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Fibronectin-Binding Protein of *Streptococcus equi* subsp. *zooepidemicus*

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By screening a genomic λ library of *Streptococcus equi* subsp. *zooepidemicus*, we have cloned and sequenced a gene, termed *fnz*, encoding a fibronectin (Fn)-binding protein called FNZ. On the basis of the deduced amino acid sequence of FNZ, the mature protein has a molecular mass of ~61 kDa. Analysis of FNZ reveals a structural organization similar to that of other cell surface proteins from streptococci and staphylococci. The Fn-binding activity is localized to two domains in the C-terminal part of FNZ. One domain is composed of five repeats, which contain a motif similar to what has earlier been found in other Fn-binding proteins in streptococci and staphylococci. The first and second repeats are separated by a short stretch of amino acids, including the motif LAGESGET, which is an important part of the second Fn-binding domain. This motif is also present in an Fn-binding domain (UR) in protein F of *Streptococcus pyogenes*. A fusion protein covering the Fn-binding domain of FNZ inhibits the binding of the 29-kDa N-terminal fragment of Fn to cells of various streptococcal species as well as to *Staphylococcus aureus*.

Fibronectin (Fn), a dimeric glycoprotein with a molecular mass of ~450 kDa, is considered an important ligand involved in the binding of gram-positive cocci to the host tissue (7, 27). The protein is present in a soluble form in plasma and various body fluids and in a fibrillar form in the extracellular matrix of connective tissue. The main function of Fn is to mediate substrate adhesion of eukaryotic cells, which involves the binding of specific cell surface receptors to certain domains of the Fn molecule (9). The protein also interacts with several other macromolecules, such as DNA, heparin, fibrin, and collagen (21, 30).

Pathogenic bacteria such as *Staphylococcus aureus*, coagulase-negative staphylococci, and serological groups A, B, C, and G of streptococci have been reported to bind Fn (15, 22, 28, 36). Genes encoding Fn-binding proteins have been cloned and sequenced from *Staphylococcus aureus* (*fnbA* and *fnbB*) (4, 14, 35), *Streptococcus dysgalactiae* (*fnbA* and *fnbB*) (16), and *Streptococcus equisimilis* (*fnb*) (17), and for two different strains of *Streptococcus pyogenes* two highly homologous genes (*sfb* and *prtF*) have been characterized (34, 37). Another gene from *S. pyogenes* encoding a protein with both serum opacity and Fn-binding activity has also been described (29). Comparisons between these Fn-binding proteins reveal that they all consist of a C-terminal repetitive domain which binds Fn. Furthermore, another protein, FBP54, from *S. pyogenes*, different from the proteins mentioned above, was recently described to bind both Fn and fibrinogen through a domain located in the N-terminal end of the protein (2). In addition, Pancholi and Fischetti (26) have reported that a glyceraldehyde-3-phosphate dehydrogenase from a group A streptococcal strain binds several host proteins, like lysozyme, myosin, actin, and Fn.

Streptococcus equi subsp. *zooepidemicus*, an opportunistic commensal organism on the mucosa, is frequently associated with diseases in horses (38). The bacterium has a broad host spectrum and can occasionally also infect humans. It has earlier been reported that different strains within this subspecies have the ability to bind Fn as well as various other plasma proteins and extracellular matrix components (18, 22).

In the present study, we report on the cloning, sequencing, and expression of a gene encoding an Fn-binding protein from *S. equi* subsp. *zooepidemicus*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *S. dysgalactiae* S2 and *S. equi* subsp. *zooepidemicus* Z5 are animal clinical isolates obtained from the State Veterinary Institute, Uppsala, Sweden. The plasmids pUC18 and pUC19 were used together with the *Escherichia coli* strains DH5α and TG1 for cloning purposes. The *E. coli* strain P2392 (Stratagene, La Jolla, Calif.) was used for propagation of λ phages. The plasmid pEZZ 18 (24) was used for expression of extracellular fusion proteins with immunoglobulin G (IgG)-binding capacity in *E. coli* TG1. *S. aureus* Cowan I was grown in tryptone soy broth (Oxoid, Basingstoke, Hampshire, United Kingdom), and streptococcal strains were grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 1% yeast extract (Oxoid). The *E. coli* strains were grown in Luria-Bertani (LB) medium supplemented in appropriate cases with 50 μg of ampicillin per ml. All incubations were at 37°C.

Proteins and reagents. Bovine serum albumin (fraction V, radioimmunoassay grade) was obtained from U.S. Biochemical (Cleveland, Ohio). Horseradish peroxidase (HRP) was obtained from Boehringer GmbH (Mannheim, Germany). Bovine Fn used to coat microtiter wells and to conjugate HRP was obtained from Bional (Tartu, Estonia). Isolation of the 29-kDa N-terminal fragment of Fn and ^{125}I labelling of this fragment and whole Fn have been described previously (16). Molecular weight markers used in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad (Richmond, Calif.). Nitrocellulose (NC) filters were obtained from Schleicher and Schüll (Dassel, Germany). The enhanced chemiluminescence (ECL) system (Amersham, Little Chalfont, Bucks, United Kingdom) was used to visualize bound HRP-conjugated Fn.

