, d). This finding is similar to the cooperative activities of five IgG-binding repeats in binding to IgG (Vasi *et al.*, 1999).

Among streptococcal H-IgA-binding proteins, the group A streptococcal receptors bind both serum IgA and sIgA, the group B streptococcal receptor mainly binds serum IgA, and *S. pneumoniae* receptors only bind the IgA secretory component (SC) region (Hammerschmidt *et al.*, 2000). Probably due to the binding activities to sIgA, the group A streptococcal IgA-binding receptors might be involved in anti-phagocytic activities, and a few studies have suggested that binding to IgA interfered with interactions between bacteria and host cells (Fluckiger *et al.*, 1998). Since IgA is the predominant immunoglobulin defence against microbial infection of human mucosal surfaces, sequestering of IgA by the bacterium may be a mechanism for avoiding the first line of host defence. The binding to IgA might also block the interactions between the IgA Fc part and the host receptors (Johnsson *et al.*, 1999). In our work, when B-sIgA was immobilized on the membrane, no binding activities were found to labelled Mig. However, when Mig was immobilized on the membrane and tested with soluble B-sIgA, the purified α_2-M receptors of Mig bound B-sIgA (data not shown). This observation is in contradiction to previous studies, where soluble H-sIgA displayed a much lower binding activity than the immobilized H-sIgA to the streptococcal group B receptor; probably due to the fact that the SC molecule interferes with binding to that type of receptor (Faulmann & Boyle, 1991; Faulmann *et al.*, 1991; Lindahl & Stenberg, 1990). Although the binding activities of Mig and B-sIgA have to be further characterized, our data suggest that binding of Mig to B-sIgA might be different from the interaction of human streptococcal pathogens with H-sIgA.

The Mig protein of *S. dysgalactiae* expresses α_2-M and IgG-binding receptors (Jonsson & Müller, 1994), and is involved in resistance to phagocytosis (Song *et al.*, 2001). Here we report that Mig also binds B-IgA and B-sIgA. *S. dysgalactiae* infects cows in the dry period when the IgA levels in the mammary secretions are higher, and is capable of adhering, colonizing, invading and surviving inside bovine mammary gland epithelial cells (Calvinho & Oliver, 1998). Binding to B-IgA and B-sIgA by *S. dysgalactiae* may mask the bacteria, allowing avoidance of the first line of host defence. The factors of *S. dysgalactiae* involved in the invasion of MAC-T cells by a receptor-mediated endocytosis mechanism remain undetermined (Calvinho & Oliver, 1998). Thus, it is conceivable that the IgA receptor on the MAC-T cell surface binds to IgA-covered *S. dysgalactiae* cells, allowing the bacterium to be internalized into the epithelial cell. Experiments are being carried out to investigate the role of B-sIgA and B-IgA in the interaction of *S. dysgalactiae* with mammary gland host cells.

Cleary & Retnoningrum (1994) proposed a very exciting role for immunoglobulin-binding proteins of streptococci. These authors suggested that by binding to different immunoglobulins, streptococci encoding M-like proteins might be able to act as macromolecules that distinguish specific tissues or fluids in the body. In the case of Mig, this protein could act as the sensory component of a multiple component system whereby binding of IgG and/or IgA to Mig could trigger a conformational change on this protein resulting in the activation of secondary proteins with histidine-kinase activities that result in modulation of gene expression of factors involved in virulence.

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