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Fibronectin-Binding Protein of *Streptococcus pyogenes*: Sequence of the Binding Domain Involved in Adherence of Streptococci to Epithelial Cells

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The sequence of the fibronectin-binding domain of the fibronectin-binding protein of *Streptococcus pyogenes* (Sfb protein) was determined, and its role in streptococcal adherence was investigated by use of an Sfb fusion protein in adherence studies. A 1-kb DNA fragment coding for the binding domain of Sfb protein was cloned into the expression vector pEX31 to produce an Sfb fusion protein consisting of the N-terminal part of MS2 polymerase and a C-terminal fragment of the streptococcal protein. Induction of the vector promoter resulted in hyperexpression of fibronectin-binding fusion protein in the cytoplasm of the recombinant *Escherichia coli* cells. Sequence determination of the cloned 1-kb fragment revealed an in-frame reading frame for a 268-amino-acid peptide composed of a 37-amino-acid sequence which is completely repeated three times and incompletely repeated a fourth time. Cloning of one repeat into pEX31 resulted in expression of small fusion peptides that show fibronectin-binding activity, indicating that one repeat contains at least one binding domain. Each repeat exhibits two charged domains and shows high homology with the 38-amino-acid D3 repeat of the fibronectin-binding protein of *Staphylococcus aureus*. Sequence comparison with other streptococcal ligand-binding surface proteins, including M protein, failed to reveal significant homology, which suggests that Sfb protein represents a novel type of functional protein in *S. pyogenes*. The Sfb fusion protein isolated from the cytoplasm of recombinant cells was purified by fast protein liquid chromatography. It showed a strong competitive inhibition of fibronectin binding to *S. pyogenes* and of the adherence of bacteria to cultured epithelial cells. In contrast, purified streptococcal lipoteichoic acid showed only a weak inhibition of fibronectin binding and streptococcal adherence. These results demonstrate that Sfb protein is directly involved in the fibronectin-mediated adherence of *S. pyogenes* to epithelial cells.

Streptococcus pyogenes is a common cause of pyogenic infections, some of which may result in serious sequelae, such as rheumatic fever and acute glomerulonephritis (18). Adherence to, colonization of, and invasion of mucosal surfaces are prerequisites of streptococcal infections (1). Fibronectin, a glycoprotein present in soluble form in plasma and various body fluids (33, 35) and in insoluble form in the extracellular matrix and basement membrane (23), has the ability to bind to both streptococci and host cells (9, 11, 44) and is considered an important mediator of streptococcal adherence (2). The nature of the cognate streptococcal component involved in fibronectin binding has been a controversial topic for some time, and both lipoteichoic acid and streptococcal surface proteins have been proposed as fibronectin receptors (4, 9, 12, 40). In our previous study, however, we presented biochemical and genetic evidence showing that protein components of *S. pyogenes* act as the principal fibronectin receptors (42). This evidence was based on the results of cloning and expression in *Escherichia coli* of streptococcal fibronectin-binding (Sfb) proteins from two clinical isolates and one defined strain of *S. pyogenes*. The fibronectin-binding domain was thereby localized to a 40-kDa peptide encoded by the 1,000-bp C-terminal region of the gene.

In the present study, we sequenced this gene fragment and showed that the fibronectin-binding domain of the Sfb protein possesses no similarity to other *S. pyogenes* surface ligand-binding proteins and thus may represent a novel type of surface protein. We also present evidence supporting a direct involvement of Sfb protein in the adherence of streptococci to epithelial cells.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *E. coli* K-12 strains HB101 (F$^-$ *hsd20 recA13 ara-14 proA2 lacY1 galK2 rpsL20* Sm^r *xyl-5 mtb-1 supE44*) and 537 (28, 34, 41) were used. *E. coli* 537 was obtained from H. Schaller and harbors plasmid pcI857, encoding kanamycin resistance and the temperature-sensitive *c*I repressor of bacteriophage lambda. The construction of the Sfb protein-expressing plasmid pST11 has been described previously (42). The *E. coli* expression vector pEX31 was constructed by Strebel et al. (41). Streptococcal strain DSM2071 (M serotype 23) was obtained from the German Culture Collection (Braunschweig, Germany); *S. pyogenes* strains A45 and A75 were clinical isolates obtained from patients with acute tonsilitis. Streptococci were grown in Todd-Hewitt broth (Oxoid, Basingstoke, England); *E. coli* strains were grown in LB broth.

Standard techniques. Isolation of human fibronectin from blood was performed by affinity chromatography with gelatin-agarose and heparin-agarose (30). Purified fibronectin was radiolabeled with ^{125}I by the method of Hunter and Greenwood (25). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (27) was carried out with a 5%

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acrylamide stacking gel and a 10% acrylamide running gel. Proteins separated on SDS gels were electrophoretically transferred to nylon membranes (Immobilon; Millipore, Bedford, Mass.) with a semidry blotting cell (Bio-Rad, Munich, Germany). The colony blot procedure and testing for fibronectin-binding activity of blotted proteins were done as described earlier (42). Transformation of *E. coli*, isolation of plasmid DNA, and agarose gel electrophoresis were performed as described by Sambrook et al. (36). Restriction enzymes and T4 ligase were purchased from GIBCO/BRL (Eggenstein, Germany) and used according to the instructions of the manufacturer. Fusion protein-expressing cultures of *E. coli* 537 harboring pSTX2 were grown and heat induced as previously described by Jerlström et al. (26).

Subcloning of the fibronectin-binding domain into pEX31. Plasmid DNA from pST11 and DNA from pEX31A, -B, and -C (representing all reading frames in the polylinker region) were digested with *Bam*HI and *Xba*I. The resulting fragments were separated on agarose gels, and the 3.3-kb fragment of pEX31 and the 1.4-kb fibronectin-binding domain-encoding fragment of pST11 were isolated with a DNA extraction kit (Quiaex; Diagen, Hilden, Germany), ligated together, and transformed into competent cells of *E. coli* 537. After growth at 28°C, the colonies were incubated at 42°C for 2 h and tested for expression of Sfb fusion proteins. Fusion protein-expressing plasmid pSTX1 was isolated, digested with *Hin*dIII, and religated to eliminate the 400-bp internal *Hin*dIII fragment to produce pSTX2.

