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Cloning and Expression of Two Different Genes from *Streptococcus dysgalactiae* Encoding Fibronectin Receptors*

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Binding of bacteria to fibronectin has been implicated as a mechanism of bacterial adhesion to the host tissue. In this report we have analyzed the binding of a strain of *Streptococcus dysgalactiae* to fibronectin. The cells bind to a site in the NH_2-terminal domain of the protein via trypsin-sensitive cell surface components. Furthermore, a lysate prepared by sonication of streptococcal cells contained fibronectin-binding proteins that inhibit the binding of the ligand to intact bacteria. When the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted to an Immobilon-P filter, and probed with ^{125}I-labeled fibronectin, a 140-kDa fibronectin-binding protein was identified along with a number of smaller binding proteins. A genomic DNA library was constructed and screened for the expression of fibronectin-binding proteins. Two clones were isolated and shown to contain unrelated inserts by restriction mapping and cross-hybridization experiments. The two encoded proteins were also immunologically distinct although both bound to the same region of the fibronectin molecule, and both effectively inhibited the binding of ^{125}I-fibronectin to bacterial cells. Immunological analyses showed that only one of the two proteins tentatively identified as fibronectin receptors was expressed in detectable quantities in the *Streptococcus dysgalactiae* strain under the culture conditions employed.

Fibronectin belongs to the family of adhesive matrix proteins which serve as a substratum for the adhesion and migration of eukaryotic cells. Fibronectin is distributed throughout the animal body and is found as a soluble protein in high concentrations in body fluids such as blood and in an immobilized form in the extracellular matrix of various tissues (Yamada, 1983; Hynes, 1985; Rouslahti, 1988). Adhesion of eukaryotic cells to fibronectin primarily involves a family of cell surface receptors known as the integrins, which recognize and bind to specific sites in the central part of the protein. A primary recognition site for the integrin receptors is defined by the amino acid sequence RGD, and synthetic peptides based on this motif inhibit the attachment of many cell types to fibronectin substrates.

Studies during the last decade have shown that also several different genera of bacteria recognize and bind fibronectin and other adhesive matrix proteins (Höök *et al.*, 1989). However, in contrast to eukaryotic cells, little is known about bacterial receptors for fibronectin. A staphylococcal fibronectin receptor protein has been isolated (Mosher and Proctor, 1980; Espersen and Clemmensen, 1982; Fröman *et al.*, 1987), and a gene encoding a protein with fibronectin binding activity has been cloned (Flock *et al.*, 1987) and sequenced (Signäs *et al.*, 1989). Recently described protein structures "curli," present on the surface of some *Escherichia coli*, also appear to bind fibronectin (Olsen *et al.*, 1989).

Various strains of group A, C, and G streptococci have been shown to bind fibronectin (Switalski *et al.*, 1982; Myhre and Kuusela, 1983). However, the nature of the bacterial receptor(s) involved in these interactions remains unclear. Previous studies suggest that a protein structure (or structures) located on the surface of most fibronectin-binding streptococcal cells recognizes a site in the NH_2-terminal part of the fibronectin molecule (Kuusela *et al.*, 1984). *Streptococcus dysgalactiae* cells have also been reported to bind fibronectin at a site located in the COOH-terminal half of the protein. This interaction was mediated by a trypsin-insensitive surface component (Chhatwal and Blobel, 1987; Valentin-Wiegand *et al.*, 1988). Furthermore, lipoteichoic acid present on the surface of group A streptococci has been proposed to bind fibronectin in a hydrophobic interaction (Beachey and Simpson, 1982).

In the present study we have identified two genes coding for fibronectin-binding proteins in a bovine mastitis isolate of *S. dysgalactiae*. The two genes were cloned and expressed in *E. coli*. Recombinant proteins encoded by the cloned genes are shown to bind both intact ^{125}I-fibronectin and its 29-kDa NH_2-terminal fragment and to inhibit the binding of ^{125}I-fibronectin to cells of *S. dysgalactiae*. However, under standard culture conditions only one of the genes seems to be expressed.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Media

S. dysgalactiae strain S2 was obtained from W. Mamo (Dept. of Veterinary Microbiology, Swedish University of Agricultural Sciences, Uppsala, Sweden). *E. coli* K12 strain TG1 (Carter *et al.*, 1985) was used as a host for transformations with the plasmid vector pUC18 (Norrander *et al.*, 1983). A strain of *Streptomyces albus* with lytic activity toward streptococci was obtained from Dr. W. Köhler (Insti-

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tute für Mikrobiologie und Experimentelle Therapie, Jena, GDR) (Prauser and Köhler, 1965). The *Streptococcus* and *Streptomyces* strains were grown in Todd-Hewitt broth (Difco), and *E. coli* was grown in Luria broth. All cultures were grown at 37 °C. Bacteria were stored at −70 °C in their respective media supplemented with 20% glycerol.

Enzymes and Other Proteins

Restriction enzymes and T4 DNA ligase were purchased from International Biotechnologies Inc. (New Haven, CT), New England Biolabs (Beverly, MA), Promega (Madison, WI), or Boehringer Mannheim and used according to the suppliers' recommendations. For exonuclease III digestions an Erase-a-Base system kit from Promega was used following a standard protocol from the supplier. Other methods involving DNA techniques were essentially as described (Maniatis *et al.*, 1982).

Collagen type II was prepared from bovine nasal cartilage. Fetuin and bovine IgG were purchased from Sigma, and ovalbumin was from Pharmacia LKB Biotechnology Inc.

Purification and Radiolabeling of Fibronectin and Fibronectin Fragments

Purification of human fibronectin from plasma was based on affinity chromatography on gelatin-Sepharose (Vuento and Vaheri, 1979; Miekka *et al.*, 1982). The 29-kDa NH_2-terminal fibronectin fragment was generated by trypsin digestion of fibronectin and purified as described by Garcia-Pardo *et al.* (1983). The proteins were labeled with ^{125}iodine (Amersham Corp.) using the chloramine-T method (Hunter, 1978). The specific activity of the labeled native fibronectin used for screening of recombinants was 0.19 MBq/μg; native fibronectin and its 29-kDa NH_2-terminal fragment had activities of 0.07 and 0.05 MBq/μg, respectively.