Isolation of clones expressing Fn-binding activity. Cells of *S. equi* subsp. *zooepidemicus* Z5 were tested for binding of a ^{125}I-labelled 29-kDa N-terminal fragment of Fn as described previously (16). A genomic λEMBL3 library of this strain (12) was screened as described earlier for isolation of IgG-binding clones (6) but exchanging labelled IgG for the ^{125}I-labelled 29-kDa N-terminal fragment of Fn. Among the positive clones obtained, one clone, called λSZF1, was chosen for further studies. Subclones from λSZF1 were generated by digestion of purified phage DNA with appropriate restriction enzymes and subsequent ligations of fragments into pUC18 and -19. After screening for subclones expressing Fn-binding activity, one clone, designated pSZF20, was chosen for further studies. Phage and plasmid DNA were prepared by standard methods (31).

DNA sequencing. The nucleotide sequence of pSZF20 was partially determined by the dideoxy chain termination method with a Sequenase version 2.0 kit (U.S. Biochemical). The sequencing samples were analyzed on wedge-shaped 6% acrylamide–7 M urea gels, which were subsequently dried and subjected to

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autoradiography. The sequencing strategy included the generation of subclones suitable for double-stranded sequencing with commercial primers, and in appropriate cases, specific synthetic primers were used. Computer programs from the PC GENE, DNA, and protein sequence analysis software package (Intelligenetics, Inc., Mountain View, Calif.) were used to record and analyze the sequence data.

Construction and purification of peptides derived from protein FNZ. To construct clones expressing various parts of FNZ, two DNA fragments were PCR amplified. Fragment 2 was constructed by digestion of the PCR product obtained with the template pSZF20 together with the primers 303B (5′-GGCGATGTCT CCTTGGAAAAGCT-3′) and LAGES I (5′-TATCTAGAACCGCCGCCGAT CCC-3′) with *Eco*RV. Fragment 3 was constructed with the primers LAGES II (5′-TATCTAGAACACCTAAACCAGGACAAA-3′) and LAGES III (5′-TAG GATCCTTATGGCGCAGGTGCAGGT-3′). The underlined nucleotides in the respective primers correspond to complementary sequences in the *fnz* gene. Fragments 2 and 3 were cleaved with *Xba*I and ligated, resulting in fragment 4. These three fragments (fragments 2 to 4) together with the 633-bp *Eco*RV-*Hha*I fragment, called fragment 5 (see Fig. 1, positions 1117 to 1749), were cloned separately into the vector pEZZ 18, resulting in the four plasmids pZZ-2 to pZZ-5 encoding the proteins ZZ-2 to ZZ-5 (see Fig. 4). These four proteins were purified from the culture supernatants by affinity chromatography with IgG-Sepharose columns mainly as described by Lindgren et al. (16). Bound material was eluted with 0.25 M acetic acid (pH 2.8) and dialyzed against phosphate-buffered saline (PBS; pH 7.4 [137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.4 mM KH_2PO_4]).

SDS-PAGE and Western blot (immunoblot) analysis. *E. coli* cells harboring various plasmid constructs were grown overnight, pelleted, washed once in PBS, and resuspended in PBS. The samples were mixed and boiled in an equal volume of sample buffer, containing 5% β-mercaptoethanol and 2.5% SDS, before application onto an 8 to 25% gradient SDS-PAGE gel by use of the PHAST system (Pharmacia Biotech, Uppsala, Sweden). For transfer of the separated proteins to NC filters, a filter was put on top of the gel and the temperature was raised to 65°C to increase the diffusion. After 30 min, the filter was removed and saturated in PBS supplemented with 0.05% Tween 20 (PBST) for 1 h under gentle agitation at 37°C. The filter was then incubated for 1 h in PBST supplemented with HRP-labelled bovine Fn. After washing with PBST, the bound HRP-labelled Fn was detected with the ECL system.

Inhibition assays. Cells from overnight cultures of *S. equi* subsp. *zooepidemicus* Z5, *S. dysgalactiae* S2, and *S. aureus* Cowan I were collected by centrifugation, washed twice in PBS, and suspended in PBS–1% Tween 20 to an optical density at 600 nm of 0.2. The bacteria (500 μl) were incubated with 9.5×10^4 cpm of ^{125}I-labelled whole Fn, aliquots of purified fusion proteins were added to the mixture, and the volume was adjusted to a total of 1 ml with PBS–1% Tween 20. The mixtures were incubated for 2 h at room temperature (RT) and centrifuged at 3,000 × *g* for 3 min. After removal of the supernatants, the radioactivity associated with the pellets was quantified in a gamma counter (LKB Wallac, Turku, Finland). Radioactivity (550 cpm) recovered from a control (tubes that contained no streptococci) was subtracted in each test.