Cell fractionation procedure. Cells of an induced culture of *E. coli* 537 harboring plasmid pSTX2 were harvested by centrifugation. The pellet was resuspended in lysis buffer (50 mM Tris-HCl [pH 8.0], 25% sucrose, 1 mM EDTA, 0.4% lysozyme) and incubated for 30 min on ice. $MgCl_2$ was added to a final concentration of 10 mM, $MnCl_2$ was added to 1 mM, and DNase I was added to 10 μg/ml. After the suspension had been incubated on ice for 30 min, 2 volumes of detergent mix I (20 mM Tris-HCl [pH 7.5], 200 mM NaCl, 1% sodium deoxycholate, 1% Nonidet P-40, 2 mM EDTA) were added, and the lysate was incubated on ice for 5 min. After centrifugation for 15 min, the supernatant fluid containing the cytoplasmic fraction was collected, and the pellet was suspended in 1/10 of the original culture volume of detergent mix II (0.05% Triton X-100, 1 mM EDTA). The pellet mixture was incubated on ice for 30 min and periodically vortexed. After centrifugation for 15 min, inclusion body (pellet) and membrane (supernatant) fractions were obtained. The inclusion body fraction was then resuspended in 1/10 of the original culture volume of TE buffer (10 mM Tris-HCl [pH 7.5] containing 1 mM EDTA), and all fractions were subjected to SDS-PAGE and immunoblotting with fibronectin and antifibronectin antibodies, as described previously (42).

Purification of Sfb fusion protein. Cells from a 1-liter induced culture of *E. coli* 537 harboring plasmid pSTX2 were harvested by centrifugation and resuspended in 10 ml of phosphate-buffered saline (PBS, pH 7.5). Iodoacetic acid (10 mM) and 10 mM benzamidine chloride were added to inhibit proteases, and after the suspension was cooled to 4°C, the cells were disrupted in a French press. The suspension was centrifuged, and the particle-free supernatant was collected and equilibrated with 4 M urea in 20 mM Tris-HCl, pH 7.0. The fusion protein was then purified by fast protein liquid chromatography (FPLC) with a Mono Q ion-exchange column (HR 10 by 10; Pharmacia LKB, Uppsala, Sweden), eluted by a linear salt gradient. The fractions under each peak were pooled and dialyzed against PBS before use in binding and adherence experiments.

DNA sequencing. Supercoiled DNA from plasmid pSTX2 was used as the template for the sequencing reaction with synthetic oligonucleotides, as described by Chen and Seeburg (7). The sequence was determined by the dideoxy chain termination method (37), and the reactions were performed with the Sequenase sequencing kit (USB, Cleveland, Ohio) according to the protocol of the manufacturers. The sequencing samples were analyzed by PAGE with 6% acrylamide gels containing 48% urea. Computer programs were used to record and analyze the sequence data.

PCR. Plasmid pSTX2 was used as the template to generate DNA fragments coding for the first repeat of the Sfb protein via the polymerase chain reaction (PCR). Primer A (5′-AGGTTTTTCAGGATCCATGGTTGAG-3′) was designed to be complementary to the noncoding strand right before the first repeat, representing nucleotides 135 to 159, including three one-base mismatches for introducing a *Bam*HI cloning site. Primer B (5′-CTTCTGTCGGATCCTAAGG AGTTGTT-3′) is complementary to nucleotides 252 to 277 of the coding strand at the end of the first repeat, containing four one-base mismatches that were necessary for introduction of a *Bam*HI site and a stop codon, replacing the last amino acid (glutamine) of the repeat. The reaction mixture contained 0.2 ng of template DNA, 20 pmol of each primer, 0.08 mM deoxynucleoside triphosphate mix, 0.5% Tween 20, 1× *Taq* polymerase buffer, and 2 U of *Taq* polymerase (Boehringer). PCR reactions (40 cycles) were performed, each cycle consisting of denaturation (94°C, 20 s), annealing (51°C, 30 s), and extension (72°C, 15 s) except for the first cycle, in which the denaturation time was increased to 2 min, and the last cycle, in which the extension time was increased to 5 min for complete extension.

Subcloning of the PCR fragment. To determine the size and quantity of the PCR product, 5% of the reaction mixture was loaded on a 1.7% agarose gel, electrophoresed, and stained. To purify the PCR fragment, the remainder of the reaction mixture was separated on a preparative agarose gel, and the 145-bp fragment was recovered from the agarose with Quiaex (Diagen). After purification, the DNA was cut with *Bam*HI to create cohesive ends, ligated to *Bam*HI-digested pEX31A vector DNA, and transformed into *E. coli* 537. The resulting transformants were incubated for 5 h at 42°C for heat induction and tested for expression of fibronectin-binding activity by colony blotting.

Binding experiments. Cells of streptococcal cultures were harvested by centrifugation, washed twice with PBS, and suspended in PBS containing 0.1% Tween 20 to give 10% transmission at 600 nm (8, 10). Then 200 μl of the cell suspension was added to 10 ng of ^{125}I-labeled fibronectin (specific activity, 2.91 mCi/mg) which had been incubated for 5 min with either 20-μl quantities of increasing concentrations (0.05 to 2.5 mg of total protein per ml) of lysate from *E. coli* overexpressing Sfb fusion protein or 50-μl quantities (2.5 to 100 μg/ml) of purified fusion protein. The suspension, containing streptococci, labeled fibronectin, and lysate or purified fusion protein, was incubated for another 40 min at room temperature. Nonbound fibronectin was separated by centrifugation, and the amount of cell-bound fibronectin was determined in a gamma counter. In parallel, 25 μg of nonlabeled fibronectin and 50 μg (total protein) of lysate from bacteria harboring pSTX2 without the insert were used as positive and negative controls, respectively. The fibronectin binding of *S. pyogenes* was also studied in the presence of purified lipoteichoic acid (LTA) (at 1 μM phos-

phorus per assay; kindly provided by Werner Fischer, Universität Erlangen, Erlangen, Germany).