Preparation of DNA

Streptococcal cells (final concentration 2.5×10^9 colony-forming units/ml) were incubated for 3 h in a mixture of equal volumes of 10 mM Tris-HCl buffer (pH 7.9) containing 150 mM NaCl, 100 mM EDTA, and 1 mg/ml lysozyme, and streptolytic culture medium from *S. albus*. Chromosomal DNA was then prepared according to Marmur (1961). Plasmid DNA from *E. coli* clones was prepared using a boiling method and LiCl extraction (Monstein and Geijer, 1986).

Construction of a Genomic Library from S. dysgalactiae, Strain S2, in E. coli TG1 and Detection of Clones Expressing Fibronectin Binding Activity

Chromosomal DNA from *S. dysgalactiae* S2 was partially digested with *Sau*3A1 and fractionated on a 1% agarose gel. Fractions containing DNA of 3–9 kilobase pairs (kb)[1] were mixed with pUC18 after the plasmid had been digested with *Bam*HI and alkaline phosphatase. The mixture was ligated with T4 DNA ligase and used for transformation of *E. coli* TG1 to ampicillin resistance. Seven hundred white colonies collected from LB agar plates supplemented with 50 μg/ml ampicillin, 0.004% 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (Sigma), and 0.1 mM isopropyl β-D-thiogalactopyranoside (Sigma) were replica plated to nitrocellulose filters. The bacteria bound to the nitrocellulose filters were lysed in chloroform vapor for 5 min and then washed 3 × 10 min in 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 80 (buffer A). Nonspecific protein binding sites on the filters were blocked by incubation of the filters for 2 h at 37 °C in 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1% fat-free milk powder. Subsequently, ^{125}I-labeled fibronectin was added, and the incubation was continued overnight at 22 °C. The filters were washed 3 × 10 min in buffer A. Positive clones were detected on an x-ray film (β-max, Amersham Corp.) after a 2-day exposure.

Dot-Blot Hybridization

Plasmid DNA was denatured, diluted, and bound to a nitrocellulose filter. The probes were prepared from the plasmids containing streptococcal DNA by digestion with appropriate restriction enzymes (see legend to Fig. 5), gel purified, and labeled with [α-^{32}P]dATP by a random-priming method (Multiprime DNA labeling system, Amersham Corp.). The labeled probes were allowed to hybridize with the immobilized DNA at 42 °C overnight. Subsequently, the filters were washed with 0.1 × SSC (1 × SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7.0) supplemented with 0.1% SDS at 65 °C and analyzed by autoradiography.

Assays for Fibronectin Binding

Direct Binding Assay

S. dysgalactiae S2 was grown overnight in Todd-Hewitt broth. Bacteria were collected by centrifugation and suspended in phosphate buffered saline (PBS; 140 mM NaCl, 10 mM phosphate, pH 7.4) to a density of 1×10^{10} cells ml^{-1}. 5×10^8 bacteria were incubated with 5×10^4 cpm of ^{125}I-labeled fibronectin in 0.5 ml of PBS containing 0.1% bovine serum albumin and 0.1% Tween 80 to block nonspecific binding to cells and tubes. The mixture was rotated in an end-over-end mixer for 1 h at 22 °C. Incubation was stopped by the addition of 2.5 ml of ice-cold 0.1% Tween 80 in PBS, and the tubes were centrifuged at 1,400 × *g* for 15 min. After removal of the supernatant, the radioactivity associated with the cells was quantified in a counter (LKB Wallac, Turku, Finland). Bacteria incubated with 5×10^4 cpm of ^{125}I-labeled intact fibronectin or the 29-kDa NH_2-terminal fragment bound 6,000–6,500 and 12,000–12,500 cpm, respectively. Radioactivity recovered from incubation mixtures lacking bacteria (background) was 300–500 cpm and subtracted in each test.

Inhibition Assay

Solubilized proteins were analyzed for fibronectin binding activity by measuring their ability to compete with streptococcal cells for binding of ^{125}I-labeled fibronectin. The assay was performed in the same way as described above for the direct binding assay, with the exception that aliquots of soluble protein were added to the assay mixture.

Solubilization of Fibronectin-binding Proteins

S. dysgalactiae S2 was grown at 37 °C for 18 h. The cells were collected by centrifugation and suspended in 50 mM Tris-HCl (pH 7.6) containing 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (Merck), and 1 mM *N*-ethylmaleimide (Sigma) to a density of 5×10^{10} cells/ml. Cell densities were estimated by comparing the absorbance of the sample with a previously prepared standard curve relating A_{600} to the cell number determined by counting cells in a Petroff Hausser chamber. The suspension was sonicated (3 × 1 min), and bacterial debris was removed by centrifugation (30 min, 10,000 × *g*). The supernatant containing the solubilized proteins was stored at −70 °C until used.

Lysates of *E. coli* clones were prepared by sonication as described above or by incubating the bacteria (2.5×10^9 cells/ml) in 50 mM Tris-HCl buffer (pH 7.9) containing lysozyme (1 mg/ml) and 100 mM EDTA at 37 °C for 30 min. The lysates were clarified by centrifugation and stored at −70 °C until used.

Isolation and Purification of Fibronectin-binding Proteins from E. coli Clones

E. coli clones containing the plasmids pSDF100 or pSDF200 were grown in LB medium at 37 °C for 18 h. Bacteria were collected by centrifugation and lysed by sonication as described. A four-step procedure was developed for the purification of fibronectin-binding proteins.

Step 1. Ion-exchange Chromatography

After the addition of solid urea to a final concentration of 2.0 M, the lysate was applied to a column of Q-Sepharose Fast Flow (Pharmacia), equilibrated with 50 mM Tris-HCl (pH 7.6) containing 2.0 M urea, 1 mM phenylmethylsulfonyl fluoride, and 1 mM *N*-ethylmaleimide. The column was washed with the same buffer and eluted with an NaCl gradient (0.0–0.6 M) in the same buffer. Fractions showing fibronectin binding activity as indicated in the inhibition assay were pooled and dialyzed against water.

Step 2. Ammonium Sulfate Precipitation

Fibronectin-binding components in the pooled fractions were precipitated by adding solid ammonium sulfate to 60% saturation. The precipitate was dialyzed against water before further fractionation.

Step 3. Fast Protein Liquid Chromatography

Further purification of the fibronectin-binding proteins was ob-

[1] The abbreviations used are: kb, kilobase pair(s); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; bp, base pairs.

tained by ion-exchange chromatography using a Mono Q column, fitted to a fast protein liquid chromatography system (Pharmacia), and equilibrated with 25 mM Tris-HCl (pH 7.6). Bound material was eluted from the column with an NaCl gradient (0.0–0.6 M). Samples containing fibronectin-binding proteins were dialyzed against water and lyophilized.

