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M-Like Proteins of *Streptococcus dysgalactiae*

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***Streptococcus dysgalactiae* is one of the most important bacterial species isolated from bovine mastitis. To identify potential virulence factors of this species we prepared chromosomal DNA from strain 8215 and constructed a phage display library. By affinity selection of the library against fibrinogen (Fg), we isolated and characterized a gene, called *demA*, encoding a protein with the molecular mass of ~58 kDa, called DemA, displaying both plasma protein binding properties and sequence similarities with the M and M-like proteins of other streptococcal species. Purified recombinant DemA protein was found to completely inhibit Fg-binding to cells of *S. dysgalactiae*. A continued sequence analysis revealed that the *demA* gene was preceded by an open reading frame (*dmgA*) coding for a putative protein, called DmgA, with high similarities to the Mga proteins of *Streptococcus pyogenes*. By additional cloning, the corresponding *dmgA* and *demA* genes from another strain, called Epi9, were isolated and analyzed. These genes, called *dmgB* and *demB*, respectively, revealed a high degree of similarity to the corresponding genes in strain 8215. Increased binding of Fg by cells of strain Epi9, grown in an atmosphere with 10% CO_2, was correlated to an enhanced transcription of the *demB* gene as shown in a Northern blot. Strain 8215 did not respond to CO_2, which could be explained by a nonfunctional *dmgA* gene due to insertion of an insertion sequence element. Based on sequence similarities of the described proteins to Mga, M, and M-like proteins and the response to elevated level of CO_2, we suggest that the *dmg* and *dem* genes are members of a regulon similar to the described *mga* regulon in *S. pyogenes*, which encodes several virulence factors in this species.**

Streptococcus dysgalactiae, a Lancefield group C α-hemolytic streptococcus species, is a common pathogen in subclinical and clinical mastitis causing substantial economic losses in dairy herds (6, 41). The bacterium expresses various extracellular and cell surface bound proteins which specifically interact with plasma or connective tissue proteins of the host (21, 26, 35, 39) and are assumed to play an important role for the colonization and persistence of the pathogen in the host. Genes of various *S. dysgalactiae* isolates coding for proteins binding α_2-macroglobulin (α_2M), albumin, immunoglobulin G (IgG), and fibronectin have been cloned and characterized (15–17, 22, 43). The interaction between the α_2M-protease complex and cells of streptococci reduces the phagocytosis of the bacteria by polymorphonuclear leukocytes (42). Binding of IgG from various animal species in a nonimmune fashion via the constant region of the molecule is considered to interfere in various ways with the recognition of the streptococcal cells by the host immune system (3, 9, 34). Recent publications on colonization and invasion of host epithelial cells by group A streptococci revealed the importance of the fibronectin-binding property and the M1 protein in this process (10, 31). The invasion of epithelial cells of bovine mammary glands by *S. dysgalactiae* has also been shown, although the bacterial factors involved have not been identified (1, 4, 5).

Searching for additional potential virulence factors in *S. dysgalactiae*, a phagemid library was made from strain 8215, the strain from which the *mag* gene (which encodes a protein binding α_2M, albumin, and IgG) was cloned (16). Since fibrinogen (Fg) binding is common among mastitis isolates of *S. dysgalactiae* (26, 35) and is also a feature expressed by M or M-like proteins, the library was affinity selected against this protein. Phagemid clones binding to Fg were isolated, and the inserts of chromosomal DNA in the corresponding phagemid vectors were sequenced. A gene called *demA*, coding for both Fg- and IgG-binding capacity, was isolated and analyzed. By additional cloning, a gene similar to *demA* was isolated from another strain of *S. dysgalactiae* and named *demB*. Directly upstream of the respective *dem* genes we recognized genes, called *dmgA* and *dmgB*, which, when deduced, revealed similarities to Mga proteins in *Streptococcus pyogenes* known to be involved in the regulation of the expression of various virulence factors (7, 33). Using Northern blot assays, the transcription of the *dmg* and *dem* genes was studied under different growth conditions.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *S. dysgalactiae* strains isolated from bovine mastitis cases from different parts of Sweden were obtained from the National Veterinary Institute, Uppsala, Sweden. The strains were kept on blood agar plates and were cultured overnight in Todd-Hewitt broth (Oxoid, Basingstoke, Hampshire, United Kingdom) supplemented with 0.3% (wt/vol) yeast extract (Oxoid) under slow agitation at 37°C. In some experiments, the bacteria were grown in an incubator (Forma Scientific Inc., Marietta, Ohio) with an atmosphere enriched with 10% CO_2. *Escherichia coli* TG1 [*supE hsd*Δ*5 thi* Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*)] was used for the construction of phagemid library and for the production of phage stocks. *E. coli* DH5α [*supE44* Δ*lacU169* (ϕ80 *lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] was used for the expression of recombinant proteins. The *E. coli* strains were grown in Luria-Bertani (LB) medium supplemented, when appropriate, with 100 μg of ampicillin per ml, and alternatively on LA plates (LB medium supplemented with 1.5% agar and 100 μg of ampicillin per ml). All incubations were at 37°C. The phagemid vector pG8H6 (14) was used to construct the phage display library. For additional cloning, the plasmid vectors pUC19 and pGEX-2T (Amersham Pharmacia Biotech, Uppsala, Sweden) were used.

Proteins and reagents. Restriction endonucleases and *Taq* DNA polymerase were either from Amersham Pharmacia Biotech or MBI Fermentas (Vilnius, Lithuania). Vent$_R$ DNA polymerase with 3′→5′ proofreading exonuclease activity was from New England Biolabs (Beverly, Mass.). Human Fg was obtained from IMCO Corporation Ltd. (Stockholm, Sweden) and rabbit anti-human Fg IgG conjugated to horseradish peroxidase (HRP) was purchased from Dakopatts A/S, Glostrup, Denmark. Rabbit anti-sheep IgG-HRP was the product of Southern Biotechnology Associates, Inc., Birmingham, Ala. Bovine serum albumin (BSA; fraction V) was from USB (Cleveland, Ohio). Reagent-grade human IgG

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was from Sigma Chemical Co. (St. Louis, Mo.). Human IgG Fab and Fc fragments were the products of Rockland (Gilbertsville, Pa.). Human Fg and the recombinant protein GDEMA8 were iodinated with ^{125}I (Amersham, Little Chalfont, England) by the Iodo-Beads labelling method according to the description of the manufacturer (Pierce, Rockford, Ill.). Molecular weight markers used in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad (Richmond, Calif.). Nitrocellulose (NC) filters, BA-S 85, and Hybond-C used for Western blotting were from Schleicher and Schuell (Dassel, Germany) and Amersham Pharmacia Biotech, respectively. Nylon membrane (Hybond-N$^+$) used for Southern blotting was the product of Amersham Pharmacia Biotech. Sterile filters (Minisart N; pore size, 0.45 μm) were obtained from Sartorius AG (Göttingen, Germany).