Cell culture and adherence experiments. A human epithelial cell line (HEp2; ATCC strain CCL 23) was used to study the effects of the Sfb fusion protein on fibronectin-mediated adherence of streptococci to epithelial cells. Preliminary studies in our laboratory had shown that *S. pyogenes* adhered to these cells mainly through a fibronectin-mediated mechanism. Epithelial cells were grown in Dulbecco's modified Eagle's medium (DME; GIBCO) containing 10% fetal calf serum (GIBCO), 5 mM glutamine (Flow Laboratories Inc., McLean, Va.), 1 mM pyruvate (Flow), 100 IU of penicillin per ml, and 100 μg of streptomycin (Flow) per ml at 37°C in an atmosphere containing 8% CO_2. For adherence assays, cells were harvested by trypsinization and seeded on 96-well microtiter plates (Nunc, Roskilde, Denmark) at a concentration of approximately 10^5 cells per well. The cells were grown to confluency for 18 to 20 h and then used for adherence experiments.

A previously described fluorometric microassay (45) was used to directly determine streptococcal adherence to epithelial cells in the microtiter plates. Briefly, 100-μl samples of fluorescein isothiocyanate-labeled streptococci containing 2.5×10^7 bacteria in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered DME were added to each well. After 45 min at 37°C, nonadherent streptococci were removed by three washings with HEPES-buffered DME, and adherent bacteria were quantitated by fluoroscanning (Fluorescan II Titertek; Flow). Controls contained buffer without streptococci. Each test was done in quadruplicate, and results are expressed as means ± standard deviations.

Inhibition experiments were done with crude particle-free supernatants of the French press extracts from *E. coli* cultures harboring either Sfb fusion protein-expressing plasmid pSTX2 or a plasmid without an insert (control) at concentrations of 1 to 50 μg of total protein per assay. Adherence was also studied in the presence of FPLC-purified Sfb fusion protein (0.125 to 5 μg per assay) and of purified streptococcal LTA at concentrations similar to those used in the binding assays.

Nucleotide sequence accession number. The EMBL accession number is X67947.

RESULTS

Construction of plasmids which express Sfb fusion proteins. In a previous study, the position and transcriptional direction of the gene coding for the Sfb protein of *S. pyogenes* DSM 2071 were determined, and deletion derivatives were constructed that revealed that the binding domain is encoded by a 1-kb *Bam*HI-*Hin*dIII fragment and is located within the C-terminal part of the protein (42). In this study, the *Bam*HI-*Xba*I fragment of plasmid pST11, comprising the relevant *Bam*HI-*Hin*dIII fragment and an additional 300 bp of streptococcal DNA, was cloned into pEX31A, -B, and -C in an oriented fashion downstream of the inducible lambda promoter p_L so that the C-terminal binding domain of the Sfb protein was fused to the N-terminal part of MS2 polymerase in one of the three possible reading frames represented in the three vectors (Fig. 1). After temperature induction of the transformants, clones expressing Sfb protein were isolated at high frequency from each reaction. Western immunoblot analysis of lysates from positive clones showed that only insertion of the *Bam*HI-*Xba*I fragment into reading frame B resulted in expression of an Sfb fusion protein of 55 kDa (as a result of its fusion with the N-terminal fragment of MS2 polymerase); positive transformants from the two other experiments expressed only a 40-kDa truncated Sfb nonfusion protein. The hybrid plasmid pSTX1 thereby generated was further reduced in size by deletion of the 300-bp *Hin*dIII-*Hin*dIII fragment, creating plasmid pSTX2. Western blot analysis of lysates of *E. coli* 537 cells harboring pSTX2 showed that the sizes of the fusion proteins expressed by pSTX1 and pSTX2 are identical (data not shown).

Sequence determination of the coding region of the fibronectin-binding domain of Sfb. Both strands of the insert in pSTX2 were sequenced by the dideoxy chain termination method in combination with synthetic oligonucleotide primers homologous to vector DNA regions 30 nucleotides up- and downstream of the inserted streptococcal DNA. Further oligonucleotide primers for sequencing within the streptococcal DNA region were designed on the basis of sequence information thereby obtained.

Since the construct represents an active in-frame fusion with MS2 polymerase, the translational reading frame of the sequenced region (Fig. 2) was inferred from that of the polymerase. The sequence exhibits an open reading frame comprising 804 nucleotides that codes for a polypeptide of 268 amino acids and that terminates with a TAA stop codon at nucleotide 805. One hundred twenty-six nucleotides downstream of the stop codon is located a region with the potential to form a hairpin structure characteristic of transcriptional terminators. One characteristic feature of the sequence is the existence of a region that starts at nucleotide 154 and is composed of four nearly identical repeat sequences, designated R1 through R4. Downstream of the region of repeats follows a stretch of 40 amino acids exhibiting no special structural features, which might constitute the cell wall-spanning region of the protein. This stretch is followed by an LPATG consensus sequence. This sequence has been found in all surface proteins of gram-positive cocci characterized so far and is thought to be a signal sequence for anchorage in the membrane (17). The LPATG sequence is followed by a hydrophobic region of 20 amino acids (Fig. 3), which probably forms the transmembrane segment of the protein, and a stretch of six mainly basic amino acids at the C terminus, which is thought to be located on the cytoplasm side of the membrane (Fig. 2 and 3).

Analysis of the repeats. R1 through R3 consist of 37 amino acids each, whereas R4 contains only 32 amino acids. The repeats are highly conserved with respect to their amino acid and nucleotide sequences; the only differences in the amino acid sequences are found in repeat R2 at positions 35 and 36, and the only differences in the nucleic acid sequences are some two or three variations per repeat in the codon wobble positions. Figure 3 gives detailed information on the distribution of hydrophilic and hydrophobic regions within the repeats. Each repeat exhibits two internal domains in which charged amino acids are concentrated; these domains are formed by three or four negatively charged and one positively charged amino acid. The alternation of charged and uncharged domains results in a continuous periodicity within one repeat that is maintained over the whole repeated region.