Step 4. Affinity Chromatography

Fibronectin-binding material from the Mono Q column was dissolved in PBS and applied to a column of Sepharose 4B, which was previously substituted with the 29-kDa fibronectin fragment and equilibrated with PBS. The column was washed with PBS containing 1.0 M NaCl, and proteins adsorbed to the affinity matrix were eluted with 2.0 M guanidine hydrochloride in PBS, dialyzed against water, and lyophilized.

This purification procedure resulted in a 174- and 42-fold increase in the specific activity of fibronectin-binding proteins in lysates of *E. coli* harboring pSDF100 and pSDF200, respectively. However, when these proteins were examined by SDS-PAGE they appeared to have undergone extensive degradation, and the preparations consisted of several polypeptides with M_r values of 15,000–140,000. Essentially all of these peptides bound ^{125}I-labeled fibronectin when analyzed in a Western blot type assay.

Production and Purification of Antibodies

The purified pSDF100 and pSDF200 gene products were used as antigens for the immunization of male New Zealand White rabbits. After preimmune bleeding, rabbits were immunized intramuscularly with 50 μg of either pSDF100- or pSDF200-encoded purified protein emulsified in Freund's incomplete adjuvant. Additional injections of 50 μg were given after 10 and 20 days, with immune serum being collected on day 30.

Affinity matrices for isolation of monospecific antibody were prepared by directly passing *E. coli* pSDF100 and pSDF200 cell lysates through a column of Sepharose 4B substituted with the 29-kDa fibronectin fragment, as described in the protein purification protocol. Protein retained on this matrix was eluted and after dialysis was coupled to 2 ml of cyanogen bromide-activated Sepharose CL-4B (Pharmacia) according to the method recommended by the manufacturer. The amounts of protein used for coupling were 4 mg for pSDF200 and 0.7 mg for pSDF100. Immune serum (10 ml) was passed twice through a column packed with the affinity matrix, previously equilibrated in 10 mM Tris-HCl (pH 7.5). After washing with this buffer, retained antibodies were eluted by consecutively applying 100 mM glycine (pH 2.5) and 100 mM triethylamine (pH 11.5). An intermediate wash with 10 mM Tris-HCl (pH 8.8) was included between the elutions. The eluted fractions were immediately neutralized by adding 1/10 of the fraction volume of 1 M Tris-HCl (pH 8.0). Prior to use for Western blotting, affinity-purified anti-pSDF100 antibody was passed through the pSDF200 protein affinity column to adsorb antibodies recognizing common epitopes. Likewise, anti-pSDF200 was passed through the pSDF100 protein matrix.

SDS-PAGE, Western Blotting, and Autoradiography

For SDS-PAGE, the gel and electrophoresis buffers were those of Laemmli (1970). Resolving gels were cast with a 3–15% gradient of acrylamide. The Bio-Rad Trans-Blot apparatus was used for transfer of proteins to Immobilon-P membranes (Millipore; Bedford, MA) using the transfer buffer of Towbin *et al.* (1979). After transfer, the membranes were incubated in 5% (w/v) non-fat milk powder to block additional protein binding sites.

For immunoblots, the membranes were incubated overnight at 4 °C with affinity-purified antibody diluted 1,000-fold in Tris-buffered saline (20 mM Tris-HCl, 500 mM NaCl, pH 7.5) containing 3% bovine serum albumin. The secondary antibody was Bio-Rad goat anti-rabbit alkaline phosphatase conjugate, used as recommended by the supplier with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium as the color development substrate.

For detection of fibronectin binding activity, the blocked membranes were incubated overnight at 4 °C, under gentle agitation, in 30 ml of Tris-buffered saline containing 5×10^5 cpm of ^{125}I-labeled fibronectin or 29-kDa NH_2-terminal fibronectin fragment. After thorough washing, the membranes were dried at 37 °C and placed into a film cassette with Fuji RX100 x-ray film. The film was developed after overnight exposure at 4 °C.

Demonstration of Cell Surface Localization of Fibronectin-binding Proteins

One hundred mg (wet weight) of *S. dysgalactiae* S2 was suspended in 0.5 ml of PBS and digested with trypsin (Sigma, type XIII 75 μg/ml) for 1 h at 37 °C. Trypsin was inactivated with soybean trypsin inhibitor (300 μg/ml), and the cells were pelleted by centrifugation. The supernatant was taken as the trypsin-released fraction while the cell pellet was suspended in SDS-PAGE reducing buffer and boiled to release remaining cell-associated proteins. Cells that had not been treated with trypsin were also boiled in reducing buffer for use as a control.

Protein solubilized from the control cells, trypsin-treated cells, and protein released from the cell surface by trypsin digestion was subjected to SDS-PAGE, transferred to Immobilon-P, and probed with affinity-purified antibody to pSDF200 antigen. The blot was developed using the Bio-Rad amplified Immune-Blot assay kit. In a parallel experiment, trypsin-treated cells were assayed for fibronectin binding relative to control cells with ^{125}I-labeled 29-kDa fibronectin fragment as ligand.

RESULTS

Characterization of ^{125}I-Labeled Fibronectin Binding to S. dysgalactiae Strain S2—In agreement with previous studies (Mamo *et al.*, 1987) strain S2 expresses a fibronectin receptor. When 5×10^4 cpm of ^{125}I-labeled fibronectin are added to 5×10^8 cells 6,000–6,500 cpm of ligand typically bind to the cells whereas only 300–500 cpm were recovered from incubation mixtures not containing bacteria. A binding site for *S. dysgalactiae* is located in the NH_2-terminal domain of fibronectin since a ^{125}I-labeled fragment encompassing this region also bound to the bacterial cells. Binding of ^{125}I-labeled fibronectin of its NH_2-terminal fragment to bacterial cells was a time-dependent process. Furthermore, the bacteria could be saturated with labeled ligand, and digestion of cells with trypsin resulted in the reduction of the ability to bind fibronectin (data not shown). The bacteria were also incubated with ^{125}I-labeled intact fibronectin or the 29-kDa NH_2-terminal tryptic fragment of fibronectin in the presence of increasing amounts of the corresponding cold protein or peptide. The cold 29-kDa fragment inhibited the binding of both ^{125}I-labeled molecules to the same degree as cold intact fibronectin (Fig. 1). A number of unrelated proteins were tested for their ability to interfere with the binding of ^{125}I-labeled fibronectin to bacteria. At the concentrations tested, these proteins, which included collagen, ovalbumin, fetuin, and nonimmune IgG, did not interfere with the bacterial binding of ^{125}I-labeled intact fibronectin or its NH_2-terminal fragment (Table I). Taken together these results indicate that a protein (or proteins) on the surface of *S. dysgalactiae* acts as a specific fibronectin receptor(s) recognizing the NH_2-terminal domain in the protein.

