Contribution of Fibronectin-Binding Protein to Pathogenesis of \textit{Streptococcus suis} Serotype 2

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In the present study we investigated the role of the fibronectin (FN)- and fibrinogen (FGN)-binding protein (FBPS) in the pathogenesis of \textit{Streptococcus suis} serotype 2 in piglets. The complete gene encoding FBPS from \textit{S. suis} serotype 2 was cloned in \textit{Escherichia coli} and sequenced. The occurrence of the gene in various serotypes was analyzed by hybridization studies. The FBPS protein was expressed in \textit{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 \textit{S. suis} infection. Therefore, the FBPS mutant was considered as an attenuated mutant.

\textit{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, \textit{S. suis} causes septicaemia and meningitis in humans (3). The pathogenesis of an \textit{S. suis} infection is rarely understood. Sows are symptomless carriers of \textit{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 \textit{S. suis} infection. Until now, 35 capsular serotypes of \textit{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 \textit{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 \textit{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 \textit{S. suis}, we cloned promoters and their downstream sequences that are “on” during experimental \textit{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 \textit{S. suis} serotype 2 strains (30). The other (ivs-31) showed homology to genes encoding fibronectin (FN)- and/or fibrinogen (FGN)-binding proteins of \textit{Streptococcus gordonii} (GenBank accession no. X65164) and \textit{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 \textit{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 \textit{S. pyogenes} challenge in mice (13).

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

\textbf{MATERIALS AND METHODS}

\textbf{Bacterial strains and growth conditions.} The bacterial strains and plasmids used in this study are listed in Table 1. \textit{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. \textit{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 \textmu g/ml for \textit{E. coli} and 100 \textmu g/ml for \textit{S. suis}), ampicillin (Boehringer, Mannheim, Germany) (100 \textmu g/ml for \textit{E. coli}), and kanamycin (Boehringer) (25 \textmu g/ml for \textit{E. coli}).

\textbf{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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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 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).

Strains

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<td>S. suis 10</td>
<td>Virulent serotype 2 strain</td>
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<td>10ΔFBPS</td>
<td>Isogenic fbps mutant of strain 10</td>
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Plasmids

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<td>pFBPS7-46 in which 382-bp SalI-SalI fragment is replaced by 1.2-kb Spc' from pIC-spc</td>
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*a Tetr', tetracycline resistant; Cm', chloramphenicol resistant; Amp', ampicillin resistant; Spc', spectinomycin resistant; Kan', kanamycin resistant.

Construction of an fbps knockout mutant. To construct the mutant strain 10ΔFBPS, the pathogenic strain 10 (27, 29) of S. suis serotype 2 was 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 were used as described in Table 1. To obtain an insertional knockout mutant in S. suis serotype 2. A 5-kb EcoRI fragment was cloned in pGEM7Zf(+) yielding pFBPS7-46. In pFBPS7-47, the 382-bp SalI-SalI 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.

**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 EcoRI fragment was cloned in pGEM7Zf(+) yielding pFBPS7-46. In pFBPS7-47, the 382-bp SalI-SalI 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.
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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 Fpa and FBPS, 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 EcoRI 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 FpaA 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 FBPS4, 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 FBP4 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 FBP4 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-
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 FBPS4 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. 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 FBPS 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 FBPS; Mw, molecular size marker (in kilodaltons).

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).
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 incubated at an actual ratio of 0.65 (1.63 × 10^6 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 4668) 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 4668 low numbers of both wild-type and mutant bacteria were reisolated in a ratio of 0.84 (1.0 × 10^8 CFU of wild-type bacteria and 5.2 × 10^7 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 4668 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
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 10ΔFBPS 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.

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