INFECTION AND IMMUNITY, Mar. 2002, p. 1319–1325 Vol. 70, No. 3
0019-9567/02/$04.00+0 DOI: 10.1128/IAI.70.3.1319–1325.2002

Contribution of Fibronectin-Binding Protein to Pathogenesis of *Streptococcus suis* Serotype 2

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Received 19 July 2001/Returned for modification 27 August 2001/Accepted 15 November 2001

In the present study we investigated the role of the fibronectin (FN)- and fibrinogen (FGN)-binding protein (FBPS) in the pathogenesis of *Streptococcus suis* serotype 2 in piglets. The complete gene encoding FBPS from *S. suis* serotype 2 was cloned in *Escherichia coli* and sequenced. The occurrence of the gene in various serotypes was analyzed by hybridization studies. The FBPS protein was expressed in *E. coli* and purified, and binding to human FN and FGN was demonstrated. The induction of antibodies in piglets was studied upon infection. An isogenic mutant unable to produce FBPS was constructed, and the levels of virulence of the wild-type and mutant strains were compared in a competitive infection model in young piglets. Organ cultures showed that FBPS was not required for colonization of the tonsils but that FBPS played a role in the colonization of the specific organs involved in an *S. suis* infection. Therefore, the FBPS mutant was considered as an attenuated mutant.

Streptococcus suis causes severe infections in piglets. The bacterial infections include meningitis, septicemia, and arthritis, and the animals often do not survive the infection (6, 28). Occasionally, *S. suis* causes septicemia and meningitis in humans (3). The pathogenesis of an *S. suis* infection is rarely understood. Sows are symptomless carriers of *S. suis* on their tonsils and pass the bacteria on to their piglets. The piglets cannot cope with the bacterium and subsequently develop the specific symptoms of an *S. suis* infection. Until now, 35 capsular serotypes of *S. suis* have been described (26), but serotype 2 strains are most often isolated from diseased piglets. Capsule is an important virulence factor, since piglets infected with an acapsular mutant of *S. suis* serotype 2 strains do not develop any clinical symptoms (22). Bacterial proteins have been suggested to play a role in the pathogenesis as well (1, 26). The expression of muramidase-released protein (MRP), extracellular factor (EF), and suilysin was shown to be strongly associated with pathogenic strains of *S. suis* serotype 2 (2, 29, 30). Since isogenic mutants lacking MRP and EF and isogenic mutants lacking suilysin were still pathogenic for young piglets, these proteins are not absolutely required for virulence (1, 23). Recently, a new virulence factor was identified (21) by using a complementation approach. The function of this virulence factor in the pathogenesis has to be further investigated.

Many important virulence factors are environmentally regulated and are induced at specific stages of the infection process (15). To identify these genes in *S. suis*, we cloned promoters and their downstream sequences that are "on" during experimental *S. suis* infection of piglets (20). Twenty-two in vivo-selected (*ivs*) genes were found. Two of the *ivs* genes were directly linked to virulence, since homology to genes in the database that encode for known virulence factors was found. One of these *ivs* genes (*ivs-21*) was identical to the *epf* gene of virulent *S. suis* serotype 2 strains (30). The other (*ivs-31*) showed homology to genes encoding fibronectin (FN)- and/or fibrinogen (FGN)-binding proteins of *Streptococcus gordonii* (GenBank accession no. X65164) and *Streptococcus pyogenes* FBP54 (8). A considerable number of FN-binding proteins of various bacterial species have been shown to be important virulence factors (12). In *S. pyogenes*, FBP54 was shown to be expressed in the human host and to preferentially mediate adherence to human buccal epithelial cells (7). It was recently shown that the FBP54 protein induces protective immunity against *S. pyogenes* challenge in mice (13).

In the present study we describe an FN- and FGN-binding protein of *S. suis* (FBPS). The sequence of *fbps* was determined. Binding studies showed that purified FBPS bound FN and FGN. A contribution of FBPS to the pathogenesis of *S. suis* serotype 2 was found.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *S. suis* strains were grown in Todd-Hewitt broth (code CM 189; Oxoid, Ltd., London, United Kingdom) and plated on Columbia blood base agar plates (code CM 331; Oxoid, Ltd.), containing 6% (vol/vol) horse blood. *Escherichia coli* strains were grown in Luria broth (17) and plated on Luria broth containing 1.5% (wt/vol) agar. If required, the following antibiotics were added at the indicated concentrations: spectinomycin (Sigma, St. Louis, Mo.) (50 μg/ml for *E. coli* and 100 μg/ml for *S. suis*), ampicillin (Boehringer, Mannheim, Germany) (100 μg/ml for *E. coli*), and kanamycin (Boehringer) (25 μg/ml for *E. coli*).