Construction of the phagemid library. A phagemid library was constructed by the method described by Jacobsson and Frykberg (10). In short plasmid DNA from the clone pSZF20, encoding the Fn-binding protein FNZ, was randomly fragmentated by sonication (MSE, Loughborough, England), and fragments with the approximate size of 500 bp were isolated by preparative agarose gel electrophoresis. The isolated fragments were treated with T4 DNA polymerase in the presence of free nucleotides to obtain blunt ends and thereafter ligated into the vector pHEN1 (8) with a Ready To Go ligation kit from Pharmacia. The ligated material was electrotransformed into 10 50-μl aliquots of competent *E. coli* TG1 cells. The transformed cells were diluted to 80 ml with LB broth plus 2% glucose and incubated for 1 h at 37°C before ampicillin was added to a final concentration of 50 μg/ml. The next day, the cells were pelleted and resuspended in 10 ml of LB broth. From the resuspended pellet, 0.5 ml was infected with the helper phage R408 at a multiplicity of infection of 20. After 1 h, the cells were diluted to 40 ml with LB broth and the culture was incubated for another 6 h. Finally, the bacteria were pelleted, and the supernatant, containing both wild-type phages

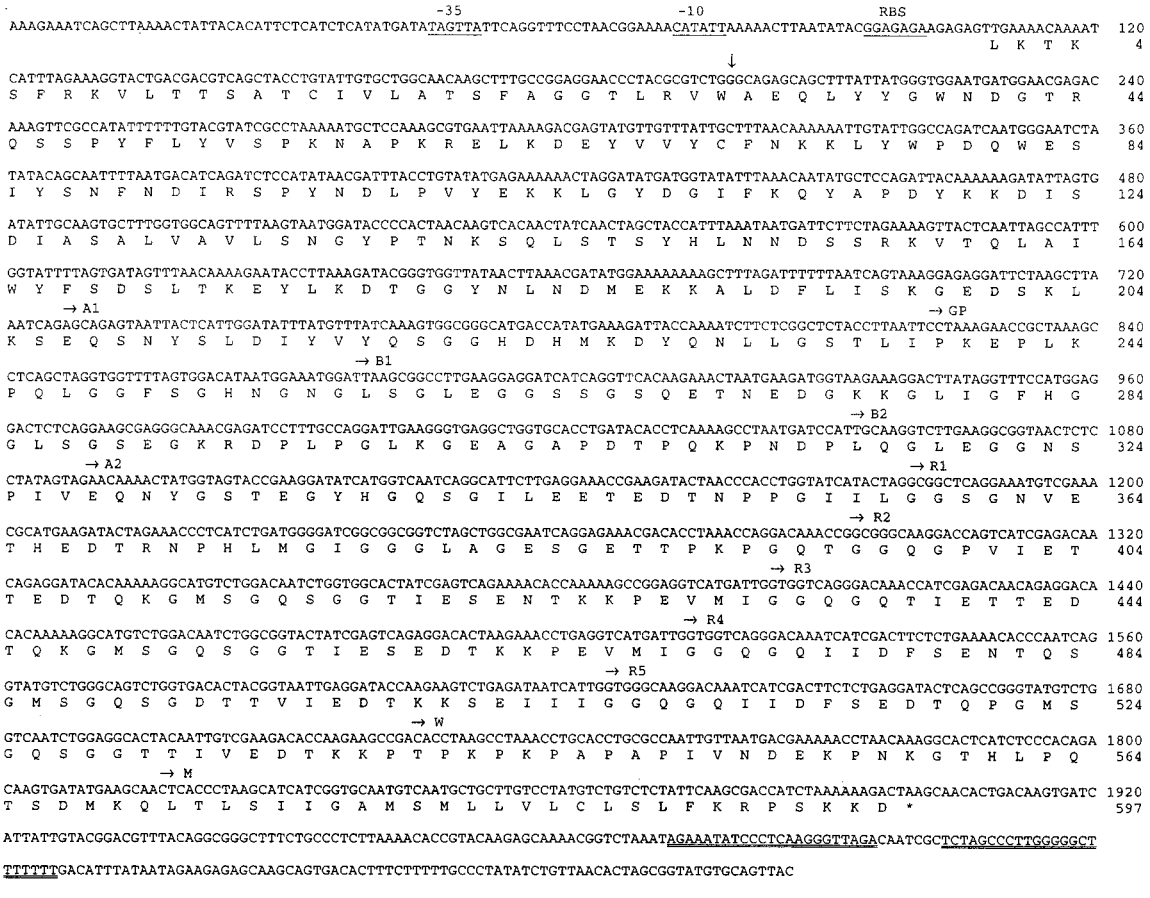

FIG. 1. The complete nucleotide sequence of the *fnz* gene from *S. equi* subsp. *zooepidemicus* Z5 and the deduced amino acid sequence of FNZ. A putative ribosome-binding site (RBS) and possible −10 and −35 promoter signals are underlined with thin lines. A putative signal sequence (S) cleavage site is marked with a vertical arrow, the translational stop codon is marked with an asterisk, and a possible transcription termination hairpin loop is underlined with thick lines. The putative wall- and membrane-spanning regions (W and M, respectively) as well as a glycine-proline-rich domain (GP) and the Fn-binding repeats (R1 to R5) are indicated. The repeated motif LXGLEGGXS (B1 and B2) and the two imperfect repetitive sequences of 15 amino acids (A1 and A2) are also indicated.

