Identification of a Novel Virulence Determinant with Serum Opacification Activity in Streptococcus suis

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Received 6 March 2006/Returned for modification 13 April 2006/Accepted 2 August 2006

STREPTOCOCCUS SUII serotype 2 is an important invasive porcine pathogen worldwide. It also causes meningitis and other diseases in humans. Processing of pork is considered to be a major risk factor of this zoonosis (3). In pigs, septicaemia, meningitis, polyarthritis, polyserositis, and pneumonia are common clinical manifestations. These diseases have been reproduced experimentally by infections with S. suis serotype 2. In addition to epidemiological data, experimental infections demonstrated that wild-type serotype 2 strains, which express a 136-kDa muramidase-released protein (MRP) and an 80-kDa extracellular factor (EF), are highly virulent, in contrast to MRP EF+ serotype 2 strains from Europe (32, 35).

Smith et al. (27) previously showed that the capsule of S. suis protects against phagocytosis. Other factors such as sulycin and the fibronectin- and fibrinogen-binding protein of S. suis (FBPS) may contribute to the pathogenesis of S. suis (1, 9, 19), but so far, the capsule is the only major virulence factor to be identified (27).

In other streptococci such as Streptococcus pyogenes and Streptococcus dysgalactiae, a number of large surface-associated proteins, including SfbI, serum opacity factor (SOF), and fibronectin-binding protein A (FnBA), belong to a group of adhesins called MSCRAMMs (microbial surface components recognizing adhesive matrix molecules). Repetitive sequence elements that are located at the C termini of MSCRAMMs function as a fibronectin-binding domain (13, 17). Structural features like these repeats and the LPXTG anchor motif distinguish the large MSCRAMMs from the fibronectin-binding proteins FBP54 of S. pyogenes and FBPS of S. suis (6, 9). In addition to the homology of the fibronectin-binding repeats, SOF of S. pyogenes and FnBA of S. dysgalactiae show homology in their large N-terminal domains, which are responsible for opacification of mammalian serum (5, 15, 16). Recently, Courtney et al. (7) demonstrated that this N-terminal domain of SOF triggers the disruption of high-density lipoprotein (HDL) particles and that the concomitant formation of lipid droplets accounts for the opacification of serum. SOF knockout mutants were attenuated in virulence in a mouse model (5). As HDL executes anti-inflammatory functions, the N-terminal domain of SOF has been postulated to contribute to the pathogenesis of S. pyogenes in addition to the fibronectin binding of the C-terminal repeats (7). Here, we describe the identification and characterization of a novel virulence factor of S. suis called OFS (opacity factor of S. suis) with structural and functional properties similar to those of SOF and FnBA.

MATERIALS AND METHODS

Bacterial strains and growth conditions. In this study, only serotype 2 S. suis strain 10 and its isogenic mutants were used. Strain 10 has been shown to be highly virulent in experimental infections of pigs (31). Through insertional mutagenesis of genes involved in the biosynthesis of the capsule, Smith et al. constructed the capsule-deficient isogenic mutant 10cps∆EF (27), which was, as was strain 10, kindly provided by H. Smith (DLO-Lelystad, The Netherlands). All S. suis strains including the different ofs mutants investigated in this study are mrp− eff− shy−. S. pyogenes strains M1, M2, and M49, kindly provided by B.
The erythromycin resistance gene of pUCerm aligns.