DNA techniques and sequence analysis. Routine DNA manipulations were performed as described by Sambrook et al. (19). DNA sequences were determined on a 373A DNA Sequencing System (Applied Biosystems, Warrington, Great Britain). Samples were prepared by use of an ABI Prism dye terminator cycle sequencing ready reaction kit (Applied Biosystems). Sequencing data were

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
Strains		
E. coli		
XL2-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}ZΔM15 Tn*10*(Tet^r) *amy* Cm^r]	Stratagene
M15	Nal^s Str^s Rif^s Thi$^-$ Lac$^-$ Ara$^+$ Gal$^+$ Mtl$^-$ F$^-$ RecA$^+$ Uvr$^+$ Lon$^+$	Qiagen
S. suis		
10	Virulent serotype 2 strain	29
10ΔFBPS	Isogenic *fbps* mutant of strain 10	This work
Plasmids		
pGEM7Zf(+)	Replication functions pUC, Amp^r	Promega Corp.
pKUN19	Replication functions pUC, Amp^r	14
pIC19R	Replication functions pUC, Amp^r	16
pDL282	Replication functions of pBR322 and pVT736-1, Amp^r Spc^r	25
pIC-spc	pIC19R containing Spc^r gene of pDL282	Laboratory collection
pQE-30	Replication functions pBR322, Amp^r, expression vector, six-His tag	Qiagen
pQE-30-FBPS	pQE-30 containing the 1.8-kb *fbps* gene	This work
pREP4	Replication functions pACYC, Kan^r, *lacI* gene	Qiagen
pE194	Em^r	11
pIVS-E	Replication functions of pWV01, Spc^r, promoterless *erm* gene of pE194	20
pIVS-31	pIVS-E containing 200 bp showing homology to *Streptococcus gordonii flpa*	20
pFBPS7-46	pGEM7Zf(+) containing *Eco*RI-*Eco*RI fragment of *fbps*	This work
pFBPS7-47	pFBPS7-46 in which 382-bp *Sal*I-*Sal*I fragment is replaced by 1.2-kb Spc^r from pIC-spc	This work

[a] Tet^r, tetracycline resistant; Cm^r, chloramphenicol resistant; Amp^r, ampicillin resistant; Spc^r, spectinomycin resistant; Kan^r, kanamycin resistant.

assembled and analyzed using the Lasergene program (DNASTAR). The BLAST software package was used to search for protein sequences homologous to the deduced amino acid sequences in the GenBank and EMBL databases.

Southern blotting and hybridization. Chromosomal DNA was isolated as described by Sambrook et al. (19). DNA fragments were separated on 0.8% agarose gels and transferred to GeneScreen Plus hybridization transfer membrane (NEN Life Science Products, Boston, Mass.) as described by Sambrook et al. (19). DNA probes of the *fbps* and *spc* genes were labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham Life Science, Little Chalfont, Buckinghamshire, Great Britain) by use of a random primed DNA labeling kit (Boehringer). The DNA on the blots was prehybridized for at least 30 min at 65°C and subsequently hybridized for 16 h at 65°C with the appropriate DNA probes in a buffer containing 0.5 M sodium phosphate (pH 7.2), 1 mM EDTA, and 7% sodium dodecyl sulfate (SDS). After hybridization, the membranes were washed twice with a buffer containing 40 mM sodium phosphate (pH 7.2), 1 mM EDTA, and 5% SDS for 30 min at 65°C and twice with a buffer containing 40 mM sodium phosphate (pH 7.2), 1 mM EDTA, and 1% SDS for 30 min at 65°C. The signal was detected on a phosphorimager (Storm; Molecular Dynamics, Sunnyvale, Calif.).

Construction of an *fbps* knockout mutant. To construct the mutant strain 10Δ FBPS, the pathogenic strain 10 (27, 29) of *S. suis* serotype 2 was electrotransformed (24) with the plasmid pFBPS7-47. In this plasmid, the *fbps* gene was inactivated by the insertion of a spectinomycin resistance gene. To create pFBPS7-47 (Fig. 1) the 382-bp *Sal*I-*Sal*I fragment of pFBPS7-46 was replaced by the 1.2-kb *Eco*RV-*Sma*I fragment of pIC-Spc, containing the spectinomycin resistance gene, after the *Sal*I sites of the vector were made blunt (Fig. 1). After electrotransformation of strain 10 with pFBPS7-47, spectinomycin-resistant colonies were selected on Columbia agar plates containing spectinomycin (100 μg/ml). Southern blotting and hybridization experiments were used to select for double-crossover integration events (data not shown).

FBPS expression construct. To construct an FBPS expression plasmid the QIA *expressionist* kit (Qiagen GmbH, Hilden, Germany) was used. The primers corresponded to positions 250 to 272 and positions 1911 to 1892 of the *fbps* gene. The sequences of these primers were 5′-GCGGATCCGATGACGATGACAA ATCTTTTGACGGATTTTTTTTAC-3′ and 5′-CCCAAGCTTGGGCATGAA CTAGATTTTCATGG-3′. The primers contained restriction sites for *Bam*HI and *Hin*dIII, respectively, to amplify the *fbps* gene from pFBPS7-47. The amplified PCR product was digested with *Bam*HI and *Hin*dIII, and the 1.8-kb *fbps* gene was cloned into pQE-30 digested with *Bam*HI and *Hin*dIII, yielding pQE-30-FBPS. pQE-30-FBPS was transformed to M15[pREP4].