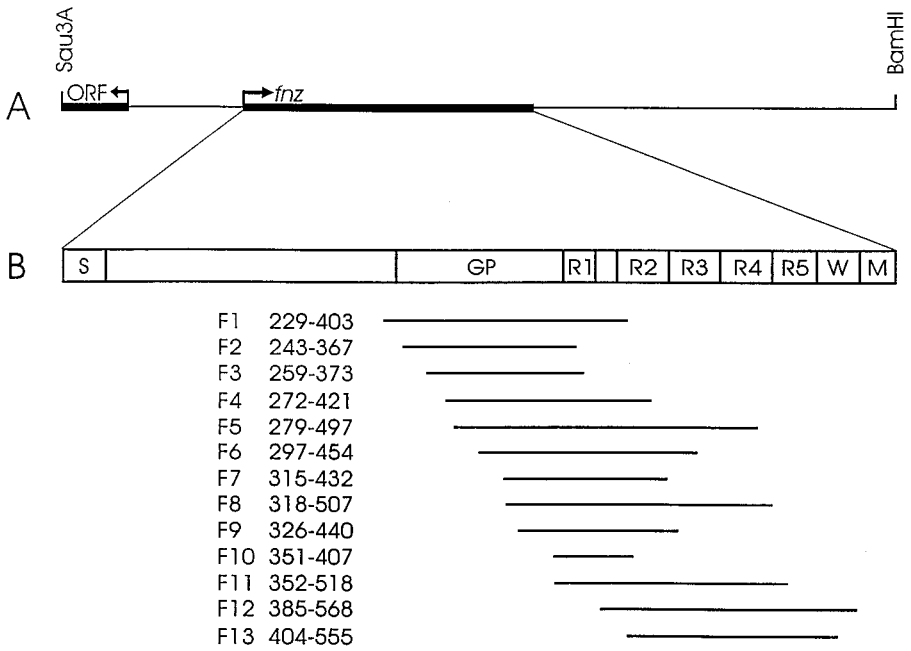

FIG. 2. (A) Map of pSZF20 with the gene *fnz* and the upstream open reading frame (ORF). (B) Schematic representation of protein FNZ with the different functional domains indicated and defined as described in the legend to Fig. 1. The bars correspond to the amino acid sequences of the phagemid clones F1 to F13, isolated by panning against Fn. The numbers refer to the amino acid positions in protein FNZ as shown in Fig. 1.

and recombinant phagemid particles, was sterile filtered and stored at 4°C. The library consisted of 6×10^5 clones, and after propagation, the phagemid titer was 6×10^9 CFU/ml.

Panning of the phagemid library and identification of Fn-binding clones. Microtiter wells (Maxisorp; Nunc, Copenhagen, Denmark) were coated overnight at 4°C with 200 μl of bovine Fn at a concentration of 100 μg/ml in 0.05 M $NaHCO_3$ (pH 9.7). The wells were then incubated with PBST for 1 h at RT. After washing with PBST, 200 μl of the phagemid library was added to the coated wells, and the plates were incubated for 4 h at RT. Before elution, the wells were washed extensively with PBST and then stepwise eluted with 200-μl buffer solutions consisting of 50 mM Na citrate and 150 mM NaCl with decreasing pH values (5.4, 3.7, and 2.1). The eluates were neutralized by the addition of 50 μl of 1 M Tris (pH 8.0). From the eluates, 20 μl was used to infect 50 μl of *E. coli* TG1 cells. After 30 min of incubation at RT, the cells were spread on LA plates (LB medium with 1.5% agar) containing 2% glucose and 50 μg of ampicillin per ml. The plates were incubated overnight at 37°C, and the next day the colonies were transferred to NC filters, which were placed on LA plates containing ampicillin (50 μg/ml) and IPTG (isopropyl-β-D-thiogalactopyranoside; 100 μg/ml). After 4 h, the filters were removed and placed for 5 min in chloroform vapor to lyse the colonies. The filters were saturated with PBST for 1 h at 37°C and then washed in PBST to remove cell debris. After washing, the filters were incubated for 1 h at 37°C in PBST containing HRP-labelled Fn. After a thorough washing with PBST, the bound Fn was detected with the ECL system. Positive clones were grown on a small scale for preparation of phagemid DNA to sequence the inserts encoding Fn-binding activity.

Nucleotide sequence accession number. The nucleotide sequence of *fnz* has been deposited in the EMBL sequence data bank under accession number X99995.