Construction of phagemid libraries of *S. dysgalactiae*. All DNA manipulations were performed by standard methods (37). The shotgun phage display library was constructed essentially as described by Jacobsson and Frykberg (13, 14). Short chromosomal DNA of *S. dysgalactiae* strain 8215 was prepared and fragmented by sonication. After different time intervals, aliquots were analyzed on an agarose gel. A sample containing a majority of DNA fragments with sizes of ~1,000 bp and without prior size fractionation was made blunt ended with T4 DNA polymerase. The DNA fragments were ligated, with the Ready-To-Go T4 DNA ligase kit (Amersham Pharmacia Biotech), into an *Sma*I-digested and dephosphorylated pG8H6 vector. Electrotransformation of the ligated material into *E. coli* TG1 cells yielded $\sim 1.2 \times 10^8$ ampicillin-resistant transformants. A 5-ml sample of an overnight culture of the electroporated bacteria was infected with helper phage R408 at a multiplicity of infection (MOI) of 20, melted agar was added to 0.5%, and the mixture was distributed over 20 LA plates. After incubation overnight, the phage particles were eluted from the soft agar by vigorous shaking. The suspension was centrifuged (15,000 × *g*), followed by sterile filtration, and the titer of the phage display library was determined to be 3×10^{10} CFU/ml.

Panning of phagemid library and identification of Fg-binding clones. Microtiter wells (Maxisorp Nunc, Copenhagen, Denmark) were coated overnight at 4°C with 200 μl of human Fg at a concentration of 100 μg/ml in 0.05 M $NaHCO_3$ (pH 9.7). The wells were blocked by incubation with phosphate-buffered saline (PBS) and 0.05% Tween 20 (PBST) containing BSA (final concentration, 1 mg/ml) for 1 h at room temperature (RT). After washing with PBST, 600 μl of the phagemid library was added to each of three coated wells and the wells were incubated overnight at 4°C. Before elution, the wells were extensively washed with PBST at RT, and then they were eluted stepwise with 200-μl buffer solutions consisting of 50 mM Na citrate and 150 mM NaCl with decreasing pH (5.4, 3.4, and 1.9). The eluates were neutralized by the addition of 75 μl of 2 M Tris-HCl (pH 8.6). From the eluates, 50 μl was used to infect 20 μl of *E. coli* TG1 cells (overnight culture) and 100 μl of fresh LB was added. After a 20-min incubation at 37°C, the cells were spread on LA plates containing 2% glucose. The plates were incubated overnight, and colonies derived from fractions eluted at pH 3.4 and 1.9 were resuspended in LB medium and pooled. After infection with helper phage R408 at a MOI of 20, the sample was mixed with 5 ml of 0.5% LB soft agar and poured onto an LA plate. After incubation overnight, the phagemid particles were eluted and subjected to another round of panning as described earlier (13, 14). Finally, after the second panning, individual clones were grown in small scale for preparation of phagemid DNA in order to sequence the inserts.

Panning of selected phagemid particles against IgG and BSA. Stocks from phagemid clones harboring fragments of different size of the *demA* gene were prepared and analyzed for the presence of additional binding capacity to human IgG, bovine IgG, and BSA. The phagemid stocks were prepared as follows. A volume of 500 μl of *E. coli* TG1 cells harboring the appropriate phagemid was infected with helper phage R408 (MOI of 20). After propagation in soft agar on an LA plate, the phagemid particles were eluted as described above. The generated phage stocks (3×10^7 CFU/ml) were panned in duplicate (200 μl/microtiter well) against immobilized human IgG, bovine IgG, and BSA, and as a positive control, they were panned against human Fg for 2 h at RT. Wells coated with hen egg ovalbumin served as a negative control. The wells were extensively washed with PBST and eluted with 200 μl of buffer solution containing 50 mM Na citrate–150 mM NaCl (pH 2). The eluate was immediately neutralized by addition of 15 μl of 1 M Tris-base (pH 11). Aliquots of the eluted phagemid particles were used to infect *E. coli* TG1 cells and plated on LA plates supplemented with 2% glucose to determine the CFU.

Production and purification of GST fusion proteins. Two DNA fragments were PCR amplified from the pDEMA6 clone harboring the *demA* gene with a combination of primer pairs of O1 (5′-CGCGGATCCAAGGCTAATGACGATATTTAC-3′), O2 (5′-CCGAATTCCGCTATCCACAGTATTGAGAA-3′), and O3 (5′-CCGAATTCAACCTGTTGATGGTAATTGTA-3′). The underlined sequences of the primers hybridize to the following positions at the 5′ end of the *demA* gene: O1, 118 to 138; O2, 818 to 837; and O3, 1554 to 1535. The primers were designed in such a way that the forward primer included the *Bam*HI site while the reverse ones contained the *Eco*RI restriction site in the 5′ end. The amplifications were made with $Vent_R$ DNA polymerase in order to minimize the possibility of errors in the nucleotide sequence produced by the PCR. pGEX-2T was double digested with the *Bam*HI and *Eco*RI restriction endonucleases and dephosphorylated with calf intestine alkaline phosphatase. The two PCR products were also cleaved with *Bam*HI and *Eco*RI and then ligated into the linearized pGEX-2T vector, resulting in the plasmid constructs pGDEMA7 and pGDEMA8, respectively. The constructs were electroporated into *E. coli* DH5α cells, and positive colonies were identified by colony blotting with ^{125}I-Fg. The production and purification of the two glutathione *S*-transferase (GST) fusion proteins from the isolated transformants were performed by following the manufacturer's recommendations. GST protein alone was prepared as a control.

SDS-PAGE and Western blot analysis. The purity of the recombinant proteins was analyzed by SDS-PAGE, using precasted 8 to 25% gradient gels and the PhastSystem (Amersham Pharmacia Biotech). The gels were stained with Coomassie blue. To test the binding activities, four parallel sets with the two recombinant proteins (GDEMA7 and GDEMA8) and the control (GST protein alone) were run on gels and diffusion blotted to NC membranes for 30 min at 65°C. The membranes were cut in such a way that every strip contained all three proteins. The filter strips were subsequently blocked in PBS–0.5% (wt/vol) ovalbumin–0.05% Tween 20 for 2 h at RT. Each filter was then incubated for 2 h with a different ligand: HRP-conjugated rabbit IgG (1:1,000 dilution), HRP-conjugated human IgG (1:250 dilution), or ^{125}I-labelled human Fg or unlabelled horse IgG (10 μg/ml). The interaction of the unlabelled horse IgG with the proteins was detected by additional incubation of the filters with HRP-conjugated Protein G (Amersham Pharmacia Biotech) for 2 h. After extensive washing, the bound HRP-labelled ligands were detected with 4-chloro-1-naphthol (Serva, Heidelberg, Germany) as substrate. The captured ^{125}I-Fg was visualized by exposing the blot to Biomax MS (Kodak) film for 24 h at −70°C.

Binding of ^{125}I-labelled GDEMA8 protein to separated chains of human Fg and to Fab and Fc fragments of human IgG was analyzed in a similar Western blot assay. After boiling Fg in a reducing sample buffer (62 mM Tris-HCl, pH 6.8, 9% glycerol, 2% SDS, 40 mM dithiothreitol, and 0.025% bromophenol blue), the resulting chains were separated on a 12.5% homogeneous gel. The IgG fragments were run under nonreducing conditions on the same gel. The proteins were transferred to an NC membrane and probed with ^{125}I-GDEMA8 protein, and the membrane was exposed to Biomax MS (Kodak) film for 48 h at −70°C.