Identification of Fibronectin-binding Protein(s) in S. dysgalactiae Strain S2—A lysate of *S. dysgalactiae* S2 prepared by sonicating the bacterial cells in a buffer containing protease inhibitors was found to inhibit the binding of ^{125}I-labeled intact fibronectin or the 29-kDa fibronectin fragment in a concentration-dependent manner (Fig. 2). When the lysate was passed over a column of fibronectin-Sepharose, the inhibitory activity was removed. On the other hand, when the lysate was passed through a column of albumin-Sepharose, the activity was only marginally reduced (data not shown). These results suggest that the inhibitory activity of the lysate was caused by the presence of fibronectin-binding proteins.

The lysate was fractionated by SDS-PAGE, transferred to an Immobilon-P filter, and probed with ^{125}I-labeled intact fibronectin or the 29-kDa NH_2-terminal fragment. Autoradiographic analyses revealed that a major fibronectin-binding component reacting with the ^{125}I-labeled 29-kDa fragment had migrated in the electrophoresis as a protein of 140 kDa

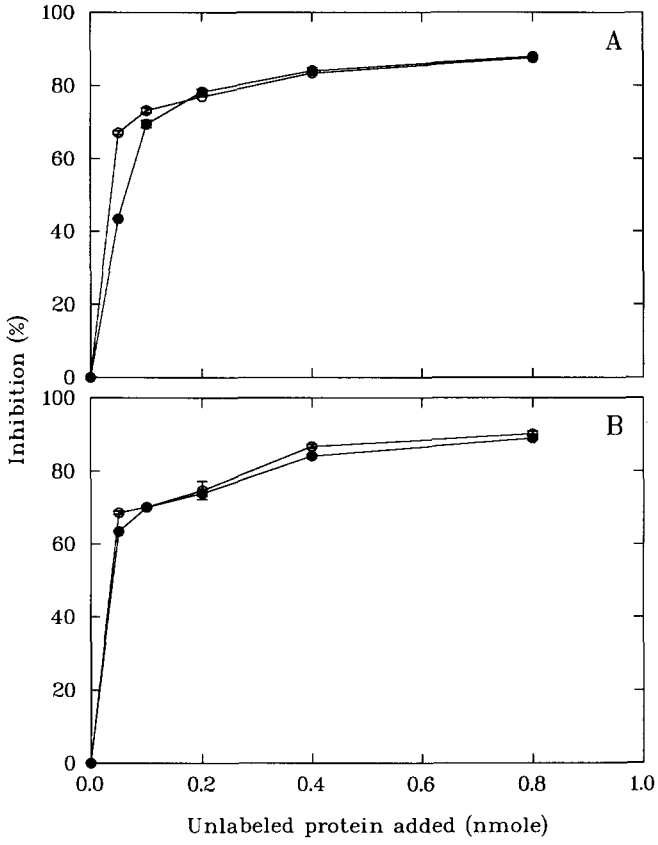

FIG. 1. **Inhibition of binding of ^{125}I-labeled native fibronectin or the labeled 29-kDa fragment of fibronectin to *S. dysgalactiae* S2 by adding unlabeled fibronectin or 29-kDa fragment.** Cells (5×10^8) were incubated with 5×10^4 cpm of labeled fibronectin (*A*) or labeled 29-kDa fragment of fibronectin (*B*) in the presence of unlabeled fibronectin (●) or 29-kDa fragment of fibronectin (○). After 1 h of incubation with end-over-end mixing at room temperature, the radioactivity bound to the bacteria was determined. The inhibition is expressed as percentage of binding. Labeled ligand bound in the absence of unlabeled fibronectin was set to 100% (equivalent to 0% inhibition). Radioactivity recovered in the absence of bacterial cells was considered background.

(Fig. 3). This component reacted weakly or not at all when the filter was incubated with intact ^{125}I-fibronectin, which might reflect a difference in affinity for the two ligand forms. Additional fibronectin-binding polypeptides of smaller size were also present. These might represent degradation products or different gene products.

The growth medium of strain S2 was tested for the presence of extracellular fibronectin-binding proteins by measuring the inhibitory activity of the culture medium on the binding of ^{125}I-labeled fibronectin to cells of S2. No inhibitory activity was detected.

Construction of a Genomic Library from S. dysgalactiae Strain S2 in E. coli TG1 and Detection of Clones Expressing Fibronectin Binding Activity—A genomic library from *S. dysgalactiae* S2 in *E. coli* TG1 was prepared as described under "Materials and Methods." Three positive clones, with recombinant plasmids called pSDF100, pSDF200, and pSDF300, were identified. Since the restriction cleavage patterns of the inserts in two of the recombinant plasmids, pSDF100 and pSDF300, were partially overlapping, only pSDF100 (with an insert of 4.9 kb), encoding the highest fibronectin binding activity of the two, and pSDF200 (with an insert of 6.9 kb) were studied further. Fig. 4, *a* and *b*, shows the completely different restriction maps of the inserts in pSDF100 and pSDF200.

TABLE I

Inhibition of the binding of ^{125}I-labeled fibronectin or the 29-kDa NH_2-terminal fibronectin fragment to S. dysgalactiae S2 by different amounts of unlabeled proteins

Bacteria (5×10^8) were incubated with 5×10^4 cpm of ^{125}I-labeled fibronectin or ^{125}I-labeled 29-kDa fibronectin fragment in the absence or presence of unlabeled proteins. Values represent means ± standard errors of three separate experiments.