RESULTS

Identification and characterization of *fnz*. Probes derived from the *fnbA* and *fnbB* genes from *S. dysgalactiae* and the *sfb* gene from *S. pyogenes* have been shown not to hybridize to chromosomal DNA from *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus* (17, 40). This indicated that the Fn-binding activity in both subspecies of *S. equi* is encoded by genes not closely related to *S. dysgalactiae* and *S. pyogenes*. Therefore, we decided to screen a λEMBL3 library of chromosomal DNA from *S. equi* subsp. *zooepidemicus* Z5 for clones expressing Fn-binding activity by use of a ^{125}I-labelled 29-kDa N-terminal Fn fragment. A positive clone, called λSZF1, was subcloned into a plasmid vector, and an Fn-binding subclone containing a 5.2-kb *Sau*3A-*Bam*HI fragment was isolated. This clone was designated pSZF20 (see Fig. 2A). By additional subcloning and DNA sequence analysis, it was found that the Fn-binding activity was encoded by an open reading frame of 1,791 bp, starting with a TTG codon at nucleotide position 108 and ending with a TAA codon at position 1898 (Fig. 1). The open reading frame is preceded by sequences typical for promoter- and ribosome-binding sites of gram-positive cocci and is followed by a sequence resembling a transcriptional terminator, suggesting that the gene product is translated from a monocistronic messenger. The gene, termed *fnz*, encodes a protein called FNZ, which is composed of 597 amino acids with a calculated molecular mass of ~64 kDa. Since FNZ is predicted to be a cell surface protein, the deduced amino acid sequence should include an N-terminal signal sequence. Analysis of the sequence by the method of von Heijne (42) revealed a possible signal cleavage site between amino acids 32 and 33, resulting in a mature protein of 565 amino acids with a calculated molecular mass of ~61 kDa. After the signal sequence, there is a region rich in aromatic amino acids followed by a region termed GP. Besides a high number of glycine and proline residues, the GP region contains two copies of the motif LXG LEGGXS, called B1 and B2, respectively (Fig. 1). The B sequences are flanked by two imperfect repetitive sequences of 15 residues, called A1 and A2, respectively (Fig. 1). Following the GP region, there is a repetitive region called R (Fig. 1, R1 to R5). The C-terminal part of the protein contains all the typical features of cell surface proteins from gram-positive cocci, a proline-rich region, which is typical for the cell wall-spanning domain, followed by ~20 hydrophobic amino acids, which probably form the transmembrane segment of the protein. The hydrophobic domain is directly followed by eight, mainly positively charged amino acids in the C-terminal end. Furthermore, the wall-spanning domain contains the consensus sequence LPXTG, involved in cell wall anchoring (32, 33),

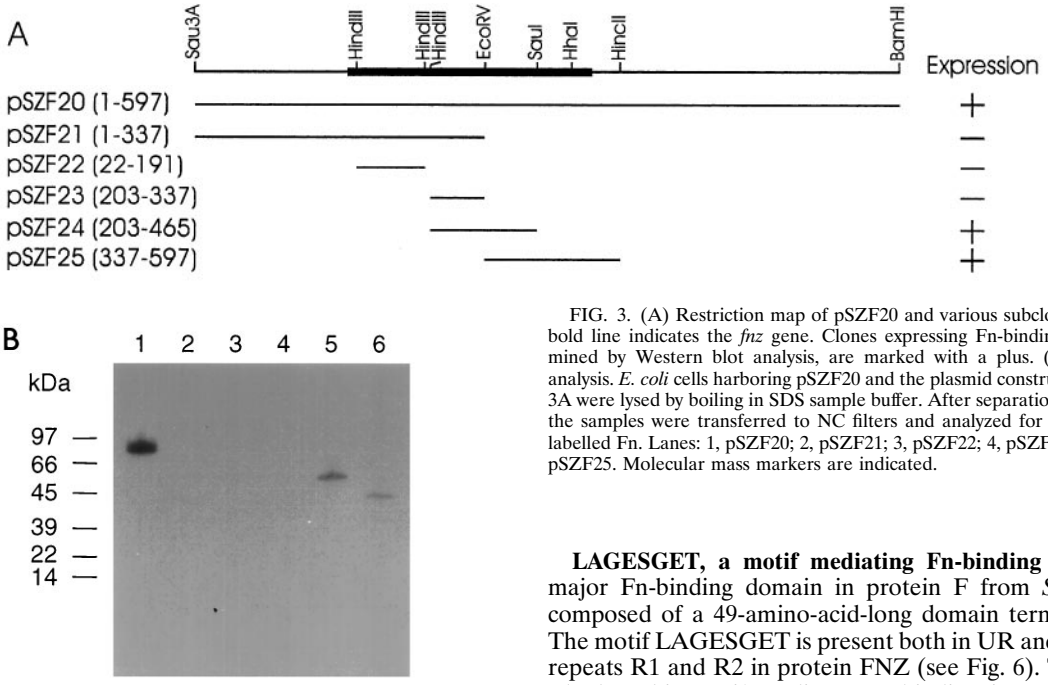

FIG. 3. (A) Restriction map of pSZF20 and various subclones thereof. The bold line indicates the *fnz* gene. Clones expressing Fn-binding activity, determined by Western blot analysis, are marked with a plus. (B) Western blot analysis. *E. coli* cells harboring pSZF20 and the plasmid constructs shown in Fig. 3A were lysed by boiling in SDS sample buffer. After separation by SDS-PAGE, the samples were transferred to NC filters and analyzed for binding of HRP-labelled Fn. Lanes: 1, pSZF20; 2, pSZF21; 3, pSZF22; 4, pSZF23; 5, pSZF24; 6, pSZF25. Molecular mass markers are indicated.

although in protein FNZ, there is a serine instead of a glycine in position five. A similar exception is also found in protein SCP from *S. pyogenes*, where glycine is exchanged for asparagine (1).