The functionally related fibronectin-binding protein (FnBP) of *Staphylococcus aureus* (38) shows similar features in its binding region, which also consists of three complete 38-amino-acid repeat sequences and one incomplete repeat exhibiting alternating charged and uncharged domains. Recent studies showed that synthetic peptides representing the single repeat of the *S. aureus* FnBP bound fibronectin and that interaction of the peptides with fibronectin was due to the presence of the charged amino acids of the repeat structure (29). An alignment of the amino acid sequences of

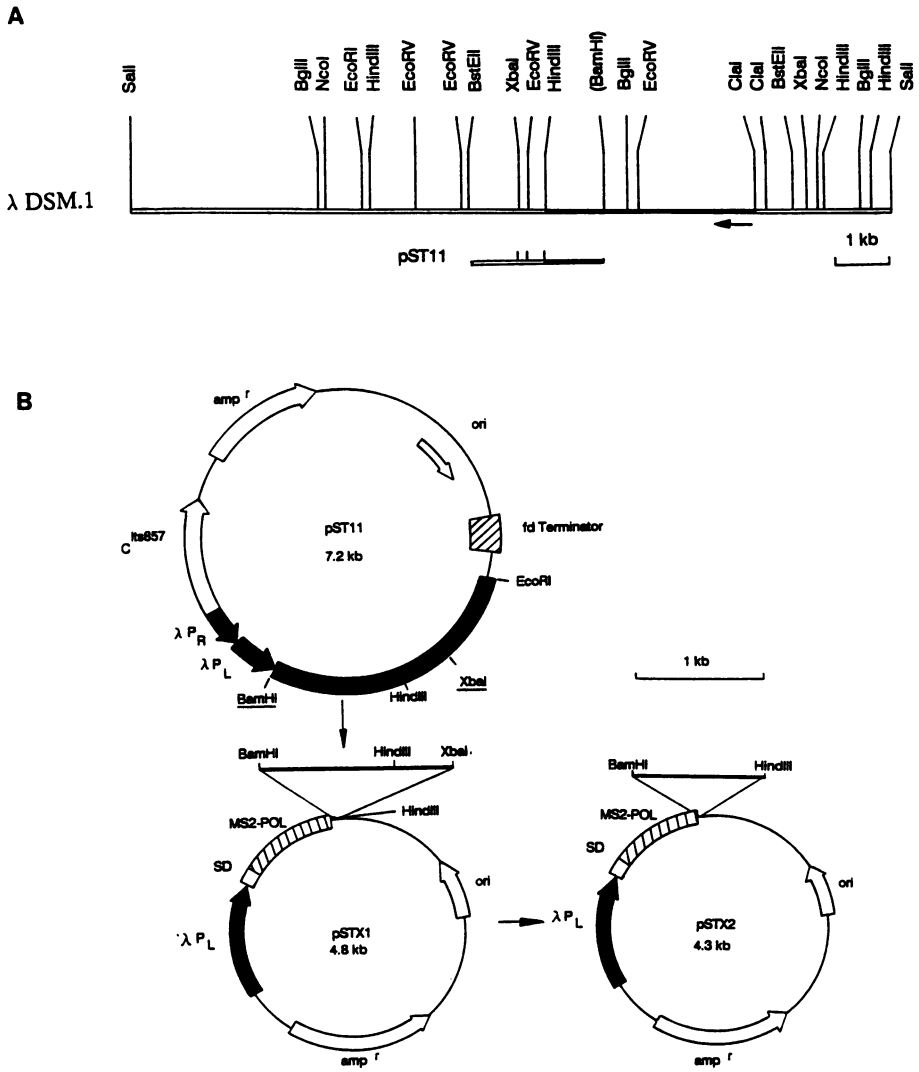

FIG. 1. (A) Partial restriction map of the lambda insert containing the Sfb protein gene of *S. pyogenes* DSM 2071 and insert of the subclone pST11. The transcriptional direction is indicated by an arrow. The *Bam*HI site indicated in brackets is present only in pST11. (B) Cloning scheme for the construction of plasmids pSTX1 and pSTX2 expressing fusion proteins with fibronectin-binding activity. POL, polymerase; SD, Shine-Dalgarno sequence.

the repeats of the fibronectin-binding proteins of *S. pyogenes* and *S. aureus* is shown in Fig. 4. The whole region exhibits about 50% homology, and a higher degree of homology exists between the charged domains (60 to 70%). The distance between the charged domains is also the same in the two proteins. The average degree of homology at the DNA level was about 60%, again with the highest conservation in the coding regions of the charged domains (data not shown).

Localization of the binding domain by cloning of the R1 repeat. The characteristic features of the repeats of Sfb protein and the high degree of homology they show with the binding domain of the staphylococcal FnBP support the notion that repeats R1 through R4 constitute the fibronectin-binding structures. To confirm this hypothesis, we cloned a single repeat in pEX31 in order to test the resulting peptide fusions for their ability to bind fibronectin. Since the streptococcal DNA fragment coding for the repeat region did not contain suitable restriction sites, the coding region for repeat R1 was amplified via PCR to introduce *Bam*HI restriction sites up- and downstream of the repeat. To ensure that translation stops at the end of R1, a stop codon was introduced by altering the last codon of R1. Cloning of the PCR fragment coding for amino acids 1 to 36 of R1 into pEX31A resulted in transformants that expressed fibronectin-binding activity after heat induction of the vector promoters, as revealed by colony blotting. The positive clones contained the 123-bp PCR fragment, and Western blot analysis of total cell lysates from heat-induced liquid cultures showed expression of a 16-kDa fusion peptide with fibronectin-binding activity. These results indicate that the binding domain for fibronectin is localized within amino acids 1 to 36 of repeat R1. Moreover, the fact that the four repeats of Sfb protein are nearly identical supports the idea that each of the repeats constitutes at least one binding domain for fibronectin.

Lack of homology to M protein. M proteins have previously been implicated in streptococcal adherence (14). In order to determine whether the Sfb protein of *S. pyogenes* is