Competing protein	^{125}I-Labeled fibronectin	^{125}I-Labeled 29-kDa fibronectin fragment
	% inhibition of binding	
Control, no added protein	0.0	0.0
Fibronectin		
50 μg	95.6 ± 1.1	98.8 ± 1.2
100 μg	95.1 ± 1.1	98.0 ± 2.2
NH_2-terminal 29-kDa fragment of fibronectin		
50 μg	90.3 ± 1.3	99.5 ± 1.0
100 μg	86.9 ± 0.1	99.2 ± 0.2
Collagen type II		
50 μg	3.1 ± 1.3	2.2 ± 2.0
100 μg	5.7 ± 1.9	12.3 ± 2.3
Ovalbumin		
50 μg	2.3 ± 1.9	5.0 ± 4.5
100 μg	2.6 ± 2.4	4.7 ± 2.7
Fetuin		
50 μg	3.2 ± 1.2	4.9 ± 3.0
100 μg	2.9 ± 0.2	6.1 ± 1.4
IgG		
50 μg	2.7 ± 2.0	2.7 ± 2.1
100 μg	7.0 ± 4.9	2.3 ± 1.9

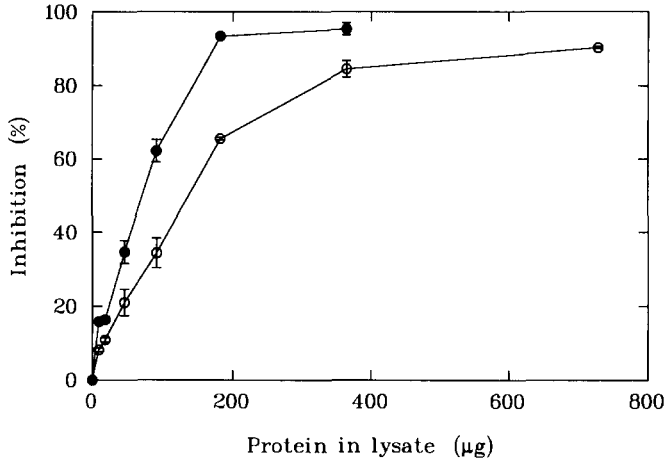

FIG. 2. **Inhibition of binding of ^{125}I-labeled native fibronectin or 29-kDa fragment of fibronectin to *S. dysgalactiae* S2 in the presence of increasing amounts of lysate from *S. dysgalactiae* S2.** Cells of *S. dysgalactiae* S2 were incubated with labeled fibronectin (●) or labeled 29 kDa fragment (○) in the presence of increasing concentrations of lysate produced by sonication of live *S. dysgalactiae* S2 cells. Data are expressed as percentage of control, where binding to bacteria incubated in the absence of lysate was set as 0%.

To localize the domains in the inserts of pSDF100 and pSDF200 which encode the fibronectin binding activity, subcloning was performed in pUC18 using restriction sites of the inserts (Fig. 4, *a* and *b*). Cleavage of the insert in pSDF100 with *Xba*I generated a 1.4-kb fragment, which, after ligation into pUC18 (pSDF102), coded for a protein with fibronectin binding activity. Digestion of the insert in pSDF102 with exonuclease III for different time periods resulted in a number of subclones with inserts of decreasing size. The corresponding proteins retained the ability to bind fibronectin until only approximately 500 bp remained of the pSDF102 insert. A

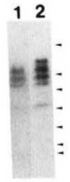

FIG. 3. **Identification of fibronectin-binding proteins in cell lysates of *S. dysgalactiae* S2.** Cell lysate protein (80 μg) was subjected to SDS-polyacrylamide gradient gel electrophoresis (3–15%), transferred to Immobilon-P, and probed with ^{125}I-fibronectin (*lane 1*) or ^{125}I-29 kDa fragment of fibronectin (*lane 2*). The migration position of prestained molecular mass standards (Bethesda Research Laboratories) is indicated. The molecular masses of the protein standards, in daltons are: myosin heavy chain, 200,000; phosphorylase *b*, 97,400; bovine serum albumin, 68,000; ovalbumin, 43,000; chymotrypsinogen, 25,700; β-lactoglobulin, 18,400; and lysozyme 14,300. The ratio of the different reactive proteins varied from one experiment to another, which possibly was the consequence of varying cleavage of a native protein by proteolytic enzymes present in the lysate.

further shortening of the insert resulted in a loss of fibronectin binding by the encoded protein. The negative test results for subclones pSDN102 and pSDN103 concerning the ability to bind fibronectin are not conclusive since one cannot exclude the possibility that the inserts are in the wrong reading frame or are inserted in the wrong orientation. However, the results locate at least one domain encoding fibronectin binding activity to the insert in pSDF102.

By using a similar strategy the region in pSDF200 coding for fibronectin binding activity was localized to a 1-kb *Xho*I-*Hin*dIII fragment (pSDF203) in the 3′-end of the insert. The *Hin*dIII site is within the multilinker. Digestion of the insert in pSDF203 with exonuclease III gave a series of subclones with inserts of decreasing size. Subclone pSDF203c3 with an insert of approximately 750 bp coded for a protein with fibronectin binding activity. Subclones pSDF203c6 and pSDF203c8 with inserts of 600 and 500 bp, respectively, coded for proteins with reduced fibronectin binding activities. Subclones with shorter inserts coded for inactive proteins.

To investigate possible relationship between the inserts in pSDF100 and pSDF200, DNA fragments from the domains in the respective inserts, which code for fibronectin binding activity, were selected as probes to be used in cross-hybridization experiments. A 0.6-kb *Xba*I-*Bst*XI fragment, originating from pSDF100, present in pSDF102 and the *Xho*I-*Hin*dIII fragment, subcloned in pSDF203, were used as probes (marked as *bold lines* in Fig. 4, *a* and *b*) in hybridizations to decreasing amounts of plasmid DNA from pSDF100, pSDF200, pUC18, and chromosomal DNA from *S. dysgalactiae* S2 on a nitrocellulose filter (Fig. 5). The probe from pSDF102 gave strong signals when hybridized to pSDF100. A very weak signal was recorded when the probe from pSDF102 was hybridized to pSDF200, and no signal was obtained in hybridizations to pUC18. Analyses using the probe from pSDF202 gave a strong signal with pSDF200 and no signals with pSDF100 and pUC18. These data support the results obtained by restriction analyses (Fig. 4, *a* and *b*), indicating that the streptococcal DNA inserts in pSDF100 and pSDF200 originate from two different genes. Both probes also hybridized to chromosomal DNA from strain S2 verifying that the two genes were derived from this strain.