Seven hundred base pairs upstream from the *fnz* gene, we have identified and partially sequenced (data not shown) another open reading frame, going in the opposite direction (Fig. 2A). Preliminary analysis shows that this open reading frame encodes a protein resembling ROF A of *S. pyogenes*, which acts as a positive *trans*-acting regulator of protein F (5).

Identification of the Fn-binding domains. To map the Fn-binding domain in protein FNZ, a phage display cloning and mapping technique was used (10). Briefly, the plasmid pSZF20 (Fig. 2A) harboring the *fnz* gene was randomly disintegrated by sonication, and purified fragments were selected by size, ligated into the phagemid vector pHEN1, and subsequently transformed into *E. coli* cells. The transformed *E. coli* cells (representing 6×10^5 independent clones) were grown overnight in the presence of ampicillin and the following day were infected with helper phage R408 to obtain recombinant phagemid particles. The generated phagemid library (6×10^9 CFU/ml) was panned (affinity selected) against Fn-coated microtiter wells. After sequence analysis of 13 positive clones, the Fn-binding domain was localized to the C-terminal part of protein FNZ (Fig. 2B). To confirm that no Fn-binding activity is located in the N-terminal part of the protein, Western blot experiments were done with different subclones of pSZF20 (Fig. 3A). In agreement with the results from the phage display experiments, no Fn-binding activity was found to be encoded from the region upstream from the *Eco*RV site (Fig. 1, nucleotide position 1116) of the *fnz* gene (Fig. 3). As determined from Western blot analysis (Fig. 3B, lane 1), the apparent relative molecular mass of the mature FNZ is larger than the calculated ~61 kDa, which is based on the deduced amino acid sequence. Differences between calculated and determined molecular masses have been reported earlier for other Fn-binding proteins as well (29, 35).

LAGESGET, a motif mediating Fn-binding activity. The major Fn-binding domain in protein F from *S. pyogenes* is composed of a 49-amino-acid-long domain termed UR (25). The motif LAGESGET is present both in UR and between the repeats R1 and R2 in protein FNZ (see Fig. 6). To determine whether this motif mediates Fn binding in protein FNZ, a series of fusion proteins was constructed (Fig. 4A). The purified fusion proteins (Fig. 4B) were tested for Fn binding in a Western blot (Fig. 4C) and for their inhibitory activity (Fig. 5). In both experiments, the protein missing LAGESGET (ZZ-4) showed a considerably lower binding activity than that of the fusion protein where the motif is intact (ZZ-5). In contrast, no difference was detected between proteins ZZ-4 and ZZ-5 in their ability to inhibit the binding of the 29-kDa fragment of Fn to cells of *S. equi* subsp. *zooepidemicus* (data not shown). The protein ZZ-5 also efficiently inhibited the binding of the 29-kDa fragment of Fn to cells of *S. aureus* Cowan I and *S. dysgalactiae* S2 (data not shown).

DISCUSSION

Although the overall organization of the Fn-binding protein FNZ from *S. equi* subsp. *zooepidemicus* Z5 resembles that of earlier reported Fn-binding proteins from *S. aureus* (14), *S. dysgalactiae* (16), *S. pyogenes* (29, 34, 37), and *S. equisimilis* (17), the amino acid sequence of FNZ displays a low degree of homology to the sequences of these proteins. Analysis of the deduced amino acid sequence of FNZ and comparisons with the sequences of earlier reported Fn-binding proteins suggested that the repetitive domain close to the putative cell wall-spanning domain should be responsible for mediating the Fn binding of the protein. In addition to the repetitive domains, a nonrepetitive Fn-binding domain has been found in both FnBPA and FnBPB from *S. aureus* (10, 13) and in protein F from *S. pyogenes* (34). To map the Fn-binding domain in FNZ, we used a shotgun phage display technique which has been used earlier in our laboratory to clone and map the binding domains of bacterial receptor proteins (10, 11). The mapping of FNZ showed that some positive phagemid clones did not contain overlapping DNA sequences (Fig. 2B), suggesting the presence of at least two Fn-binding sites. Furthermore, all positive phagemid clones contain DNA sequences corresponding to a repetitive domain, consisting of five highly homologous repeats, 33 to 37 residues long, called R1 to R5 (Fig. 6). The first repeat, called R1, has a low degree of homology to the R2