Comparative binding assays with *S. dysgalactiae* strains 8215 and Epi9. Strains 8215 and Epi9 were cultured overnight in Todd-Hewitt broth–0.3% yeast extract in either normal or 10% CO_2-enriched atmosphere. The cells were harvested by centrifugation, and the pellets were washed twice with ice cold PBS–1 mM NaN_3. The cells were resuspended in PBS–0.1% (wt/vol) ovalbumin–0.1% Tween 20–1 mM NaN_3, and the optical density (OD) of the suspensions at 600 nm (OD_{600}) was adjusted to 0.6. Four hundred microliters of each cell suspension was mixed in Eppendorf tubes with 100 μl of ^{125}I-Fg (specific activity, 113,000 cpm). The tubes were incubated on a rolling drum for 2 h at RT. The cells were pelletized by centrifugation and washed twice with PBS–0.1% Tween 20. The radioactivity associated with the cells was measured in a γ-counter. *Streptococcus zooepidemicus* ZV with high Fg-binding capacity (H. Lindmark, personal communication) was used as a positive control while *E. coli* TG1 served as a negative control in the assays.

Competitive binding assays with *S. dysgalactiae* strains 8215 and Epi9. The capacity of the pGDEMA8-encoded fusion protein, called GDEMA8, and the control GST protein to inhibit the binding of ^{125}I-Fg to cells of *S. dysgalactiae* strains was studied. The assays were performed similarly to the binding experiments, with only slight modifications. Shortly, cell suspensions of *S. dysgalactiae* 8215 and Epi9 were prepared in PBS–0.1% (wt/vol) ovalbumin–0.1% Tween 20–1 mM NaN_3 from cultures grown overnight in ambient atmosphere. The OD_{600}s of the cell suspensions were adjusted to 1.4 for strain 8215 and to 0.75 for strain Epi9. Serial dilutions of GDEMA8 and GST proteins were made in PBS–0.1% (wt/vol) ovalbumin–0.1% Tween 20–1 mM NaN_3, and 100 μl of each dilution step was mixed with 300 μl of the respective cell suspension and 100 μl of ^{125}I-Fg. The tubes were incubated on a rolling drum for 4 h at RT. After washing with PBS–0.1% Tween 20, the radioactivity associated to the pellets was determined as described above.

DNA sequencing and homology studies. The nucleotide sequences were determined with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit and the ABI Model 373A DNA sequencer. Specific synthetic primers were purchased from Life Technologies (Rockville, Md.). Computer programs from PCGENE (Intelligenetics Inc., Mountain View, Calif.), a DNA and protein sequence analysis software package, were used to record and analyze the sequence data. The databases Genbank, EMBL, Swissprot, and PIR were screened for sequence homologies. Prediction of the coiled-coil secondary structure of the protein was made on the COIL web server (http://ulrec3.unil.ch/software/COIL_form.html) as described by Meehan et al. (29).

Detection of *demA* in strains of *S. dysgalactiae*. Genomic DNA was prepared from mastitis isolates of *S. dysgalactiae* as described before (18). The DNA samples were digested with *Bam*HI and *Eco*RI restriction endonucleases, and the cleavage products were separated by agarose gel electrophoresis. The fragments were vacuum blotted to a nylon membrane and probed with an [α-^{32}P]CTP-labelled insert of pGDEMA8. The bands hybridizing with the probe were visualized by exposing the filter to Biomax MR (Kodak) film.

Cloning of the *mga*-like and *demA*-like genes from *S. dysgalactiae* strain Epi9. PCRs were performed with chromosomal DNA of 10 mastitis isolates of *S. dysgalactiae* to identify strains which contain the complete *mga*-like gene, but not the ΔIS-*dmgA* junction. Primer pair O4 (5′-CAGATTATCCAACAGACTTGAATGAA-3′) and O5 (5′-CCTTAGACTCACCTACAACA-3′), complementary to nucleotide positions 28 to 52 and 402 to 421, respectively, in the insert of the

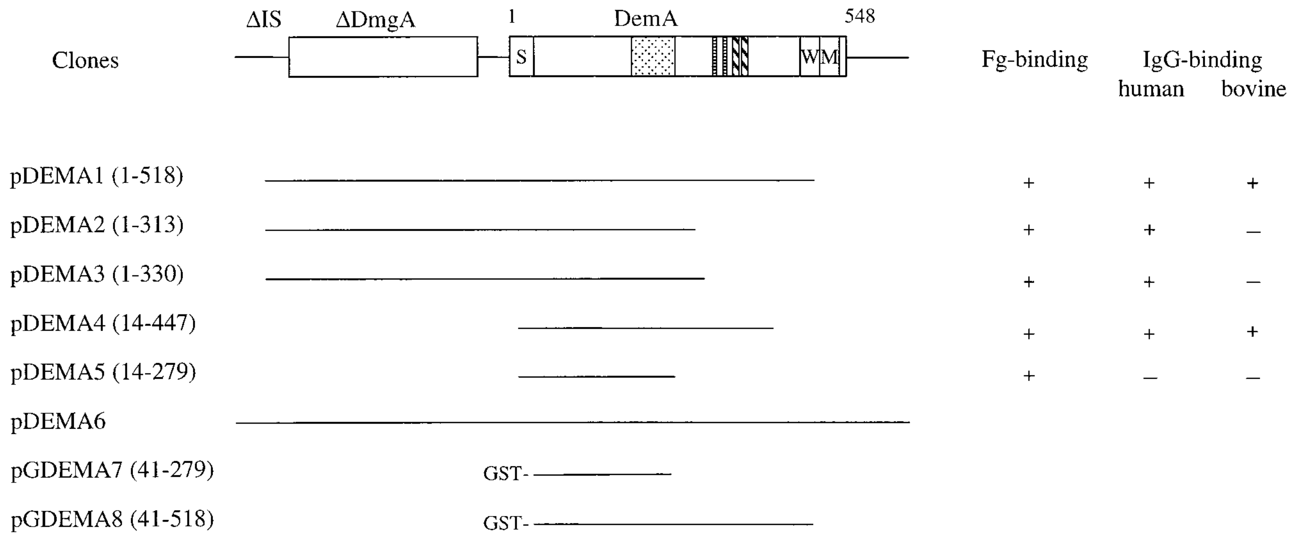

FIG. 1. Schematic presentation of deduced gene products encoded by the pDEMA6 clone derived from *S. dysgalactiae* 8215. pDEMA1 to pDEMA5 are phagemid clones isolated after panning a phage display library of strain 8215 against Fg. pGDEMA7 and pGDEMA8 are expression clones of the pGEX-2T vector with inserts corresponding to the phagemid clone pDEMA5 and a PCR fragment derived from pDEMA1 encoding amino acids 41 to 518 of DemA, respectively, yielding GST fusion proteins. The figures within parentheses after the names of the clones indicate the encoded amino acids of protein DemA. The DemA protein, including the signal peptide, consists of 548 amino acid residues as indicated above the schematic drawing. S, signal peptide, followed by the cell surface exposed part of the protein with repetitive sequences marked by different patterns; W, the cell wall spanning region; M, the membrane spanning region directly followed by five charged amino acids; ΔDmgA, a truncated protein highly homologous to the Mga regulatory protein from *S. pyogenes* (32); ΔIS, 276 bp homologous to an IS element. +, the phagemid clone can bind Fg, human IgG, and bovine IgG; −, no detectable binding.

pDEMA6 clone were used to amplify the ΔIS-*dmgA* junction. Primer pair O6 (5′-TGTTGTAGGTGAGTCTAAGG-3′) and O7 (5′-ATTGTCCCATTCTGA TCCTA-3′), hybridizing to nucleotide positions 402 to 421 and 1209 to 1228, respectively, in the insert of the pDEMA6 clone (corresponding to nucleotides 126 to 145 and 933 to 952, respectively, in the *dmgA* gene) were selected to amplify the 3′ end of the *dmgA* gene.