Characterization of the Proteins Encoded by pSDF100 and pSDF200—Lysates of *E. coli* TG1 clones containing pSDF100 and pSDF200, respectively, were found to inhibit the binding of ^{125}I-labeled intact fibronectin or the NH_2-terminal 29-kDa fibronectin fragment to S2 cells in a concentration-dependent

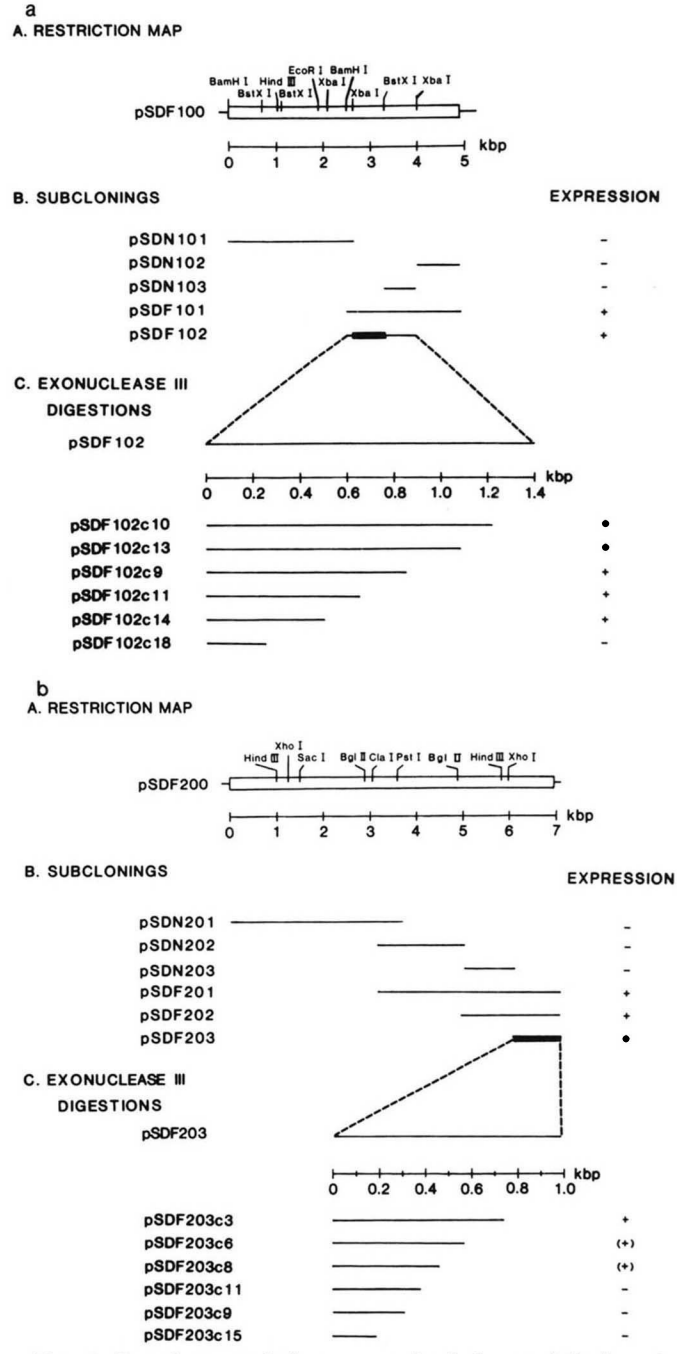

FIG. 4. ***Panel a*, restriction map and subclones of the insert in pSDF100.** *A*, restriction map of the insert in pSDF100 containing a 4.9-kb insert from *S. dysgalactiae* S2 in pUC18. *B*, various subclones constructed to determine the regions of the genes coding for the fibronectin binding activity. The *bold line* in pSDF102 indicates the probe used in dot-blot hybridization (Fig. 5). The fibronectin binding activities of lysates from the different subclones, expressed as percentage inhibition of binding of labeled fibronectin to *S. dysgalactiae* S2, are indicated. More than 85% inhibition, +; less than 15% inhibition, −. *C*, deletions made from the 3′ end of the insert in pSDF102. *Panel b*, restriction map and subclones of the insert in pSDF200. *A*, restriction map of the insert in pSDF200 containing a 6.9-kb insert from *S. dysgalactiae* S2 in pUC18. *B*, various subclones constructed in order to determine the regions of the genes coding for the fibronectin binding activity. The *bold line* in pSDF203 indicates the probe used in dot-blot hybridization (Fig. 5). The fibronectin binding activities of lysates from the different subclones, expressed as percentage inhibition of binding of labeled fibronectin to *S. dysgalactiae* S2, are indicated. More than 85% inhibition, +; between 40 and 30% inhibition (+); less than 15% inhibition, −. *C*, deletions made from the 3′ end of the insert in pSDF203.

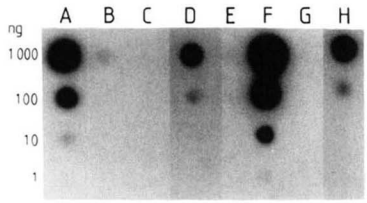

FIG. 5. **Dot-blot hybridization analysis of the *E. coli* clones expressing fibronectin binding activity.** Plasmid DNA from pSDF100 (*A* and *E*), pSDF200 (*B* and *F*), pUC18 (*C* and *G*), and chromosomal DNA from *S. dysgalactiae* S2 (*D* and *H*) was bound to a nitrocellulose filter and probed with [α-^{32}P]dATP-labeled *Xba*I-*Bst*XI fragment, 600 bp, from a pSDF102 (*lanes A, B, C,* and *D*) fragment or *Xho*I-*Hin*dIII (present within the multilinker of the vector) fragment, 1,000 bp, from pSDF203 (*lanes E, F, G,* and *H*). The probes were selected to cover the domains coding for fibronectin binding activity (see legend to Fig. 4). The amounts of plasmid DNA bound to the filter are indicated.