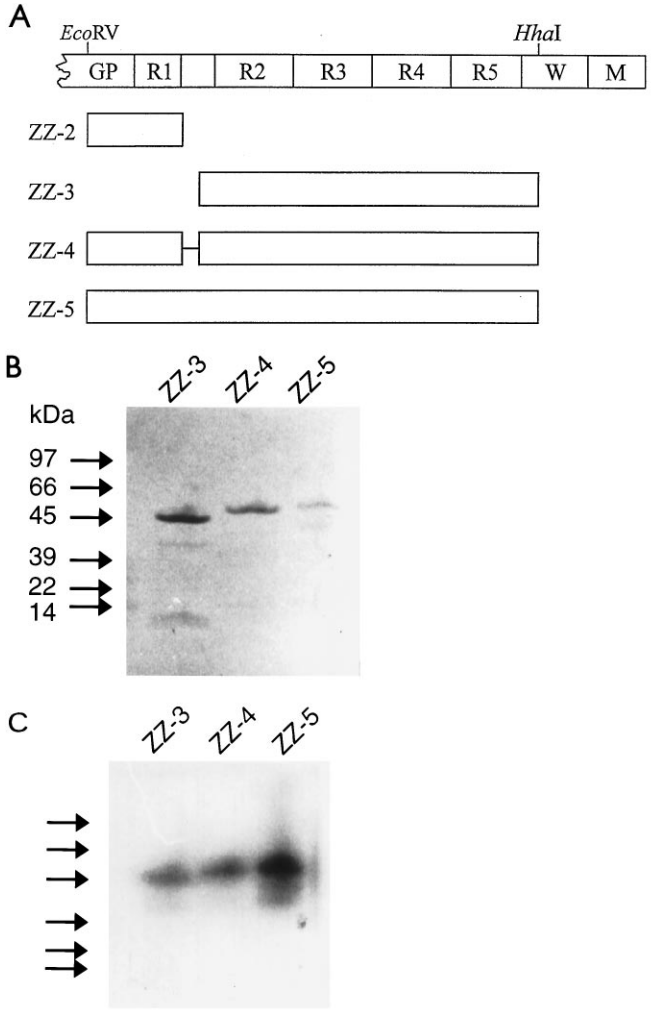

FIG. 4. (A) Parts of the fusion proteins ZZ-2 to ZZ-5 derived from the C-terminal end of protein FNZ. The DNA fragments encoding various parts of FNZ were constructed as described in Materials and Methods. (B) SDS-PAGE with affinity-purified fusion proteins. Molecular mass markers are indicated. (C) Western blot analysis. The samples from SDS-PAGE were transferred to NC filters and analyzed for binding of ^{125}I-Fn.

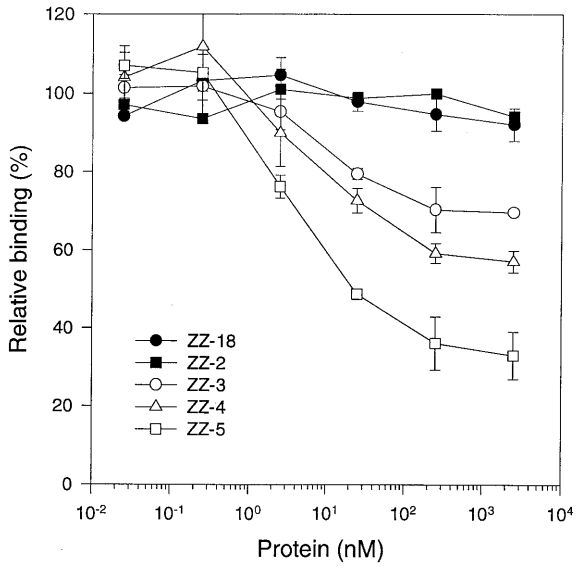

FIG. 5. Inhibition of Fn binding. Cells of *S. equi* subsp. *zooepidemicus* were incubated with ^{125}I-labelled Fn (representing 9.5×10^4 cpm) in the presence of increasing amounts of the affinity-purified fusion proteins ZZ-2 to ZZ-5. The purified IgG-binding protein (ZZ-18) encoded by the vector was used as a negative control. The radioactivity remaining associated with the bacterial cells after incubation for 2 h at RT was determined with a gamma counter. Points represent means of duplicates, and the standard deviations are indicated. The amount of bound radioactivity in the absence of inhibitory protein was 2.2×10^4 cpm, and the background (tubes without streptococci) was 550 cpm.

to R5 repeats and is separated from the latter by a stretch of 16 amino acid residues, called spacer. Comparisons of the nucleotide sequences of the R2 to R5 repeats show only minor differences between individual repeats. Analysis of the repeats (Fig. 6) reveals a pairwise relationship, i.e., the highest degree of homology is between repeats R2 and R3 and between R4 and R5, respectively. This indicates that this part of *fnz* has evolved through a gene duplication mechanism, which has also been suggested earlier for other staphylococcal and streptococcal cell surface proteins containing repetitive domains (6, 39).

Earlier reports demonstrated cross-inhibitory activities of Fn-binding proteins from different genera and species (7, 14, 16). Our results from similar experiments showed that the purified ZZ-5 fusion protein inhibits the binding of a 29-kDa N-terminal fragment of Fn to *S. equi* subsp. *zooepidemicus* Z5 and also the binding to *S. aureus* Cowan I and *S. dysgalactiae* S2 (data not shown).

McGavin et al. (20) studied the inhibitory effect of synthetic peptides corresponding to individual repeats found in the Fn-binding domains of FnBA and FnBB of *S. dysgalactiae* together with mutated peptides mimicking the A2 repeat of FnBA. From these studies, they suggested that from a core sequence, $ED^{T}_{S}X_{9\text{-}10}GGX_{3\text{-}4}{}^{I}_{V}DF$, only the amino acids $GGX_{3\text{-}4}{}^{I}_{V}DF$ together with additional acidic residues in the C-terminal part of the peptides are necessary for binding activity. The latter motif also occurs in FNZ but only in repeats R4 and R5. Furthermore, McGavin et al. (20) showed that an exchange of phenylalanine (F) for a serine in the core sequence dramatically decreased the inhibitory effect of the peptide. Interestingly, in repeat R2 and R3 (as well as in repeat R1) in FNZ, the amino acids ET are found instead of DF in this position.