Chromosomal DNA from strain Epi9 was double digested with restriction enzymes *Bam*HI and *Eco*RI. After agarose gel separation of the fragments, the DNA band containing the *dmgA*-like gene was identified in a Southern blot assay with a radioactive probe. The probe was a product of PCR amplification from chromosomal DNA with the primer pair O6 and O7. DNA fragments with the appropriate size were purified from a preparative agarose gel and ligated into a *Bam*HI- and *Eco*RI-cleaved and 5′ dephosphorylated pUC19 vector. Positive colonies were detected by colony hybridization with the same radioactive probe as before, and the inserts were sequenced. One clone, called pDMGB1, was selected for further studies.

A PCR fragment corresponding to the *demB* gene was generated from chromosomal DNA of strain Epi9 with primers O8 (5′-TATCTTAGGATCAGAAT GG-3′) and O9 (5′-ACCTGTTGATGGTAATTGTA-3′), which hybridized to nucleotides 928 to 946 of the *dmgA* gene (corresponding to nucleotides 1204 to 1222 in the insert of pDEMA6) and to nucleotides 1535 to 1554 of the *demA* gene (corresponding to nucleotides 2942 to 2961 in the insert of pDEMA6), respectively. The generated PCR product was DNA sequenced. To obtain the 3′ end of the *demB* gene, an ~3-kb chromosomal DNA fragment was cloned into the pUC19 vector and sequenced.

Detection of RNA transcripts of the *dmg* and *dem* genes in Northern blot assay. Cultures of *S. dysgalactiae* strains 8215 and Epi9 were grown to mid-log growth phase (OD_{600}, ~0.5) in ambient as well as in 10% CO_2-enriched atmosphere, and total RNA was prepared from the cells with the FastRNA kit, blue (BIO 101, Inc., Vista, Calif.) according to the manufacturer's recommendation. Twenty micrograms of total RNA from each of the four samples in two parallel sets were separated on a 1% agarose gel as described by Kihlberg et al. (19) and vacuum blotted to a nylon membrane. One-half of the membrane with one set of samples was incubated in 6× SSC (1× is 0.15 M NaCl plus 0.015 M sodium citrate)–3× Denhart–0.5% SDS overnight at 65°C with a radioactive *dmgB*-derived probe (corresponding to nucleotides 654 to 1480) while the other half was hybridized with a *demB*-derived probe (corresponding to nucleotides 1107 to 1518). After repeated washes in 2× SSC–0.1% SDS at 65°C, the membranes were exposed to x-ray film for 72 h at −70°C. The *dmgB*- and *demB*-derived probes showed 94 and 99% nucleotide sequence identity, respectively, to the corresponding regions of the *dmgA* and *demA* genes of strain 8215.

Extraction of surface proteins from *S. dysgalactiae* isolates. A mutanolysine extraction procedure was used to obtain bacterial surface proteins from overnight cultures of *S. dysgalactiae* strains 8215 and Epi9 essentially as described by Meehan et al. (29). The extracted material was subjected to SDS-PAGE analysis and used in Western blot assays for detection of Fg-binding activity.

Nucleotide sequence accession numbers. The novel nucleotide sequences have been deposited in the EMBL sequence data bank and are available under accession no. AJ243529 (harboring the *dmgA* and *demA* genes of strain 8215) and AJ243530 (harboring the *dmgB* and *demB* genes of strain Epi9).

RESULTS

Identification of phagemid clones encoding specific Fg-binding activity. A shotgun phage display library was made with fragmented chromosomal DNA from *S. dysgalactiae* 8215 and affinity selected against Fg. Phagemid clones were chosen from the second cycle of affinity selection. DNA sequence analysis of the junctions between the inserts and the vector showed that several clones had overlapping inserts, which could be arranged into five groups represented by pDEMA1 to pDEMA5 (Fig. 1). Alignment of the nucleotide sequences of the various inserts revealed a common overlapping core region. By analysis of the sequence of the ~2.8-kb insert of clone pDEMA1, two open reading frames (ORFs) were found. Alignment of the sequences of additional phagemid clones indicated that the Fg-binding activity was coded by the ORF located in the 3′ end of the insert in pDEMA1. Analysis of the deduced amino acid sequence revealed a C-terminally truncated protein. To isolate the 3′ end of the gene, Southern blot analysis was performed, in which chromosomal DNA from strain 8215 was digested with various restriction enzymes. The insert of phagemid clone pDEMA5 was used as a probe (data not shown). The result showed that the probe hybridized to a 3.5-kb *Bam*HI fragment which was subsequently cloned into the vector pUC19, resulting in the clone pDEMA6. Additional sequencing of the insert in pDEMA6 revealed that the complete gene, called *demA*, is 1,644 nucleotides long, starting with an ATG codon at nucleotide position 1408 (amino acid residue 1 of DemA in Fig. 1) and ending with a TAA codon at position 3052 of the insert

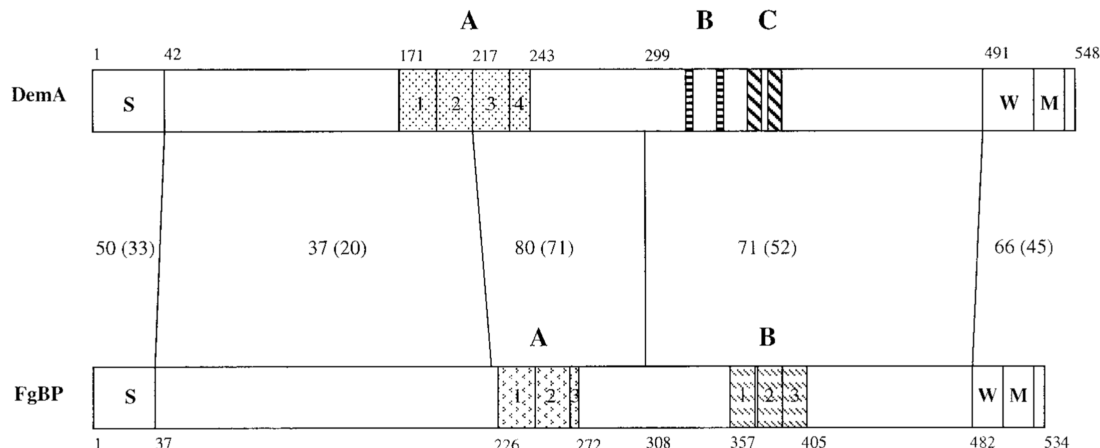

FIG. 2. Schematic presentation of the alignment of the primary structures of the Fg-binding proteins DemA from *S. dysgalactiae* 8215 and FgBP from *S. equi* TW (29). The deduced amino acid sequences of the molecules were aligned by the PALIGNE program of PCGENE and are shown as schematic bars. Numbers along the bars indicate positions of amino acids. Numbers between the bars indicate the percentage of similar residues relative to the total number of amino acids within the compared domains, marked by connecting vertical lines. The percentages of identical residues were calculated in a similar way and are shown within parentheses. S, signal peptide; A, B, and C, various repeated sequences in the respective molecules, each marked with different patterns; W, wall spanning regions; M, membrane spanning regions directly followed by five charged amino acids.