Purification of FBPS. M15[pREP4][pQE-30-FBPS] was used to express and to purify the FBPS using the QIA *expressionist* (Qiagen). In short, M15[pREP4][pQE-30-FBPS] cells were grown exponentially; 1 mM IPTG was added, and the cells were allowed to grow another 4 h at 37°C. Subsequently, cells were harvested and lysed. The cleared supernatants were loaded onto Ni^{2+}-nitrilotriacetic acid agarose columns. FBPS containing a six-His tag was bound to the Ni^{2+} column. The columns were washed and the protein was eluted. Different buffers were used for native and for denaturing purification. FBPS purified under denaturing conditions was renatured on an Ni^{2+}-nitrilotriacetic acid column by using a linear urea gradient (6 M to 1 M) in 500 mM NaCl, 20% glycerol, and 20 mM Tris-HCl (pH 7.4), containing protease inhibitors (per milliliter: 25 μg of Pefabloc, 0.7 μg of pepstatin, 1 μg of aprotinin, and 0.5 μg of leupeptin). All procedures were performed according to the manufacturer's recommendations. The six-His tag was removed from the protein by incubating purified FBPS in a solution containing 20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 2 mM $CaCl_2$, and

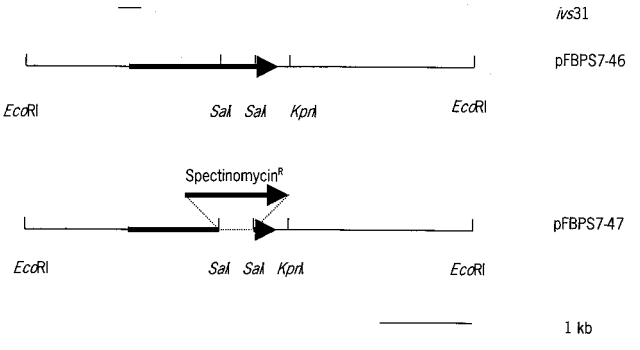

FIG. 1. Schematic presentation of the procedure used to clone the *fbps* gene of *S. suis* serotype 2 and the construction of an insertional knockout mutant in *S. suis* serotype 2. A 5-kb *Eco*RI fragment was cloned in pGEM7Zf(+), yielding pFBPS7-46. In pFBPS7-47, the 382-bp *Sal*I-*Sal*I fragment of pFBPS7-46 was replaced by a 1.2-kb spectinomycin resistance gene, after the vector was made blunt, to obtain an insertional knockout of *fbps.*

0.5 U of light-chain enterokinase (New England Biolabs, Beverly, Mass.) for 16 h at room temperature (RT).

Immunization of rabbits with FBPS. Purified and renatured FBPS was used to immunize two rabbits. To remove urea the protein was dialyzed against phosphate-buffered saline (136 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 2.79 mM KH_2PO_4 [pH 7.2]) (PBS) overnight at 4°C. Seven days before immunization, blood was collected from the rabbits to determine the natural titers of antibody against FBPS. At day 1 those rabbits with negative anti-FBPS titers were immunized intramuscularly with two injections of 0.5 ml of 100-μg/ml FBPS in a water-in-oil emulsion (Specol; ID-Lelystad, Lelystad, The Netherlands). At day 28, rabbits were immunized for the second time using the same amount of protein and the same route of immunization. Three weeks after the second immunization the rabbits were sacrificed and blood was collected. The blood was coagulated and serum was collected and used for immunodetection of FBPS.

Immunodetection of FBPS. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) by standard procedures (19). Proteins in the gel were visualized using SYPRO-orange (Molecular Probes, Sunnyvale, Calif.) staining according to the manufacturer's recommendations. Signals were detected on a phosphorimager (Storm; Molecular Dynamics). A known bovine serum albumin concentration range was used as a standard to calculate the amounts of protein present in the gel. The Molecular Dynamics program was used for the calculations.

Proteins were transferred to a nitrocellulose membrane by standard procedures (19). The membranes were blocked in Blotto–Tris-buffered saline (50 mM Tris-HCl [pH 7.5], 150 mM NaCl) (Blotto-TBS) containing 4% skim milk and 0.05% Tween 20, at RT for 1 h. To detect recombinant purified FBPS, membranes were incubated with a monoclonal antibody against the six-His tag (Clontech, Palo Alto, Calif.) in a 1:10,000 dilution in Blotto-TBS (1:1) at RT for 1 h, followed by an incubation with alkaline phosphatase-conjugated anti-mouse antibody in a 1:1,000 dilution in Blotto-TBS (1:1) at RT for 1 h. Reactivity of purified FBPS was tested by using a convalescent-phase serum of a pig that had survived an *S. suis* infection. Nitrocellulose membranes were incubated with the polyclonal pig serum in a 1:200 dilution in Blotto-TBS (1:1) at RT for 1 h, followed by an incubation at RT for 1 h with alkaline phosphatase-conjugated anti-swine antibody in a 1:2,000 dilution in Blotto-TBS (1:1). As a substrate Nitro Blue Tetrazolium (Merck, Darmstadt, Germany)-bromochloroindolyl phosphate (Sigma) was used. All washing steps were performed in Blotto-TBS (1:1).