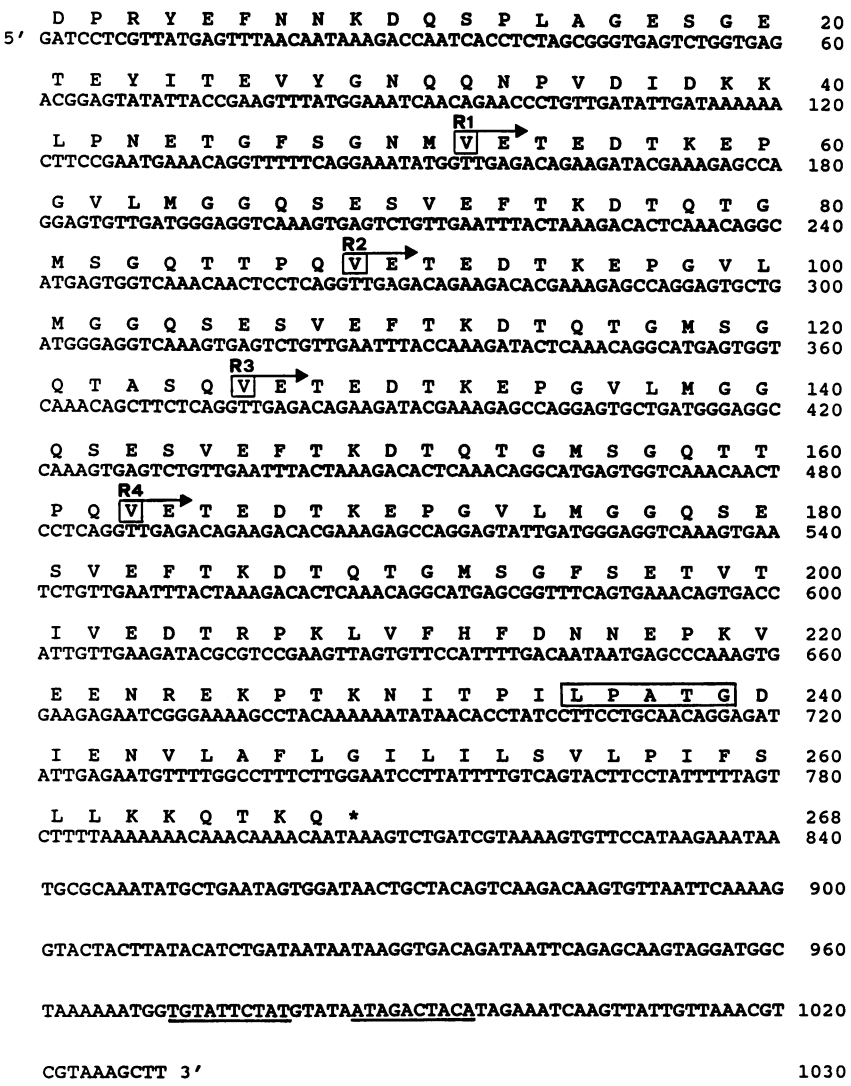

FIG. 2. Nucleotide sequence and deduced amino acid sequence of the region coding for the binding domain of Sfb protein from *S. pyogenes* DSM 2071. Amino acids are given in the one-letter code, and the stop codon is marked by an asterisk (*). The four repeated regions were designated R1 to R4, and the first amino acid (valine) from each repeat is marked with an arrow. The LPATG consensus sequence is boxed, and the potential transcription termination signal is underlined.

an M or M-like protein or is related to one, we compared its C-terminal region with that of different M-like proteins (Fig. 5). The C-terminal region of M proteins is highly conserved, with homologies between different M proteins ranging from 70 to 100% (16), whereas the Sfb protein exhibits only 18% homology with M proteins in this region. Comparison of our sequence with those of the staphylococcal surface proteins FnBP and protein A revealed 22 and 14% homology, respectively. The nucleotide sequence of the LPATG region of the Sfb protein had no features in common with the M proteins nor with the sequence N-terminal from the LPATG region (data not shown). Thus, the Sfb protein from *S. pyogenes* is homologous with M protein only to the extent that is similar to most of the previously characterized surface proteins of gram-positive cocci. We therefore conclude that the Sfb protein from *S. pyogenes* is not an M or M-like protein but represents a novel type of surface protein of group A streptococci.

Purification of Sfb fusion protein. Fractionation of recombinant *E. coli* cells revealed that the fibronectin-binding activity was equally distributed in the cytoplasm and inclusion body fractions, whereas the membrane fractions possessed only weak activity. The Sfb fusion protein was purified from the cytoplasmic fraction on an FPLC Mono Q ion-exchange column. The peak containing active fusion protein was eluted at 0.3 M salt. The Sfb fusion protein gave two main bands on SDS-PAGE gels, both of which reacted with fibronectin in Western blots (Fig. 6). This protein was used in binding and adherence assays without any further purification.

Inhibition of fibronectin binding to *S. pyogenes* by Sfb fusion protein. Increasing concentrations of French press lysates containing the fusion protein substantially inhibited the binding of ^{125}I-labeled fibronectin to *S. pyogenes* (Table 1). The 65% inhibition of binding by lysate at a concentration of 50 μg of total protein per assay was equivalent to the inhibition obtained with 25 μg of nonlabeled fibronectin per assay. Purified fusion protein was about 10-fold more active

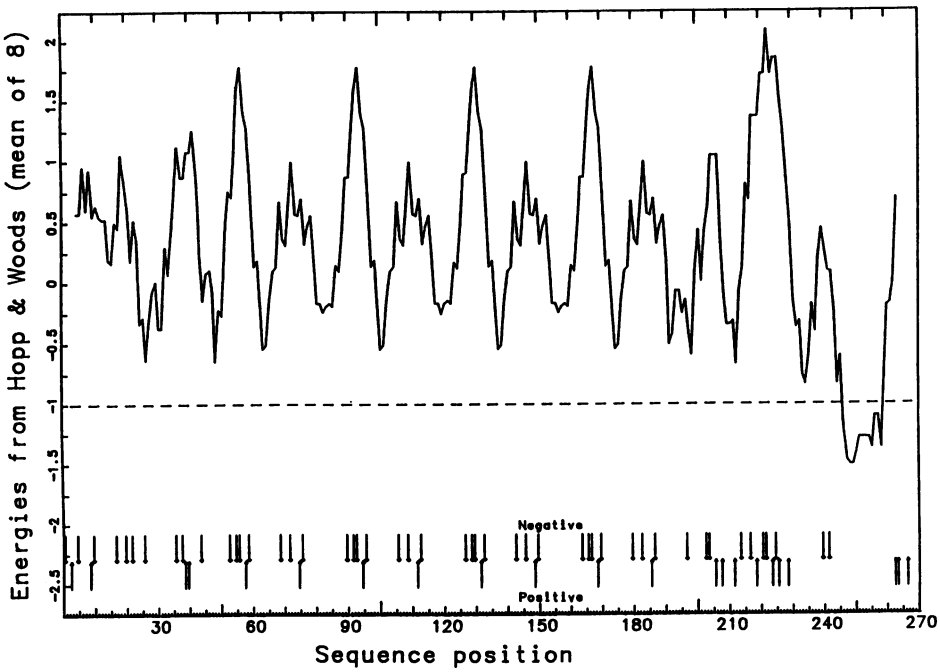

FIG. 3. Hopp and Woods hydrophobicity plot of the fibronectin-binding peptide from amino acids 1 to 268. Hydrophilic regions are represented by peaks, and the positions of charged amino acids are indicated by arrows.

in inhibition of fibronectin binding, which corresponds to the degree of purification (Table 1, Fig. 7). In control experiments, lysates from bacteria containing a plasmid without an insert and LTA purified from *S. pyogenes* showed no significant inhibition of fibronectin binding (Table 1).