(accession no. AJ243529). The ORF is preceded by a sequence typical for a ribosome-binding site of gram-positive cocci and followed by sequences resembling a transcriptional termination signal. The gene codes for a protein, called DemA, consisting of 548 amino acid residues. The deduced amino acid sequence revealed a primary structure typical for cell surface bound proteins of gram-positive cocci (Fig. 2). The N-terminal part of the protein consists of a predicted signal sequence with a possible cleavage site between amino acid residues 41 and 42, resulting in a mature protein with 507 amino acids and a molecular mass of ~54 kDa. At 128 amino acid residues downstream of the signal peptide there is a region, called A (amino acid positions 171 to 243), consisting of three complete repetitive sequences, A1, A2, and A3, and one incomplete repeat, A4. One A repeat unit contains 21 amino acids, and the truncated last unit, A4, contains only 10 amino acids. Comparisons of the individual repeats to A1 show that they are almost identical. In A2, there are three amino acids changed relative to A1, while A3 differs only in two amino acids. In both A2 and A3, one of the changes has a conserved site (amino acid residue 11). The second change in A3 is after the lysine residue at position 16, while in A2, both amino acids 15 and 17 are changed. In the C-terminal part of the protein there are also two short repetitive sequences (Fig. 2, B and C) consisting of the motifs SDLA and SEAKVA(E/K)L, which are repeated twice. The proposed cell wall spanning region, called W, is rich in serine residues and ends with the conserved cell wall anchoring motif LPSTG (38). This region is directly followed by a row of almost exclusively hydrophobic amino acids which corresponds to a cell membrane spanning region, called M. The protein ends with five charged residues in the C terminus. Secondary structure analysis of the deduced protein with the COIL program (23, 24) suggests that the protein possesses an extremely high probability (80 to 100%) of forming a coiled-coil structure, except for the first 82 residues and residues 494 to 548 within the C-terminal W and M regions.

Proteins similar to DemA. Searching for homologies to the amino acid sequence of DemA in databases revealed various levels of similarity to the M and M-like proteins in other streptococci. The highest overall identity, 41%, was observed in comparing DemA to proteins FgBP and SeM, two almost-identical M proteins of *Streptococcus equi* differing in only six amino acids (29, 40). The similarity resides mainly in the middle and in the C-terminal parts of the molecules, where stretches of identical residues are found. However, the signal sequences and the following N-terminal parts of these proteins are divergent (Fig. 2). There are also repeats present in the FgBP and SeM proteins, as shown in Fig. 2. Alignment of repeats A1 to A4 of DemA to repeats A1 to A3 of FgBP reveals a stretch of amino acids, VSKDLADKL, present in all repeat units except for repeat A3 in FgBP, which contains only the LADKL sequence.

Additional binding activities of the DemA protein. Phage stocks of clones pDEMA1 to pDEMA5 (Fig. 1) were additionally panned against human IgG, bovine IgG, and BSA in order to determine whether they, in addition to binding Fg, also bound these plasma components. Fg was used as a positive control and ovalbumin was used as a negative control in the assays. Figure 1 shows that clones pDEMA1 to pDEMA4 reacted with human IgG while clone pDEMA5, which represents the shortest Fg-binding clone coding for the N-terminal half of the DemA protein, showed no reactivity to human IgG. The interaction with bovine IgG was observed only for clones pDEMA1 and pDEMA4. Concerning the Fg-binding activity, there was no difference between the five phagemid clones, indicating that the Fg-binding region is more N terminal relative to the IgG-binding domain. The phages did not reveal any binding to BSA (data not shown).

Characterization of the recombinant proteins GDEMA7 and GDEMA8. Recombinant proteins corresponding to the shortest phagemid clone (pDEMA5, amino acids 41 to 279) and to the mature DemA protein (amino acids 41 to 518) were expressed as fusions to GST (Fig. 1 and 3a, A). The two recombinant proteins, affinity purified on glutathione-Sepharose, bound ^{125}I-labelled Fg in a Western blot assay (Fig. 3a, B). To further analyze the IgG-binding activity of DemA observed in the panning experiments with the phage particles (Fig. 1), we performed a Western blot assay with human, rabbit, and horse IgG. The result showed that the GDEMA8 protein, but not the GDEMA7 protein, bound to the different IgGs (Fig. 3a, C to E). Additional Western blot assays revealed that protein GDEMA8 interacts predominantly with the

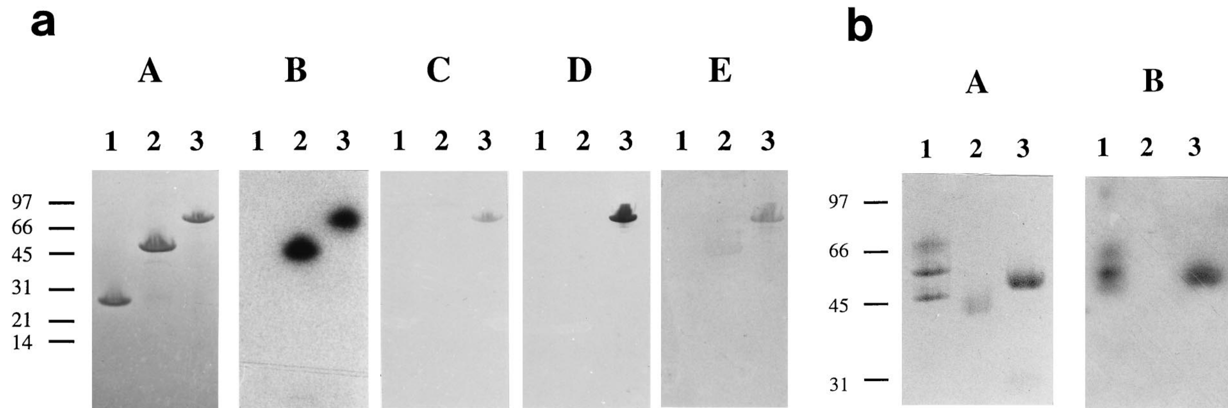

FIG. 3. SDS-PAGE and Western blotting. (a) The affinity-purified GST fusion proteins coded by pGDEMA7 and pGDEMA8 clones derived from *S. dysgalactiae* strain 8215 were separated on an SDS-PAGE gel (8 to 25% polyacrylamide) (A) and blotted to an NC membrane. Binding of Fg by the recombinant proteins was tested by probing with ^{125}I-Fg and visualized by exposing the strips to x-ray film (B). IgG binding by the recombinant proteins was probed with HRP-labelled human (C) and rabbit (D) IgG while HRP-labelled protein G was used to detect the bound unlabelled horse IgG (E). Lanes: 1, GST; 2, GDEMA7; 3, GDEMA8. (b) Human Fg in reducing sample buffer and Fab and Fc fragments of human IgG in nonreducing sample buffer were separated on a homogeneous SDS–12% PAGE gel (A), transferred to an NC membrane, and probed with ^{125}I-GDEMA8 protein. The bound probe was detected by exposing the membrane to x-ray film (B). Lanes: 1, Fg (from the top: α-, β-, and γ-chains); 2, human IgG Fab fragment; 3, human IgG Fc fragment. Molecular mass markers (kDa) are indicated.