FN and FGN binding. Binding studies were performed by indirect Western blotting. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane as described above. The membranes were blocked in MPBS (PBS containing 4% skim milk and 0.05% Tween 20). Subsequently, the membrane was incubated with human FN (5 μg/ml; Sigma) or human FGN (5 μg/ml; Sigma) in PBS containing 5% fetal calf serum, 2% NaCl, and 0.05% Tween 80 at RT for 1 h. To detect bound FN and FGN, the membranes were incubated with horseradish peroxidase-conjugated anti-FN (DAKO) or anti-FGN (DAKO) antibodies in a 1:1,000 dilution in PBS containing 5% fetal calf serum, 2% NaCl, and 0.05% Tween 80 at RT for 1 h. The signal was visualized by using an ECL^+ kit (Amersham Life Science) according to the manufacturer's recommendations. Signals were detected on a phosphorimager (Storm; Molecular Dynamics). All washing steps were performed in MPBS-PBS (1:1).

Experimental infections. Germfree piglets, crossbreeds of Great Yorkshire and Dutch Landrace, were obtained from sows by cesarean sections. The surgery was performed in sterile flexible film isolators. Piglets were allotted to groups of 4, and were housed in sterile stainless steel incubators. Housing conditions and feeding regimens were as described before (27, 29). Six-day-old piglets were inoculated intranasally with about 10^7 CFU of *Bordetella bronchiseptica* 92932, to predispose the piglets to infection with *S. suis*. Two days later they were inoculated intranasally with 10^6 CFU of *S. suis* strain 10 plus 10^6 CFU of *S. suis* strain 10Δ FBPS. To determine differences in virulence between wild-type and mutant strains, 50% lethal doses should be determined. To do this, large numbers of piglets would be required. For ethical reasons this is not acceptable. To circumvent this problem we decided to perform cocolonization studies. To monitor for the presence of *S. suis* and *B. bronchiseptica* and to check for absence of contaminants, swabs taken from the nasopharynx and the feces were cultured three times a week. The swabs were plated directly onto Columbia agar containing 6% horse blood or grown for 48 h in Todd-Hewitt broth and subsequently plated onto Columbia agar containing 6% horse blood. Pigs were monitored twice a day for clinical signs and symptoms, such as fever, nervous signs, and lameness. Blood samples from each pig were collected three times a week. Leukocytes were counted with a cell counter. The piglets were killed when specific signs of an *S. suis* infection were observed, such as arthritis or meningitis, or when the pigs became mortally ill. The other piglets were killed 2 weeks after inoculation with *S. suis* and examined in the same way as the piglets that were killed based on their clinical symptoms. All piglets were examined for pathological changes. Tissue specimens from heart, lung, liver, kidney, spleen, and tonsil and from the organs specifically involved in an *S. suis* infection (central nervous system [CNS], serosas, and joints) were sliced with a scalpel or a tissuemizer. Tissue slices from each organ or site were resuspended in 2 to 25 ml of Todd-Hewitt broth containing 15% glycerol, depending on the size of the tissue slice. The suspension was centrifuged at 1,200 δ *g* for 5 min. The supernatant was collected and serial dilutions were plated on Columbia agar containing 6% horse blood, as well as on Columbia agar plates containing 6% horse blood and 100 μg of spectinomycin per ml to quantitate the number of wild-type and mutant bacteria present. The number of mutant strain 10Δ FBPS cells was determined by counting the number of CFU on the appropriate serial dilution on the selective plates; the number of wild-type strain 10 cells was determined by counting the number of CFU on the appropriate serial dilution on the Columbia agar blood plates, from which the number of CFU counted on the selective plates was subtracted. When wild-type and mutant bacteria were found in tissues, the ratio of wild-type and mutant strain was determined again, by moving about 100 individual colonies by toothpick onto both Columbia agar plates and onto Columbia agar plates containing spectinomycin (100 μg/ml).

All animal experiments were approved by the ethical committee of the Institute for Animal Science and Health, Lelystad, The Netherlands, in accordance with the Dutch law on animal experiments.

Nucleotide sequence accession number. The nucleotide sequence data of *fbps* have been submitted to GenBank under accession no. AF438158.