Inhibition of adherence of *S. pyogenes* to HEp2 cells by Sfb fusion protein. Under our adherence assay conditions, approximately 15 bacteria of *S. pyogenes* DSM 2071 per well adhered to each HEp2 cell grown in 96-well microtiter plates. That this adherence is mediated by fibronectin was confirmed in preliminary experiments. Streptococcal adherence to HEp2 cells was competitively inhibited by up to 50% by lysates containing Sfb fusion protein, which correlated well with the ability of lysates to inhibit fibronectin binding (Table 1). Purified Sfb fusion protein also competitively inhibited the adherence of *S. pyogenes* in a concentration-dependent manner by up to 46%. As in the binding experiments, the purified material was about 10-fold more effective than the crude lysate (Table 1, Fig. 7). Control preparations (lysate from bacteria containing a plasmid without an insert and purified streptococcal LTA) reduced adherence by only 16% (Table 1).

DISCUSSION

Streptococcal adherence to host epithelial cells often represents the initiating step in the infection process. The adherence is mediated by specific interactions that occur between structures on the surfaces of streptococci and the host epithelial cells (1). Fibronectin on the epithelial cells has been shown to be involved in such interactions (5). M protein, the most prominent molecule on the surface of pathogenic group A streptococci (16), and LTA have both been implicated in fibronectin-mediated adherence of streptococci to host cells (3, 14, 31, 43). We have recently shown that protein components of *S. pyogenes* and not LTA represent the principal binding sites for fibronectin (42). The results of the present study confirm that a protein, which we have named Sfb protein, is the major group A streptococcal adhesin and demonstrate that it is not related to M protein.

We have previously localized the binding domain of the Sfb protein to a 40-kDa truncated peptide. Determination of the sequence of this domain allowed us to compare it with other ligand-binding proteins from gram-positive cocci, especially the M protein, which has received considerable

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. . . I E. EDTNKDKPS YQFGGHN. S VDFEEDTLPKV
     :  : : :  : :  :     : :     : : : :  : :
VETEDT. KE. PGVLMGGQS ES VEFTKDTQTGM. . .
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FIG. 4. Alignment of the peptides representing the repeats of the fibronectin-binding proteins from *S. aureus* (29, 38) and *S. pyogenes* DSM 2071. The upper sequence represents the D3 repeat of *S. aureus* from amino acids 8 to 38; the lower sequence shows amino acids 1 to 30 of the *S. pyogenes* repeats. Homologous amino acids are connected by a colon.

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M6          LPSTGTANPFFTAAALTVMATAGVAAVV KRKEEN
M49         LPSTGTANTFFTAAAATVMVSAGMLAL  KRKEEN
IgA-BP      LPSTGTANTFFTAAAATVMVSAGMLAL  KRKEEN
protein G   LPTTGGSNPFFTAAALAVMAGAGALAVA  SKRKED
wap A       LPSTGQAGLLLTTVGLVI VAVAGVYFY RTRR
protein A   LPETGENPLI GTTVFGGLS LAGGAALLAG RRREL
FnBP        LPTTGEESTNKGMLFGGLFSILGLALL  RRNKKNHKN
Sfb protein LPATGDI ENVLAFLGI LI LSVLPI FSLL KKQTKQ
            LPXTG   membrane spanning region  charged tail
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FIG. 5. Comparison of the C-terminal amino acid sequences of streptococcal and staphylococcal surface proteins with the C-terminal amino acid sequence of the Sfb protein of *S. pyogenes*. The proteins compared included *S. pyogenes* M6 (24), *S. pyogenes* M49 (21), *S. pyogenes* immunoglobulin A-binding protein (IgA-BP) (19), protein G from group G streptococci (32), *S. mutans* WapA (15), *S. aureus* protein A (20), and *S. aureus* fibronectin-binding protein FnBP (38). The LP-TG region is boxed.

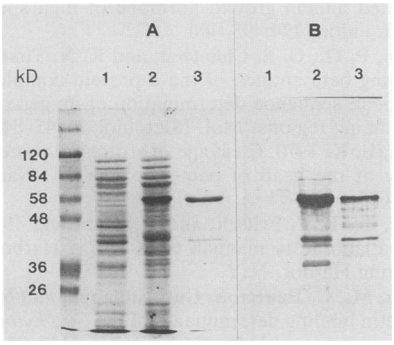

FIG. 6. Coomassie blue-stained SDS-polyacrylamide gel (A) and Western blot with fibronectin and antifibronectin antibodies (B) of lysate from *E. coli* harboring a plasmid without an insert (lane 1), lysate with Sfb fusion protein (lane 2), and purified fusion protein (lane 3).

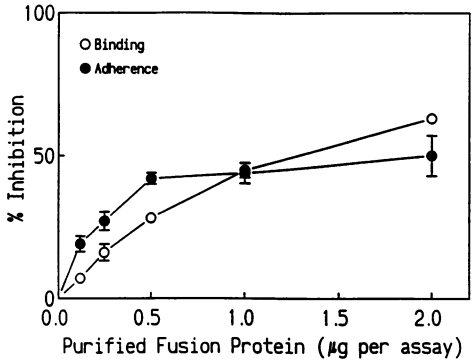

FIG. 7. Inhibition by Sfb protein of fibronectin binding and epithelial cell adherence of *S. pyogenes* strain DSM 2071. In binding experiments, streptococci were incubated for 45 min with ^{125}I-labeled fibronectin in the presence of increasing amounts of purified fusion protein, after which free fibronectin was removed and cell-bound fibronectin was determined. For adherence, streptococci were incubated with HEp2 cells in the presence of the indicated amounts of fusion protein, after which they were washed and adherent bacteria were quantitated by fluoroscanning. Data points indicate the means (± standard deviations).