β-chain of Fg and bound the Fc fragment but not the Fab fragment of human IgG (Fig. 3b, B).

Occurrence of the *demA* gene in various isolates of *S. dysgalactiae*. The presence of *demA* in 10 different strains, isolated from cases of bovine mastitis in different areas of Sweden, was investigated. A PCR-generated fragment corresponding to the insert of the pGDEMA8 clone encoding the mature DemA protein was radioactively labelled and used as a probe. Chromosomal DNA of the isolates was double digested with the restriction enzymes *Bam*HI and *Eco*RI, and the fragments were separated by agarose gel electrophoresis. The DNA was transferred to a nylon membrane and incubated with the radioactive probe. The assay revealed that a gene homologous to *demA* was present in all tested isolates (data not shown).

***demA* gene is preceded by gene resembling *mga* gene of *S. pyogenes*.** Sequencing of the phagemid clone pDEMA1 revealed that ~170 bp upstream of the *demA* gene there was an additional ORF present (Fig. 1). Comparison of the sequence to databases revealed homology to the *mga* genes of *Streptococcus pyogenes*. However, the *mga*-like gene, called *dmgA* (*mga*-like gene of *S. dysgalactiae*), of strain 8215 lacked ~500 bp of the 5′ end of corresponding *mga* genes in *S. pyogenes*, but surprisingly, the 276 bp at the 5′ end of the DNA fragment showed high homology to an earlier described insertion sequence (IS) element, IS*199*, from *Streptococcus mutans* (25). In order to clone the intact *dmgA* gene, two parallel PCRs were performed with chromosomal DNA from *S. dysgalactiae* isolates tested earlier for the presence of the *demA* gene. One primer set, O4 and O5, was designed to amplify the ΔIS-*dmgA* junction. The second set of primers, O6 and O7, was used to amplify the 3′ end of the *dmgA* gene. The results of the two PCRs revealed isolates which contained the *dmgA*-like gene without the ΔIS-*dmgA* junction.

Strain Epi9, lacking the sequence of the ΔIS-*dmgA* junction, was selected for the cloning of the expected intact *mga*-like gene. After restriction enzyme cleavage of the chromosomal DNA of Epi9 and identification by Southern blot assay of the fragment harboring this gene, an ~6-kb *Eco*RI-*Bam*HI fragment was cloned into plasmid vector pUC19. The nucleotide sequence of the insert corresponding to an *mga*-like gene, called *dmgB*, was determined (accession no. AJ243530). The deduced amino acid sequence of the *dmgB* gene revealed the highest identity (~45%) to the known *mga* gene product, Mry, of *S. pyogenes* D471, according to recent nomenclature equal to Mga (Fig. 4) (32). The N-terminal part of DmgB also contains a region highly homologous to the predicted helix-turn-helix motif of Mry (Fig. 4) (33) while the C-terminal part resembles, like in Mry (amino acids 404 to 528) (28, 32), the receiver domain of response proteins of bacterial two-component regulatory systems. The sequence of the truncated Mga-like protein, DmgA, present in strain 8215 is identical to the corresponding region in the deduced protein, called DmgB, from strain Epi9, with the exception of five amino acid residues. However, in a Northern blot assay, we could detect RNA transcript only from the *dmgB* gene, not that from the *dmgA* gene, with a specific probe (826 bp) derived from the *dmgB* gene (Fig. 5).

Analysis of the *demB* gene in strain Epi9. Chromosomal DNA of *S. dysgalactiae* strain Epi9 was PCR amplified with forward primer O8, which hybridized to the sequence corresponding to the 3′ end of the *dmgA* gene, while the reverse primer O9 annealed to the nucleotides coding for the cell wall anchoring sequence QLPSTG in protein DemA. After sequencing the PCR fragment, we found an ~1.5-kb ORF coding for a C-terminally truncated protein. By additional cloning, the missing 3′ end of the gene, called *demB*, was obtained (accession no. AJ243530). Analysis showed that the *demB* gene consists of 1,608 nucleotides encoding a protein, called DemB, of 536 amino acid residues with a molecular mass of ~57.5 kDa. The alignment of the amino acid sequence of this protein to DemA revealed high similarity. The predicted signal sequences of the two proteins are 100% homologous, but the region preceding the A repeats of both proteins (between residues 42 and 152 of DemB) showed only 44% similarity (36% identity). Comparisons of the remaining parts of the proteins revealed 97% similarity (93% identity). Furthermore, the DemB protein contains only 2.5 A repeats instead of the 3.5 repeats present in the DemA protein of strain 8215. Further analysis showed that 11 residues from the C-terminal part of A1 and 10 residues from the N-terminal part of the A2 repeat from the DemA protein in strain 8215 are deleted in DemB of strain Epi9.

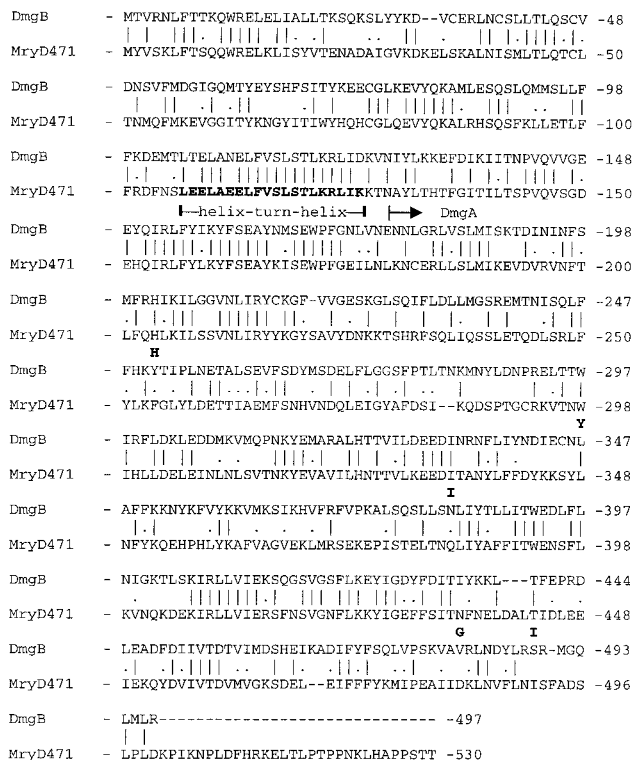

FIG. 4. Alignment of the amino acid sequences of proteins DmgB and Mry. The amino acid sequence of protein DmgB from *S. dysgalactiae* Epi9 was aligned with the Mry (later designated Mga) of *S. pyogenes* D471 (32) with the PALIGNE program of PCGENE. A vertical bar indicates identical residues while a dot indicates similar residues. Gaps introduced to improve the alignment are indicated by a dashed line. The horizontal arrow above the sequence indicates the beginning of the N-terminally truncated DmgA protein of *S. dysgalactiae* 8215. The positions of the five changed amino acids in DmgA in comparison to those in DmgB are indicated by bold letters above the sequence. The predicted helix-turn-helix motif in Mry (33) is indicated by bold letters within the sequence. The region in Mry containing amino acids 404 to 528 exhibits homology to the receiver domain of response regulators (32). The alignment reveals an overall 61% similarity (45% identity) between the DmgB and Mry proteins.