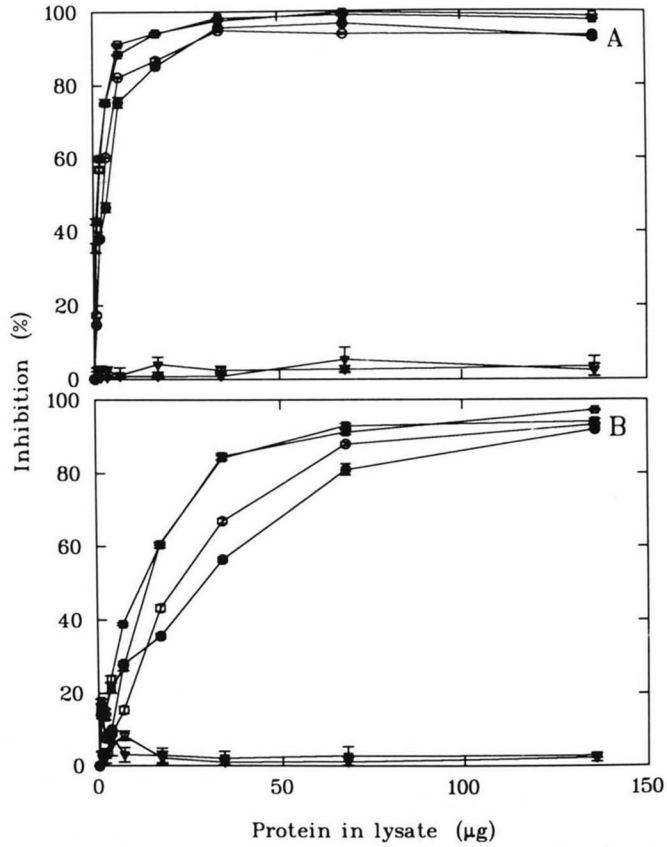

FIG. 6. **Inhibition of binding of labeled native fibronectin (*A*) or labeled 29-kDa fragment of fibronectin (*B*) to *S. dysgalactiae* S2 by gene products from the *E. coli* clones containing pSDF100 and pSDF200.** Cells of *S. dysgalactiae* S2 were incubated with labeled fibronectin (*A*) or the labeled 29-kDa fragment of fibronectin (*B*) in the presence of increasing concentrations of fibronectin receptors solubilized by sonication of live (*open symbols*) or heat-killed (*filled symbols*) *E. coli* TG1 clones with pSDF100 (*circles*) or pSDF200 (*squares*). As a negative control, lysate from cells of *E. coli* TG1 with pUC18 vector (*triangles*) was used. Data are expressed as percentage of the control, where binding to bacteria incubated in the absence of potential inhibitors was set as 0%.

fashion (Fig. 6). Heat treatment of the *E. coli* clones before preparation of the lysates had no effect on the inhibitory capacity. Both lysates could completely inhibit binding of the ligands to streptococcal cells although a somewhat higher concentration of pSDF100 lysate protein was required to obtain a level of inhibition comparable to pSDF200. This result suggests that the lysate from the pSDF100-containing clone has a lower concentration of active components, caused either by less efficient expression or by a greater sensitivity to proteolysis. Alternatively, the difference in specific activity of the two lysate preparations could reflect a difference in affinity by the corresponding receptor proteins for fibronectin. However, since complete inhibition was obtained by either lysate, the two gene products appear to bind to similar domain(s) in the fibronectin molecule. A lysate prepared from *E. coli* TG1 containing pUC18 had no inhibitory activity on the binding of ^{125}I-fibronectin to the S2 cells (Fig. 6).

The presence of fibronectin-binding components was demonstrated further in experiments in which proteins in the two lysates were fractionated by SDS-PAGE, transferred to Immobilon-P, and probed with ^{125}I-labeled intact fibronectin or the 29-kDa NH_2-terminal fragment (Fig. 7). For both pSDF100- and pSDF200-encoded proteins there was considerable heterogeneity in the size of the polypeptides which bound the two ligands. However, all bands that bound ^{125}I-fibronectin also bound ^{125}I-labeled 29-kDa fragments.

In the *E. coli* pSDF200 cell lysate, the largest polypeptide with binding activity was 140 kDa, and this was true also for the *S. dysgalactiae* S2 cell lysate. For *E. coli* pSDF100, a fibronectin-binding polypeptide with an apparent M_r of 140,000, was also observed. However, a strong signal was also obtained from a polypeptide of slightly higher molecular weight. When a lysate from *E. coli* containing pUC18 was analyzed in a similar fashion, no fibronectin-binding components were detected (Fig. 7).

Similar profiles were revealed on Western blots probed with monospecific antibodies raised against the isolated recombinant fibronectin-binding proteins from clones containing pSDF200 and pSDF100, respectively (Fig. 8). Antibodies against the pSDF200 gene product identified a protein of 140 kDa, and several smaller, presumably degradation products, in lysates of both *E. coli* clone pSDF200 and *S. dysgalactiae* S2 but did not react with polypeptides from clones containing pSDF100 or pUC18 (Fig. 8). Antibodies to fibronectin-binding proteins encoded by pSDF100 only gave a detectable signal with material from the pSDF100-containing clone revealing a polypeptide of molecular mass somewhat larger than 140 kDa. The lysate from *S. dysgalactiae* S2 did not contain any protein that could be detected with the antibody against the pSDF100-encoded fibronectin-binding protein, suggesting that this protein was not expressed in the cells in appreciable amounts. Furthermore, components in lysates of *E. coli* clones containing pSDF200 or pUC18 did not react with the pSDF100-encoded protein.

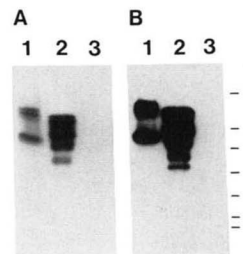

FIG. 7. **Identification of fibronectin-binding proteins in cell lysates of *E. coli* TG1 containing pSDF100 or pSDF200.** Cell lysate proteins were subjected to SDS-polyacrylamide gradient gel electrophoresis (3–15%), transferred to Immobilon-P, and probed with ^{125}I-fibronectin (*A*) or ^{125}I-labeled 29-kDa fragment of fibronectin (*B*). *Lane 1, E. coli* TG1 pSDF100 (70 μg); *lane 2, E. coli* pSDF200 (6 μg); *lane 3, E. coli* TG1 pUC18 (40 μg). The migration position of prestained molecular mass standards is indicated, and the molecular masses are as listed in the legend to Fig. 3.