attention as a potential mediator of streptococcal adherence. Sequence comparisons revealed that the C-terminal part of Sfb protein exhibits only 18% homology with the highly conserved C-terminal part of M protein, which indicates that Sfb protein is not an M or M-like protein. This supports the findings of Caparon et al. (6), who, by using an isogenic pair of M^+ and M^- strains, showed that M protein is involved in neither fibronectin binding nor streptococcal adherence to epithelial cells. Sequence comparison of the Sfb protein with a number of other ligand-binding proteins from gram-positive cocci revealed little homology, and therefore Sfb protein seems to be a novel type of functional protein of group A streptococci. Sequence comparison with the functionally related fibronectin-binding protein FnBP of *S. aureus* (38), on the other hand, revealed similarities in the binding region, which consists of four repeats. In both cases, the repeats are composed of 38 and 37 amino acids, respectively, and show a particularly high degree of conservation in the charged domains. The behavior of fibronectin-binding fusion peptides, which consist of MS2 polymerase and one single repeat of Sfb protein, clearly shows that the binding domain is located within the 37-amino-acid repeat. Since charged domains within the repeats of FnBP have been shown to represent the binding sites for fibronectin (29), the charged domains within the repeats of Sfb protein are also very likely to be responsible for this function. The characteristic distribution of charged domains, particularly the alternation of charged and uncharged domains which results in a continuous periodicity over the whole repeat region, may constitute an essential structure for fibronectin binding. The preparation and characterization of synthetic peptides representing these domains and site-specific mutations of the domain coding region, which are presently in progress, should provide crucial information in this regard. The high degree of homology between FnBP from *S. aureus* and Sfb protein from *S. pyogenes* is an interesting aspect, though it is not yet known whether the genes of these genera evolved from the same precursor or whether the convergent structures are responsible, as has been suggested in the case of the immunoglobulin A receptors of group A and group B streptococci (22).

TABLE 1. Competitive inhibition by Sfb fusion protein of fibronectin binding and adherence to HEp2 cells of *S. pyogenes* DSM 2071

Inhibitor	Amt per assay	Mean % inhibition[a]	
		Fibronectin binding	Adherence to HEp2 cells
PBS	20 µl	0	0
Control lysate	50 µg[b]	15	16
Lysate with fusion protein	50 µg[b]	65	50
Purified fusion protein	5 µg[b]	63	46
Purified streptococcal LTA	1 µM phosphorus	17	16

[a] Means were obtained from quadruplicate assays.
[b] Total protein.

To assess the potential role of the Sfb protein in the adherence of group A streptococci to epithelial cells, we constructed an expression plasmid encoding an Sfb fusion protein and hyperexpressed and purified the fusion protein. This protein not only competitively inhibited fibronectin binding to group A streptococci but also inhibited the adherence of such bacteria to HEp2 cells. In contrast, streptococcal LTA only weakly inhibited fibronectin binding or adherence. This clearly indicates that the fibronectin-binding domain of Sfb represents a major adhesin of *S. pyogenes*, and not the previously suggested LTA or M protein.

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REFERENCES

1. **Beachey, E. H.** 1981. Bacterial adherence: adhesin-receptor interactions mediating the attachment of bacteria to mucosal surfaces. J. Infect. Dis. **143**:325–344.
2. **Beachey, E. H., C. S. Giampapa, and S. M. Abraham.** 1988. Adhesin receptor mediated attachment of pathogenic bacteria to mucosal surfaces. Am. Rev. Respir. Dis. **138**:S45–S48.