RESULTS

Cloning of the *S. suis fbps* gene. One of the in vivo-selected genes (*ivs-31*) (20) showed homology to the 5′ part of genes coding for FlpA and FBP54, FN binding proteins (FnBP) of *S. gordonii* (GenBank accession no. X65164) and *S. pyogenes* (8), respectively. To clone the entire *fbps* gene of *S. suis*, *ivs-31* was subsequently used as a probe to identify a chromosomal DNA fragment of *S. suis* serotype 2 containing flanking *fbps* sequences. A 5-kb *Eco*RI fragment was identified and cloned in pGEM7Zf(+) yielding pFBPS7-46 (Fig. 1). Sequence analysis revealed that this fragment contained the entire *fbps* gene of *S. suis* serotype 2. An open reading frame of 1,659 bp coding for a polypeptide of 553 amino acids was found. The putative ATG start codon is preceded by a sequence similar to ribosome binding sites of gram-positive bacteria. Further upstream, two putative promoter sequences could be identified. Upstream of these promoter sequences of *fbps* an inverted repeat was found that could serve as a transcription terminator of the gene located 5′ of *fbps*. Downstream of *fbps* a gene was found that showed homology to an alpha-acetolactate decarboxylase was found. This gene is transcribed in the opposite direction of *fbps*. The deduced amino acid sequence was aligned with that of several previously identified FnBPs from other bacteria. As expected, FBPS was very homologous to FlpA of *S. gordonii* (76%) and also showed homology to FnBPs of other organisms, like *Streptococcus pneumoniae* (73%), *S. pyogenes* (69%), *Lactococcus lactis* (59%), and *Bacillus subtilis* (41%). Compared to the sequence of FBP54, FBPS has a longer N terminus with 76 additional amino acids. This longer N terminus was also seen in other organisms like *S. gordonii*, *S. pneumoniae,* and *B. subtilis*. In FBP54 the primary FN-/FGN-binding domain was localized to its N-terminal part, to the first 89 amino acids (8). Over this region the homology of FBPS to FBP54 is very high (80%) suggesting that FBPS can bind both FN and FGN.

Binding of FBPS to FN and FGN. To confirm the binding of FBPS of *S. suis* to FN and FGN, FBPS was purified under native conditions. A protein expression construct, which ex-

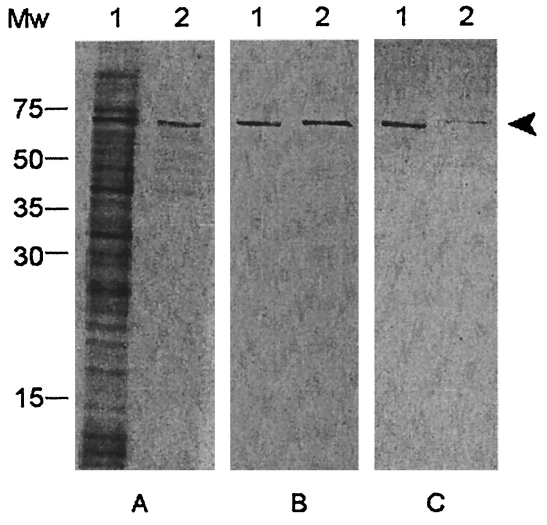

FIG. 2. Purity and immunogenicity of FBPS purified under native conditions. SDS-PAGE analysis with SYPRO-orange, a nonspecific protein-staining dye (A), and Western blot analysis with a monoclonal antibody against the six-His tag (B) of 4 μl of *E. coli* M15 [pQE-30-pREP4-FBPS] lysate (lanes 1) and 165 ng of purified FBPS (lanes 2) were carried out. Convalescent-phase serum raised against *S. suis* strain 10 was used to test immunogenicity of FPBS present in 4 μl of *E. coli* M15 [pQE-30-pREP4-FBPS] lysate and 0.5 μg of purified FBPS (C) (lanes 1 and 2). Arrowhead, 64-kDa FPBS; Mw, molecular size marker (in kilodaltons).

presses FBPS with a six-His tag fused to the N terminus, was used for this purification. Four hundred μg of FBPS was purified from 50 ml of exponential-phase *E. coli* cells after induction with IPTG. The purity of this FPBS was determined with SDS-PAGE and Western blotting (Fig. 2). The induced *E. coli* lysate contained a broad range of proteins, among which the 64-kDa protein FBPS was very clearly present (Fig. 2A, lane 1). After purification, highly purified FBPS with six-His tag was obtained (Fig. 2A, lane 2). When both samples were incubated with a monoclonal antibody against the six-His tag, FBPS was the only protein that was detected (Fig. 2B).

To determine whether FBPS binds FN and FGN, a Western blot containing purified FBPS was incubated with soluble human FN and human FGN (Fig. 3A and B). Specific binding of FN and FGN to FBPS was clearly detected. No binding of FN and FGN to BSA, a negative control protein, was observed. To exclude possible background signals due to immunoglobulin-binding of FBPS, the same experiment was performed without addition of FN or FGN. No binding was found (Fig. 3C and D), indicating that the binding was specific for FN and FGN. To control whether the binding of FN and FGN to FBPS, was not mediated by the six-His tag, the tag was removed by an enterokinase treatment. Figure 3E and F clearly show that FBPS without the six-His tag, still efficiently bound to FN and FGN. Therefore, it was concluded that FBPS can specifically bind to FN and FGN.

Immunogenicity of FBPS. Since it was shown that FBP54 induced a protective immune response in mice against a lethal dose of *S. pyogenes* (13), we next determined whether purified FBPS was recognized by convalescent-phase serum of a pig that survived an *S. suis* infection. As shown in Fig. 2 panel C, the FBPS clearly reacted with this antiserum. When the same experiment was performed with nonimmune serum of an SPF piglet, no band of the size of FBPS was detected (data not shown). These findings indicate that FBPS is expressed in vivo and that the protein is indeed immunogenic in young pigs.