When the phage display cloning and mapping technique was used, two clones, F2 and F3 (Fig. 2B and 6), which express Fn-binding activity without containing DNA encoding the regions R2 to R4, were identified. These clones cover a part of protein FNZ which includes most of the GP region and part of the R1 region (Fig. 6), showing that the complete R1 region is not necessary for binding. Interestingly, clone F2 harbors the motif $EDTX_7GGX_4ET$ (Fig. 6). Whether this motif alone is

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(348)┌>F10   ┌>R1(358) F2┐      F3┐        ┌>Spacer(380)
-EDTNPPGIILGGSGN-VETHEDTRNPHLMGIGGGLAGESGETTPKPGQT

                    F10┐
R2 (396-432)  GGQGPVIETTEDTQKGMSGQSGGTIESENTKKPEVMI
R3 (433-469)  GGQGQTIETTEDTQKGMSGQSGGTIESEDTKKPEVMI
R4 (470-506)  GGQGQIIDFSENTQSGMSGQSGDTTVIEDTKKSEIII
R5 (507-539)  GGQGQIIDFSEDTQPGMSGQSGGTTIVEDTKKP-
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FIG. 6. Alignment of the Fn-binding domains of FNZ. The start of each R repeat and the spacer region are indicated. The phagemid clone F10 and the C-terminal ends of the phagemid clones F2 and F3 within this domain are also indicated. The numbers in parentheses refer to the amino acid positions in protein FNZ as shown in Fig. 1.

mediating the binding is not yet known, but protein FNZ contains several sequences resembling this motif (Fig. 6). Further studies using synthetic peptides mimicking these motifs will answer the question if each motif interacts with Fn and how efficient each individual motif can be in its interaction with Fn.

Protein F from *S. pyogenes* JRS4 binds Fn not only to the repetitive domain called RD2 but also to an upstream adjacent domain termed UR (25). This domain, which is 49 amino acids long, mediates most of the binding of Fn to protein F. Interestingly, a motif, LAGESGET, present in UR is also found in a stretch of amino acids called spacer, separating the repeats R1 and R2 in FNZ (Fig. 6). We demonstrate here that LAG ESGET is an important part of an Fn-binding domain in protein FNZ, which like UR in protein F does not bind to the 29-kDa fragment of Fn. A fusion protein expressing LAG ESGET was several orders of magnitude more effective in inhibiting Fn binding to cells of *S. equi* subsp. *zooepidemicus* than fusion proteins lacking this motif (Fig. 5). The residues VETEDT in the C-terminal end of UR have been shown to be essential for the high-affinity binding of UR. A similar acidic motif is found at the corresponding place in FNZ. This is the only obvious homology between UR and the sequence surrounding LAGESGET in protein FNZ.

Analysis of the evolutionary relationship between *fnz* and other genes encoding Fn-binding proteins does not give a clear answer to how these genes have evolved. However, the amino acid sequence of FNZ reveals patches of homology to protein F in *S. pyogenes* such as the sequence LAGESGET. The repetitive Fn-binding domain of FNZ (R1 to R5) shows the highest degree of homology to the Fn-binding domain of protein FnBA from *S. dysgalactiae* (data not shown). We have recently cloned and characterized another gene, termed *zag*, from strain Z5 of *S. equi* subsp. *zooepidemicus* which encodes a cell surface plasma protein receptor called protein ZAG (12). This protein specifically binds, via separate domains, three different plasma proteins, IgG, α_2-macroglobulin, and albumin. Analysis of the relationship of the *zag* gene to other bacterial genes encoding cell surface proteins reveals patches of homologies to genes found in *S. dysgalactiae* encoding similar protein receptors. It is too early to suggest a model of the evolution of these genes, but one can speculate that they have evolved through mechanisms which involve intragenic and intergenic interspecies recombination of functional domains similar to what has been reported for other genes of gram-positive cocci (3, 43).

Regarding the regulation of *fnz*, preliminary results from experiments using cells of *S. equi* subsp. *zooepidemicus* grown under different environmental conditions show that a decreased oxygen level results in increased Fn-binding activity (data not shown). This is in contrast to what has been reported earlier for *prtF* (41).

Concerning the specific role of these adhesins in host-pathogen interactions, it is interesting to note that there is a common consensus sequence in the Fn-binding domain of the majority of Fn-binding proteins in staphylococci and streptococci (14, 16) and that protein F has been shown to mediate adhesion to epithelial cells (7).

The concept of using bacterial Fn-binding proteins as vaccines to protect the host from infection by the pathogen has been tested with respect to mastitis. Vaccination of mice and cows with recombinant *S. aureus* Fn-binding proteins as vaccine components resulted in protection against *S. aureus* infection in the udders of cows and the breasts of mice (19, 23). It is possible that a corresponding vaccine concept, based on specific streptococcal extracellular matrix and plasma protein receptors, can be applied to control infections caused by *S. equi.*

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