Expression of Fg binding in *S. dysgalactiae* strains 8215 and Epi9. Cells of the two isolates used for cloning of the *dem* and *dmg* genes were also tested for Fg binding. Cultured in ambient atmosphere, strain 8215 showed low (7,000 cpm) binding while Epi9 had moderate (13,000 cpm) binding to Fg in comparison to the *S. zooepidemicus* strain ZV (65,000 cpm), which served as a positive control in the radioimmunoassays. Cells of *E. coli* TG1 were used as a negative control (400 cpm). However, when the two isolates were grown in an atmosphere enriched with 10% CO_2, Epi9 showed a four- to sixfold increased capacity to bind Fg while the Fg binding of strain 8215 was unaffected (data not shown). Northern blot analysis also confirmed an increased transcription level of the *demB* gene in strain Epi9 grown in an atmosphere with elevated CO_2 (Fig. 5).

Mutanolysine extracts of both strains were subjected to SDS-PAGE, transferred to an NC membrane, and probed with ^{125}I-Fg. For both strains, positive signals were obtained in the range of 75 to 95 kDa (data not shown).

Competitive binding assays with *S. dysgalactiae* 8215 and Epi9. The capacity of the GDEMA8 recombinant protein to inhibit the binding of ^{125}I-Fg to cells was also tested in radioimmunoassays. This molecule corresponds to the mature DemA protein without the membrane spanning domain. The data in Fig. 6 show that the fusion protein was able to completely inhibit the binding of the labelled Fg to the cells of both strains while the GST protein alone had no inhibitory activity. Complete inhibition was also obtained in experiments with cells of Epi9 grown in the presence of CO_2 (data not shown).

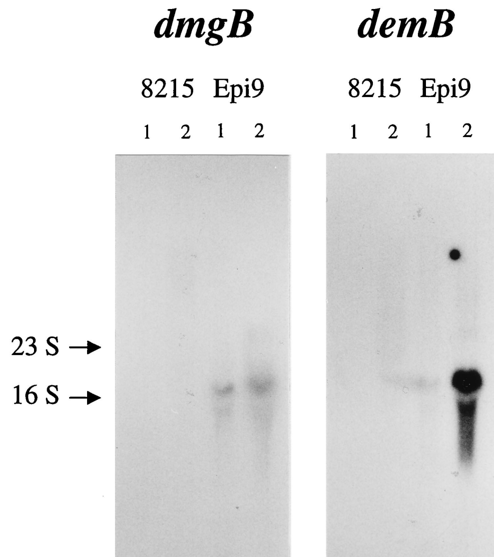

FIG. 5. Northern blot analysis of the RNA transcripts of the *dmg* and the *dem* genes. Twenty micrograms of total RNA isolated from cultures of *S. dysgalactiae* strains 8215 and Epi9 grown to mid-log phase in normal (lanes 1) or 10% CO_2-enriched (lanes 2) atmosphere were separated in an 1% agarose gel, vacuum blotted to a nylon membrane, and hybridized with specific DNA probes derived from *dmgB* and *demB* (as presented in Materials and Methods). The positions of the 23S and 16S rRNA bands are indicated.

DISCUSSION

During an infection, pathogenic bacteria like streptococci interact with their hosts by different mechanisms. Much interest has been focused on the interplay between the bacterial cell surface and the host. The M proteins have for a long time been considered one of the major virulence factors of *S. pyogenes* by mediating resistance to phagocytosis (11, 20, 36). Although not a prerequisite for inhibition of phagocytosis, Fg and IgG binding is a common feature among these proteins (9, 12). Concerning the virulence factors in *S. dysgalactiae*, our knowledge is limited (6). Using the phage display technique, we have continued to study the cell surface proteins expressed by this species. Construction of a phage display library and affinity selection of phagemid particles against Fg resulted in an enrichment of clones expressing Fg-binding activity (Fig. 1). By aligning the sequences of the inserts of these clones, it was possible to identify an ORF, which when deduced was found to encode a C-terminally truncated cell wall associated protein, called DemA, with a typical N-terminal signal sequence and a cell wall anchoring sequence (Fig. 1 and 2). By additional cloning, the complete *demA* gene was identified. The deduced amino acid sequence revealed characteristic features of a cell surface protein of a gram-positive bacterium. Further computer analysis predicted a protein with a typical coil-coiled secondary structure. Using different phagemid clones (Fig. 1) and the two GST fusion constructs (Fig. 1 and 3a, A), the Fg-binding domain was mapped to a 238-amino-acid region in the N-terminal part of the DemA protein (Fig. 1 and 3a, B). Another Western blot assay revealed that protein GDEMA8 bound predominantly to the β-chain of the Fg (Fig. 3b, B).

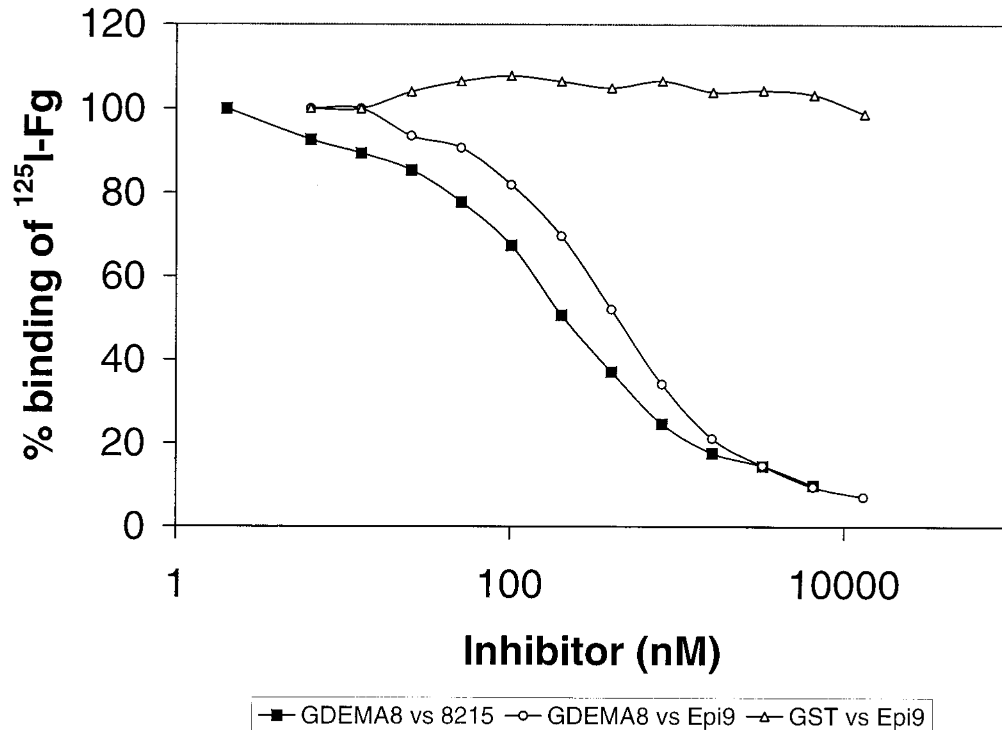

FIG. 6. Inhibition of binding of ^{125}I-Fg to *S. dysgalactiae* cells by recombinant DemA protein. Overnight cultures of *S. dysgalactiae* strains 8215 and Epi9 grown in ambient atmosphere were centrifuged and washed in PBS. The pellets were resuspended in PBS–0.1% ovalbumin–0.05% Tween 20, and the OD_{600}s were adjusted to 1.4 and 0.75, respectively. Three hundred microliters of the respective cell suspensions were mixed with 100-μl serial dilutions of the GST-DemA fusion protein GDEMA8 and 100 μl of the iodine-labelled Fg. The mixtures were incubated with end-over-end rotation for 4 h at RT. The cells were pelletized and washed twice in PBS–0.1% ovalbumin–0.05% Tween 20, and the radioactivity associated with the cells was measured in a γ-counter. A similar assay was performed with the GST protein as the inhibitor of the binding of ^{125}I-Fg to *S. dysgalactiae* Epi9 cells to exclude a possible effect of the GST part of the fusion protein on the binding.