Distribution of the *fbps* gene among the 35 *S. suis* serotypes. Since we were interested in a cross-protective vaccine candidate, we next analyzed the presence of the *fbps* gene among the various *S. suis* serotypes. *ivs-31*, the clone containing the promoter and the 5′ part of the *fbps* gene was radiolabeled, and chromosomal DNA of the reference strains of the 35 different *S. suis* serotypes was hybridized with this probe. The three different phenotypes of *S. suis* serotype 2—a pathogenic, a

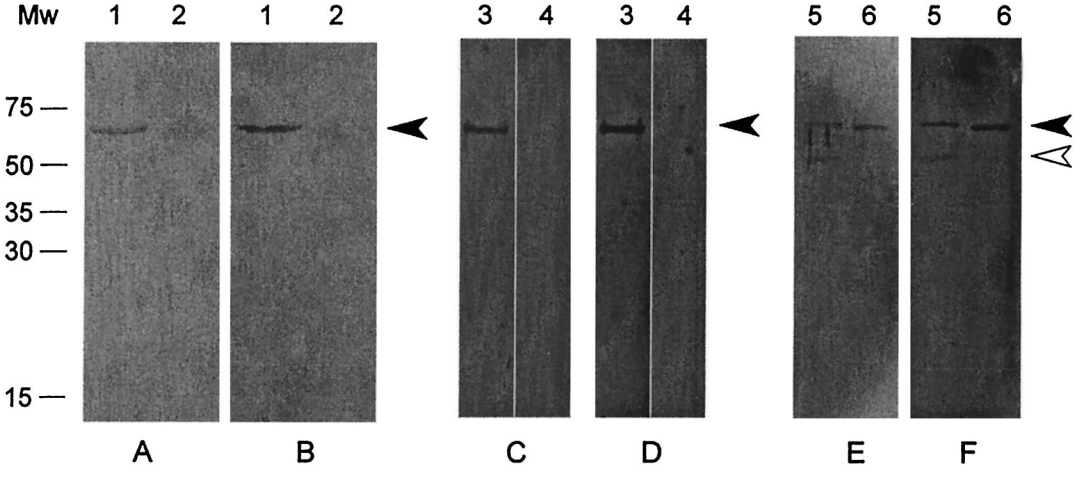

FIG. 3. Binding studies with purified FBPS. (A and B) Gels were probed with FN (A) or FGN (B) at 5 μg/ml. Lanes 1 contain 500 ng of purified FBPS, and lanes 2 contain 500 ng of BSA. (C and D) Lanes 3 and 4 contain 500 ng of purified FBPS. Lanes 3 were probed with FN (C) or FGN (D) at 20 μg/ml, and lanes 4 were only incubated with conjugate without FN or FGN. (E and F) Gels were probed with FN (E) or FGN (F) at 20 μg/ml. Lanes 5 contain 1.8 μg of purified FBPS digested with enterokinase, and lanes 6 contain 500 ng of purified FBPS. The closed arrowhead indicates 64-kDa FBPS; the open arrowhead indicates approximately 55-kDa FBPS without the six-His tag. Mw, molecular size marker (in kilodaltons).

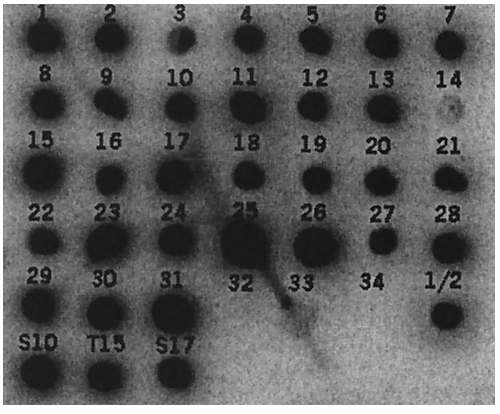

FIG. 4. Distribution of *fbps* among various *S. suis* serotypes. Chromosomal DNA (1 μg) was spotted onto nitrocellulose membrane and hybridized with a ^{32}P-labeled *fbps* probe. Serotypes were spotted as indicated. S10, *S. suis* serotype 2 MRP$^+$ EF$^+$; T15, *S. suis* serotype 2 MRP$^-$ EF$^-$; S17, *S. suis* serotype 2 MRP$^+$ EF* (23).

nonpathogenic, and a weakly pathogenic strain—were included in this study as well. The *fbps* gene was present in all *S. suis* serotypes and phenotypes, except for serotypes 32 and 34 (Fig. 4).