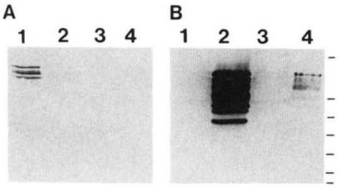

FIG. 8. **Western immunoblotting for detection of pSDF100- and pSDF200-encoded fibronectin receptors.** Whole cell lysates of *E. coli* TG1 containing pSDF100 or pSDF200, and *S. dysgalactiae* S2 were subjected to SDS-polyacrylamide gradient gel electrophoresis, transferred to Immobilon-P, and sequentially probed with monospecific antibody and Bio-Rad goat anti-rabbit IgG alkaline phosphatase conjugate as described under "Materials and Methods." *A*, pSDF100-monospecific antibody; *B*, pSDF200-monospecific antibody. The following quantities of cell lysate protein were applied: *lane 1*, 70 μg of *E. coli* pSDF100; *lane 2*, 15 μg of *E. coli* pSDF200; *lane 3*, 43 μg of *E. coli* pUC18; *lane 4*, 52 μg of *S. dysgalactiae* S2. The positions of prestained molecular mass standards are indicated. The molecular masses are as indicated in Fig. 3.

Evidence for surface localization of the pSDF200-encoded protein of *S. dysgalactiae* was obtained from an experiment in which cells were treated with trypsin followed by Western immunoblotting. Control extracts of boiled cells showed the presence of 140-kDa pSDF200 antigen as in Fig. 8*B*, and there was no evidence of this intact antigen in extracts from cells pretreated with trypsin. In the supernatant from the trypsin-treated cells three antigenic polypeptides with molecular masses of 76, 58, and 39 kDa were apparent (data not shown). This is in agreement with data from binding assays in which the trypsin-treated cells showed a 60% reduction in fibronectin binding ability relative to control cells.

Our results indicate that *S. dysgalactiae* S2 contains at least two separate genes encoding immunologically distinct fibronectin-binding proteins, both of which bind the 29-kDa NH_2-terminal domain of fibronectin. However, it appears that under the culture conditions employed, only the gene cloned in pSDF200 is expressed on the cell surface in detectable quantities by strain S2.

DISCUSSION

S. dysgalactiae belongs to group C streptococci and is an important pathogen for cattle. The strain S2 examined in this study was isolated from a case of bovine mastitis. A previous study (Mamo *et al.*, 1987) has indicated that this strain expresses fibronectin-binding cell surface receptors which are at least partly of proteinaceous nature since they are susceptible to treatment of cells with proteolytic enzymes. In the present paper we report on the identification of two separate genes in *S. dysgalactiae* S2 coding for fibronectin-binding proteins. The genes were characterized by restriction mapping (Fig. 4, *a* and *b*), and regions of the genes encoding fibronectin binding activities were localized by subcloning in pUC18. From these regions it was possible to select restriction fragments to be used as DNA probes, which in hybridization experiments specifically recognized the respective gene (Fig. 5).

Our data clearly establish the presence of cell surface protein components that mediate receptor-ligand interactions. An antibody raised against one of the expressed proteins (encoded by pSDF200) reacted with several polypeptides when a lysate of *S. dysgalactiae* S2 was analyzed by Western blot (Fig. 8). Polypeptides of similar molecular weight also bound fibronectin when the transferred peptides were probed with ^{125}I-labeled ligand (Fig. 7). Furthermore, when whole cells were incubated with trypsin there was a 60% reduction in fibronectin binding, which could be correlated with the loss of a 140-kDa antigen in Western immunoblots and the concomitant appearance of discrete antigenic polypeptides in the cell-free supernatant. Additional data in support of a cell surface localization come from preliminary nucleotide sequence analysis. The pSDF100- and pSDF200-encoded proteins were found to possess typical signal sequences for protein secretion and COOH-terminal sequences similar to those observed in several other Gram-positive cell wall proteins. This may account for the failure to obtain total loss of fibronectin binding by treatment of cells with trypsin. The fibronectin binding domain may be close to the COOH terminus, adjacent to the membrane anchor and cell wall spanning sequences as with *Staphylococcus aureus* fibronectin-binding protein (Signäs *et al.*, 1989). Polypeptides retaining binding activity could therefore remain firmly associated to the cell wall. It is likely that the many fibronectin-binding and immunoreactive polypeptides in *S. dysgalactiae* S2 (Figs. 3 and 8) and *E. coli* pSDF200 (Figs. 7 and 8) represent degradation products of a high molecular weight native receptor, possibly with an M_r of 140,000, as this is the size of the largest polypeptide identified both in immunoblots and blots probed with ^{125}I-labeled ligand. The second fibronectin-binding protein, encoded by pSDF100, is not produced at detectable levels in *S. dysgalactiae* S2 under the conditions of our experiments (Fig. 8). This observation raises the possibility that external factors may regulate the expression of the two fibronectin receptor genes in *S. dysgalactiae* which will be examined in future studies.

Previous studies on the binding site in fibronectin for *S. dysgalactiae* have resulted in apparently different conclusions. Mamo *et al.* (1987) found that strain S2 bound to a site in the NH_2-terminal region of fibronectin whereas Chhatwal *et al.* (1987) reported that a different *S. dysgalactiae* strain, also isolated from bovine mastitis, interacts with an internal site close to the RGD-containing sequence recognized by eukaryotic receptors of the integrin family. Our data confirm the previous results that strain S2 primarily binds to the NH_2-terminal domain of fibronectin. Purified unlabeled 29-kDa fragment effectively inhibits the binding of ^{125}I-labeled fibronectin to streptococcal cells (Fig. 1). The fibronectin receptor encoded by pSDF200 binds ^{125}I-labeled 29-kDa fragment of fibronectin, as does the released material from strain S2 (Figs. 7 and 3). Furthermore, it inhibits the binding of the 29-kDa fragment and intact fibronectin to streptococcal cells (Fig. 6). In view of these considerations, it is tempting to suggest that the protein encoded by the recombinant plasmid pSDF200 represents the major fibronectin receptor of *S. dysgalactiae* S2. It is possible that the strain studied by Chhatwal *et al.* (1987) expresses a different receptor with different binding specificity. However, this hypothetical receptor is not the pSDF100 product, which also recognizes the NH_2-terminal domain of fibronectin. Hence, the streptococcal fibronectin-binding proteins encoded by pSDF100 and pSDF200 have a ligand specificity similar to that of the staphylococcal fibronectin receptor characterized previously (Flock *et al.*, 1987; Fröman *et al.*, 1987). Studies are currently in progress to characterize and compare further the streptococcal and staphylococcal fibronectin-receptor proteins, their active sites, and the binding sites in fibronectin.

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