3. **Beachey, E. H., and I. Ofek.** 1976. Epithelial cell binding of group A streptococci by lipoteichoic acid on fimbriae denuded of M protein. J. Exp. Med. **143**:759–771.
4. **Beachey, E. H., and W. A. Simpson.** 1982. The adherence of group A streptococci to oropharyngeal cells: the lipoteichoic adhesion and fibronectin receptor. Infection **10**:107–110.
5. **Beachey, E. H., W. A. Simpson, I. Ofek, D. L. Hasty, J. B. Dale, and E. Whitnack.** 1983. Attachment of *Streptococcus pyogenes* to mammalian cells. Rev. Infect. Dis. **5**:S670–S677.
6. **Caparon, M. G., D. S. Stephens, A. Olsen, and J. R. Scott.** 1991. Role of M protein in adherence of group A streptococci. Infect. Immun. **59**:1811–1817.
7. **Chen, E. Y., and P. H. Seeburg.** 1985. Supercoiling sequencing: a fast and simple method for sequencing plasmid DNA. DNA **4**:165–170.
8. **Chhatwal, G. S., G. Albohn, and H. Blobel.** 1987. Novel complex formed between a nonproteolytic cell wall protein of group A streptococci and α_2-macroglobulin. J. Bacteriol. **169**: 3691–3695.
9. **Chhatwal, G. S., and H. Blobel.** 1987. Heterogeneity of fibronectin reactivity among streptococci as revealed by binding of fibronectin fragments. Comp. Immunol. Microbiol. Infect. Dis. **10**:99–108.
10. **Chhatwal, G. S., K. T. Preissner, G. Müller-Berghaus, and H. Blobel.** 1987. Specific binding of the human S protein (vitronectin) to streptococci, *Staphylococcus aureus*, and *Escherichia coli*. Infect. Immun. **55**:1878–1883.
11. **Chhatwal, G. S., P. Valentin-Weigand, and K. N. Timmis.** 1990. Bacterial infection of wounds: fibronectin-mediated adherence of group A and C streptococci to fibrin thrombi in vitro. Infect. Immun. **58**:3015–3019.
12. **Courtney, H. S., I. Ofek, W. A. Simpson, D. L. Hasty, and E. H. Beachey.** 1986. Binding of *Streptococcus pyogenes* to soluble and insoluble fibronectin. Infect. Immun. **53**:454–459.
13. **Dobson, S. R. M.** 1989. Group A streptococci revisited. Arch. Dis. Child. **64**:977–980.
14. **Ellen, R. P., and R. J. Gibbons.** 1972. M protein-associated adherence of *Streptococcus pyogenes* to epithelial surfaces: prerequisite for virulence. Infect. Immun. **5**:826–830.
15. **Ferretti, J. J., R. R. B. Russel, and M. L. Dao.** 1989. Sequence analysis of the wall associated protein precursor of *Streptococcus mutans* antigen A. Mol. Microbiol. **3**:469–478.
16. **Fischetti, V. A.** 1989. Streptococcal M protein: molecular design and biological behavior. Clin. Microbiol. Rev. **2**:285–314.
17. **Fischetti, V. A., V. Pancholi, and O. Schneewind.** 1990. Conservation of a hexapeptide sequence in the anchor region of surface proteins from Gram-positive cocci. Mol. Microbiol. **4**:1603–1605.
18. **Francis, J., and R. E. Warren.** 1988. *Streptococcus pyogenes* bacteraemia in Cambridge—a review of 67 episodes. Q. J. Med. **68**:603–613.
19. **Frithz, E., L.-O. Heden, and G. Lindahl.** 1989. Extensive sequence homology between IgA receptor and M protein in *Streptococcus pyogenes*. Mol. Microbiol. **3**:1111–1119.
20. **Guss, B., M. Uhlen, B. Nilsson, M. Lindberg, J. Sjoquist, and J. Sjodahl.** 1984. Region X, the cell-wall-attachment part of staphylococcal protein A. Eur. J. Biochem. **138**:413–420.
21. **Haanes, E. J., and P. P. Cleary.** 1989. Identification of a divergent M protein gene and an M protein-related gene family in serotype 49 *Streptococcus pyogenes*. J. Bacteriol. **171**:6397–6408.
22. **Heden, L.-O., E. Frithz, and G. Lindahl.** 1991. Molecular characterization of an IgA receptor from group B streptococci: sequence of the gene, identification of a proline-rich region with unique structure and isolation of N-terminal fragments with IgA-binding capacity. Eur. J. Immunol. **21**:1481–1490.
23. **Heedmann, K., A. Vaheri, and J. Wartiovaara.** 1978. External fibronectin of cultured human fibroblasts is predominantly a matrix protein. J. Cell Biol. **76**:748–760.
24. **Hollingshead, S. K., V. A. Fischetti, and J. R. Scott.** 1986. Complete nucleotide sequence of type M6 protein of the group A *Streptococcus*: repetitive structure and membrane anchor. J. Biol. Chem. **261**:1677–1686.
25. **Hunter, W. J., and F. C. Greenwood.** 1962. Preparation of iodine 131 labelled human growth hormone of high specific activity. Nature (London) **194**:495–496.
26. **Jerlström, P. G., G. S. Chhatwal, and K. N. Timmis.** 1991. The IgA-binding beta antigen of the c protein complex of group B streptococci: sequence determination of its gene and detection of two binding regions. Mol. Microbiol. **5**:843–849.
27. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
28. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **McGavin, M., G. Raucci, S. Gurusiddappa, and M. Höök.** 1991. Fibronectin binding determinants of the *Staphylococcus aureus* fibronectin receptor. J. Biol. Chem. **266**:8343–8347.
30. **Miekka, S. J., K. C. Ingham, and D. Menacke.** 1982. Rapid methods for isolation of human fibronectin. Thromb. Res. **27**:1–14.
31. **Ofek, I., W. A. Simpson, and E. H. Beachey.** 1982. Formation of molecular complexes between structurally defined M protein and acylated or deacylated lipoteichoic acid of *Streptococcus pyogenes*. J. Bacteriol. **149**:426–433.
32. **Olsson, A., M. Eliasson, B. Guss, B. Nilsson, U. Hellmann, M. Lindberg, and M. Uhlen.** 1987. Structure and evolution of the repetitive gene encoding streptococcal protein G. Eur. J. Biochem. **168**:319–324.
33. **Pearlstein, E., L. I. Gold, and A. Garcia-Pardo.** 1980. Fibronectin: a review of its structure and biological activity. Mol. Cell. Biochem. **29**:103–137.
34. **Remaut, E., P. Stanssens, and W. Fiers.** 1981. Plasmid vectors for high-efficiency expression controlled by the PL promoter of coliphage lambda. Gene **15**:81–93.
35. **Ruoslahti, E.** 1988. Fibronectin and its receptors. Annu. Rev. Biochem. **57**:375–413.
36. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:4767–4771.
38. **Signäs, C., G. Raucci, K. Jönsson, P.-E. Lindgren, G. M. Anantharamaiah, M. Höök, and M. Lindberg.** 1989. Nucleotide sequence of the gene for a fibronectin binding protein from *Staphylococcus aureus*: use of this peptide sequence in the synthesis of biologically active peptides. Proc. Natl. Acad. Sci. USA **86**:699–703.
39. **Simpson, W. A., and E. H. Beachey.** 1983. Adherence of group A streptococci to fibronectin on oral epithelial cells. Infect. Immun. **39**:275–279.
40. **Speziale, P., M. Höök, L. M. Switalski, and T. Wadström.** 1984. Fibronectin binding to a *Streptococcus pyogenes* strain. J. Bacteriol. **157**:420–427.
41. **Strebel, K., E. Beck, K. Strohmeier, and H. Schaller.** 1986. Characterization of foot-and-mouth disease virus gene products with antisera against bacterially synthesized fusion proteins. J. Virol. **57**:983–991.
42. **Talay, S. R., E. Ehrenfeld, G. S. Chhatwal, and K. N. Timmis.** 1991. Expression of the fibronectin-binding components of *Streptococcus pyogenes* in *Escherichia coli* demonstrates that they are proteins. Mol. Microbiol. **5**:1727–1734.
43. **Tylewska, S. K., V. A. Fischetti, and R. J. Gibbons.** 1988. Binding selectivity of *Streptococcus pyogenes* and M protein to epithelial cells differs from that of lipoteichoic acid. Curr. Microbiol. **16**:209–216.
44. **Valentin-Weigand, P., G. S. Chhatwal, and H. Blobel.** 1988. Adherence of streptococcal isolates from cattle and horses to their respective host epithelial cells. Am. J. Vet. Res. **49**:1485–1488.
45. **Valentin-Weigand, P., J. Grulich-Henn, G. S. Chhatwal, G. Müller-Berghaus, H. Blobel, and K. T. Preissner.** 1988. Mediation of adherence of streptococci to human endothelial cells by complement S protein (vitronectin). Infect. Immun. **56**:2851–2855.