Role of FBPS in pathogenesis. To test the role of FBPS in the pathogenesis of *S. suis*, an isogenic knockout mutant of FBPS was constructed in strain 10, strain 10ΔFBPS. Since upstream of *fbps* an inverted repeat was found that could serve as a transcription terminator and downstream of *fbps* a gene showing homology to an alpha-acetolactate decarboxylase was found that is transcribed in the opposite direction, polar effects on genes upstream or downstream of *fbps* are not expected. To verify that the mutant strain 10ΔFBPS did not produce FBPS, protoplasts of strain 10 and strain 10ΔFBPS were subjected to SDS-PAGE and Western blotting. FBPS was detected using a polyclonal antiserum raised against purified FBPS. It was shown that strain 10 41 FBPS expressed no FBPS, while strain 10 did (data not shown). Subsequently the virulence of this mutant strain was tested in an experimental infection in piglets. The mutant strain 10ΔFBPS was used in a competition challenge experiment with the wild-type strain to determine the relative attenuation of the mutant strain. Under in vitro conditions, the growth rates of the wild-type and mutant strain in Todd-Hewitt medium were found to be essentially identical (data not shown). Wild-type and mutant strains were inoculated at an actual ratio of 0.65 ($1.63 \times 10^6$6 CFU of wild-type bacteria ml^{-1} and 3.09×10^6 CFU of mutant bacteria ml^{-1}). During the experiment, piglets that developed specific *S. suis* symptoms (meningitis, arthritis, or mortal illness) were killed. Piglets that did not develop these symptoms were killed at the end of the experiment. From all piglets the ratio of wild-type strain to mutant strain in various organs was determined. As shown in Fig. 5A, similar numbers of wild-type and mutant bacteria were reisolated from tonsils. The ratio was similar to the input ratio (ratio varied from 0.33 to 0.85; average, 0.61). This clearly indicates that the efficiencies of colonization of wild-type and mutant strains on tonsils were essentially identical. Apparently, FBPS is not strictly required for colonization of the tonsils of the piglets. Three out of four piglets developed clinical signs specific for an *S. suis* infection. Two piglets (4664 and 4666) showed clinical signs of arthritis, and one piglet (4668) showed clear central nervous signs. The fourth piglet did not develop any clinical signs. These observations coincided with pathomorphological abnormalities of the specific organs of an *S. suis* infection in postmortem sections. As shown in Fig. 5B and Table 2, exclusively wild-type bacteria were reisolated from the joints of piglet 4664 and from the CNS of piglet 4668. The numbers of CFU of wild-type bacteria that were reisolated from these specific organs were very high, while absolutely no mutant bacteria were found. From the joints of pig 4666 low numbers of both wild-type and mutant bacteria were reisolated in a ratio of 0.84 (1.0×10^2 CFU of wild-type bacteria and 5.2×10^2 CFU of mutant bacteria), a ratio essentially identical to the input ratio (Fig. 5B and Table 2). Southern blot experiments, using the *fbps* and the *spc* genes as probes, confirmed that the mutant bacteria isolated from the joint of pig 4666 were indeed identical to the input mutant bacteria. Taken together, these data indicate that the FBPS mutant is capable of reaching and colonizing the specific *S. suis* organs (at least the joints) but that the mutant is far less efficiently recovered from organs than the wild type.

DISCUSSION

In this work we describe the first FBPS of *S. suis*. The gene encoding FBPS was cloned and sequenced, and FBPS was

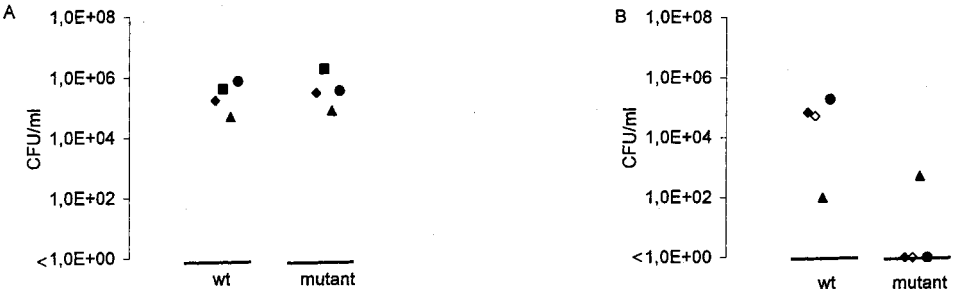

FIG. 5. Efficiency of colonization of wild-type and mutant bacteria on various organs of infected pigs. (A) Colonization of the wild-type strain 10 (wt) and the mutant strain 10ΔFBPS of the tonsils. Symbols: ◆, tonsil from pig 4664; ■, tonsil from pig 4665; ▲, tonsil from pig 4666; ●, tonsil from pig 4668. (B) Colonization of the specific organs. Symbols: ◇ and ◆, pus from joints from pig 4664; ▲, pus from a joint from pig 4666; ●, CNS specimen from pig 4668. Each symbol represents the numbers of wild-type or mutant bacteria isolated from one particular organ, from one piglet.