Furthermore, the affinity-purified GST fusion protein GDEMA8 was found to completely inhibit the binding of labelled Fg to cells of strain 8215 of *S. dysgalactiae*, which suggests that the cell-surface-displayed Fg-binding activity is encoded by the cloned gene (Fig. 6). In the Northern blot assay, there was no RNA transcript of *demA* detected from strain 8215 despite the observed Fg-binding property of the cells. This is most likely due to a low level of transcription of the *demA* gene under the experimental conditions used.

The finding that the sequence of DemA has the same overall organization and partially displays a high degree of similarity to FgBP and SeM (Fig. 2), M proteins from *S. equi* subsp. e*qui*, is important, since these proteins are claimed to be major virulence factors in this species (29, 40), similar to M proteins in *S. pyogenes* (11, 36).

Since several M or M-like proteins from *S. pyogenes* are also able to bind other plasma proteins, e.g., IgG, the IgG-binding properties of the DemA protein were tested with phage stocks from various phagemid clones and the recombinant proteins GDEMA7 and GDEMA8 (Fig. 1, 3a [A, C to E], and 3b [A and B]). The protein was found to have a higher affinity to polyclonal human IgG compared to cow IgG, while no interaction was found with pig IgG (data not shown). In Western blots, the GDEMA8 protein reacted with human, rabbit, and horse IgGs (Fig. 3a, C to E) and bound only the Fc fragment of human IgG (Fig. 3b, B), thus having IgG-binding properties similar to type IIa IgG-binding proteins of group A streptococci (2). It is therefore possible that strain 8215 expresses two unrelated IgG Fc-binding molecules, proteins MAG (16) (a type III IgG-binding protein) and DemA, on the cell surface.

Interestingly, despite the high sequence homology between the DemA protein of *S. dysgalactiae* and the FgBP protein of *S. equi* subsp. e*qui*, the FgBP did not bind horse IgG (29).

From strain Epi9 of *S. dysgalactiae*, a gene homologous to *demA* was identified and sequenced. This gene, called *demB*, encodes a protein which, except for 110 amino acids in the N-terminal part of the mature protein, shows very high identity (93%) and similarity (97%) to the DemA protein. This is in agreement with earlier findings that different M and M-like proteins from group A streptococci also have diverging amino acid sequences in their N-terminal regions, while the C-terminal regions are more conserved (11). Studies on the binding of ^{125}I-Fg to cells of Epi9 and a Northern blot analysis of total RNA confirmed the expression of DemB in Epi9 as well as the upregulation of the gene expression as a response to an elevated level of CO_2 in the atmosphere (Fig. 5). The binding of labelled Fg to cells of strain Epi9 was also completely inhibited by the recombinant Fg-binding protein derived from DemA (Fig. 6), even for cells grown in the presence of 10% CO_2 (data not shown). In addition, mutanolysine treatment of cells from both strains (8215 and Epi9) released Fg-binding proteins detected as bands in the range of 75 to 95 kDa, compared to the calculated molecular masses of the mature DemA (54 kDa) and DemB (53 kDa) proteins. The discrepancy between the calculated and observed values may be explained by the presence of other cell wall components associated with the mutanolysine-released proteins.

In *S. pyogenes*, the expression of genes encoding M or M-like proteins is regulated by the *mga* gene, which codes for a *trans*-acting positive regulator (7, 32, 33). During the identification

of the *demA* gene, which encodes the Fg-binding activity in strain 8215, an ORF directly upstream of the *demA* gene was recognized, which when deduced, showed high sequence similarity to Mga proteins (Fig. 4). However, the 5′ end of the presumed gene, called *dmgA*, was missing and instead we found part of an IS element (Fig. 1). A complete *mga*-like gene, called *dmgB*, was cloned from strain Epi9. Alignment of the region of the deduced amino acid sequence of the DmgB protein corresponding to the truncated DmgA protein showed differences in only five amino acid residues, indicating that these proteins are highly conserved (Fig. 4).

It is known that the Mga protein in *S. pyogenes* is part of a two-component regulatory system (32) involved in the regulation of genes in the *mga* regulon. The N-terminal part of the Mga protein has been reported to contain DNA-binding region(s), while at the C-terminal end (amino acids 404 to 528) it exhibits homology to the phosphorylation receiver domain of response proteins (28, 32, 33). The alignment of DemB to MryD721 shows that highly similar regions are also present in DemB (Fig. 4). Thus, if the *dmg* and *dem* genes in strains 8215 and Epi9 are part of a regulon similar to the *mga* regulon found in *S. pyogenes*, the finding that the *dmgA* gene of strain 8215 contains an inserted part of an IS element in the 5′ end after the potential helix-turn-helix motif (Fig. 4) suggests that a functional *dmgA* product is lacking in this strain. The observed low Fg binding by cells of strain 8215 is most likely the result of a basal transcription of the *demA* gene, although the presence of another gene, one that encodes an alternative Fg-binding protein with identical or overlapping binding epitope(s) with DemA on the Fg molecule, can not be fully excluded. However, all analyzed Fg affinity-selected phagemid clones contained inserts derived only from the *demA* gene. Northern blot analysis confirmed the expression of *demB* and *dmgB* genes in Epi9. Moreover, it showed that CO_2 has an enhancing effect on the transcription of *demB*, as it does on the expression of genes belonging to the *mga* regulon in *S. pyogenes*. These data indicate that *dmgB* and *demB* form a functional *mga*-like regulon, while the similar regulon in 8215 is inactivated by the inserted IS element (8, 30).

In conclusion, the finding of the overall similarity in both the deduced primary and the predicted secondary structures of DemA and DemB proteins to M proteins, the recognized divergence in amino acid sequences in the N-terminal regions of the two proteins, and the detected binding properties of DemA are all characteristics which strongly suggest that DemA and DemB are M-like proteins. Furthermore, the data from the Northern blot analyses strongly support the idea that the *dmg* and *dem* genes are members of an *mga*-like regulon in *S. dysgalactiae*. Further studies will focus on the coupling between the expression of the *dmg* and *dem* genes concerning the response to other environmental factors earlier reported to influence the expression of the genes in the *mga* regulon in *S. pyogenes* (27). It will also be interesting to find out whether Dmg controls the expression of other genes within or outside the putative regulon. The observation that a *demA*-derived probe hybridized to chromosomal DNA from all 10 tested mastitis isolates of *S. dysgalactiae* and preliminary results from PCR amplification studies with *dmg* derived primers revealing the presence of a *dmg*-like gene in 9 out of the 10 tested strains indicates that the encoded proteins are of importance for the virulence of this species.

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