TABLE 2. Numbers of reisolated wild-type (strain 10) and mutant (strain 10ΔFBPS) bacteria from organs of infected piglets (mean actual inoculation ratio 65% mutant strain)

Sample[d]	No. of bacteria (CFU/ml) for pig no.											
	4664			4665			4666			4667		
	w.t.[a]	mut.[b]	% mut.[c]	w.t.[a]	mut.[b]	% mut.[c]	w.t.[a]	mut.[b]	% mut.[c]	w.t.[a]	mut.[b]	% mut.[c]
Tonsil	1.77×10^5	3.29×10^5	65	4.35×10^5	2.42×10^6	85	5.34×10^4	8.73×10^4	61	7.94×10^5	3.96×10^5	33
Pus joint 1	6.75×10^4	<10	0				1.02×10^2	5.2×10^2	84			
Pus joint 2	5.15×10^4	<10	0									
CNS[e]										1.88×10^5	<10	0

[a] Number of wild-type bacteria found (CFU/ml).
[b] Number of mutant bacteria found (CFU/ml).
[c] Percentage of mutant bacteria calculated as follows: b/(a+b) × 100%.
[d] Only relevant organs are depicted.
[e] CNS, central nervous system.

purified. Binding of FBPS to human FN and FGN was shown. FBPS was shown to be involved in the colonization of the organs specific for an *S. suis* infection in piglets, but not in the colonization of *S. suis* on the tonsils of piglets.

Many streptococci and staphylococci have several different FnBPs, most of which are very large, about 130 kDa (12). Until now, *S. pyogenes* is the only organism having a large as well as a smaller (54-kDa) FnBP (8). The existence of more than one FnBP explains why in some organisms isogenic mutants defective in only one of the FnBPs can still bind to FN and/or FGN and are not completely attenuated in vivo (10). Although, so far no FnBP other than that described here has been described for *S. suis*, their existence could explain the fact that the FBPS mutant is not completely attenuated in vivo.

A considerable number of FN- and FGN-binding proteins of various bacterial species have been described (12). Most of these proteins were shown to be involved in adhesion to epithelial and/or endothelial cells (5, 8, 18). Therefore, it is attractive to assume a similar role for FBPS of *S. suis*. Previously, Charland and coworkers used human brain microvascular endothelial cells (HBMEC) in an in vitro blood-brain barrier model to study the pathogenesis of *S. suis* meningitis. Since *S. suis* adhered to HBMEC (4) in future experiments it would be of interest to test whether FBPS is involved in binding to HBMEC and involved in crossing the blood-brain barrier.

The role of FBPS in the pathogenesis of *S. suis* was studied in an experimental infection model in piglets. Since we were unable to determine a 50% lethal dose for the mutant strains, it was decided to compare the virulence of the isogenic FBPS mutant to that of the wild-type *S. suis* strain in a competitive infection assay in piglets. This kind of cocolonization experiment has been successfully applied to determine the virulence of mutants of *Actinobacillus pleuropneumoniae* in piglets (9). The data clearly showed that the mutant strain was capable of colonizing the tonsil as efficiently as the wild-type strain. This strongly indicates that FBPS is not involved in the colonization of the tonsil. The data also indicated that FBPS does play a role in the colonization of specific organs, since in the competition assay joints and the CNS were more efficiently colonized by wild-type than by mutant bacteria. In addition, higher numbers of wild-type bacteria were reisolated from the specific organs compared to the numbers of mutant bacteria, indicating that the mutant strain is attenuated in vivo. Although the number of pigs used for this experiment was low, these data indicate that the FBPS mutant is less virulent than the wild-type strain. Loss of virulence of *S. suis* was also described by Allen et al. (1). They constructed an isogenic knockout mutant of suilysin in a pathogenic serotype 2 strain of *S. suis*, and tested this mutant in an experimental animal model in pigs. From their findings, it was concluded that suilysin might play a role in reaching higher levels of colonization of various organs after *S. suis* has gained entrance into the bloodstream.

We were able to demonstrate that FBPS reacted with a convalescent-phase serum of a pig that survived an *S. suis* infection. Therefore FBPS is immunogenic in pigs, and this finding clearly demonstrates that FBPS of *S. suis* is expressed under in vivo conditions. Recently, it was reported that FBP54 of *S. pyogenes* is expressed in the human host (7). The in vivo expression of FBPS confirms the selection of the *fbps* gene from a gene library under in vivo conditions, as described by Smith and coworkers (20).

We showed that the *fbps* gene was present in all known serotypes of *S. suis* (except for two), as well as in all three phenotypes of serotype 2. This suggests that the *fbps* gene is present among most serotypes. However, the expression of FBPS in all serotypes and phenotypes was not studied. Therefore, it is possible that although all strains, except for serotypes 32 and 34, possess the *fbps* gene, not all strains express FBPS. Based on the facts that FBPS is immunogenic in pigs and that the *fbps* gene is present in all prevailing *S. suis* serotypes except for serotypes 32 and 34, FBPS is a very attractive candidate for a cross-protective vaccine against all serotypes. Since the mutant strain 10 Δ FBPS is not completely attenuated, this vaccine should be based on purified protein with a suitable adjuvant. This idea is supported by recent data from Kawabata et al. (13) which showed that vaccination with purified FBP54 can protect mice against a *S. pyogenes* infection.

ACKNOWLEDGMENTS

We thank the people from the Animal and Laboratory Services and the people from Pathobiology of the ID-Lelystad for their assistance during the animal experiments.

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Editor: J. T. Barbieri