**EF**
ofs::ermK5 of strain 10 and strain ofs::ermK1 and 10cps mutant strains 10 were TA cloned into pCR2.1-TOPO (Invitrogen, Karlsruhe, Germany) and cloned into pQE31 (QIAGEN, Hilden, Germany) to overexpress the OFS gene, allelic exchange was established for **ofs** in-frame deletion mutant strain 10. The allelic exchange was performed with the thermosensitive shuttle vector pSET5sΔofs. To construct this vector, a 933-bp 5′ ofs amplification product was cloned into the shuttle vector pSTrep plus 173preproBMHI plus 173postrepPstI (Table 1) were ligated after BglII digestion, cut with BamHI and SplI, and cloned into pSET5s, which was subsequently used for hybridization. Isolation of RNA, blotting, and hybridization of mutant and parental strains with pulsed-field gel electrophoresis (PAGE) and Western blot analysis, preincubation and induced samples were collected and adjusted to equal bacterial concentrations in sample buffer. Proteins were separated in 4% stacking and 8% separating gels and transferred onto nitrocellulose membrane (Protran; Schleicher & Schuell Inc., Dassel, Germany) by using standard procedures (25). Blocking of membranes was performed as described previously (9). The membranes were incubated with preimmune sera or with polyclonal antisera against pOFS or pOFSrep in a 1:2,000 dilution. After washing, detection was performed with 1,300,000-diluted horseradish peroxidase-conjugated monkey anti-rabbit immunoglobulin G (Amersham, Freiburg, Germany) and developed using the ECL Plus Western blotting detection system (Amersham) as recommended by the manufacturer. Purification of pOFS,
tOFsRep, and tOFsRep by Ni\textsuperscript{2+}-nitrilotriacetic acid affinity chromatography under denaturing conditions was carried out as recommended by the manufacturer (QiAGEN). Polyclonal antisera against purified rOFs, rOFsRep, and rOFsRep raised in rabbits were obtained from Seqlab (Göttingen, Germany).

Detection of OFS and MRP in SDS extracts. The same procedure as that used for recombinant OFS constructs was used to detect OFS in 15 \( \mu l \) of the SDS extracts, except that a 1:1,000 dilution of an antisera against tOFsRep was used. Detection of MRP was performed as described previously (26).

Detection of serum opacification activity. The 1% SDS extraction technique for streptococci was used as described previously (24), with the following modifications: the broth culture volume was increased to 10 ml for S. pyogenes and L. lactis and to 40 ml for S. suis (incubation with 0.5 ml of a 1% culture grown overnight, respectively). After 4 h of growth, 160 \( \mu l \) of a protease inhibitor cocktail (P8466; Sigma, Taufkirchen, Germany) was added to the S. suis cultures (final concentrations of 92 \( \mu M \) AEBSF [4-(2-aminoethyl)benzenesulfonyl fluoride], 400 \( \mu M \) EDTA, 8 \( \mu M \) bestatin, 1.2 \( \mu M \) pepstatin A, and 1.2 \( \mu M \) E-64). Incubation at 37°C was continued for an additional 2 h. S. pyogenes and L. lactis cultures were used for SDS extraction after 5 h of growth. The bacterial pellet was resuspended in 200 \( \mu l \) of prewarmed (42°C) 1% SDS and rotated end-over-end for 1 h at 37°C. The microwell plate assay (14) was used with horse serum (Invitrogen) as a substrate. Twenty-five microliters of the SDS extract was added to 100 \( \mu l \) of serum. To demonstrate inhibition of serum opacification by recombinant antisera, 2 \( \mu l \) of polyclonal antiserum against tOFsRep was added to the respective wells. The sealed microwell plates were incubated with moderate shaking for 40 h at 37°C. After the addition of 100 \( \mu l \) of 0.9% NaCl, the OD\textsubscript{492} was measured.

To detect serum opacity activity in E. coli lysates, noninduced and induced cultures of the pM15 strains with the different pQE constructs described above were compared in two different assays. In the drop test, 20 \( \mu l \) of lysates (sonicated phosphate-buffered saline suspensions) was placed onto 1.8% agar plates with 50% serum from horse, human, or pig (PromoCell, Heidelberg, Germany) and incubated at 37°C for 24 h. Second, SDS-PAGE was performed as described above, and gels were overlaid with 0.5% agarose containing 50% horse serum. After an incubation period of 5 days at 37°C in a sealed bag, opacification was visualized with a gel documentation system (Imago; MWG, Ebersberg, Germany).

Animal experiments. German Landrace piglets from a herd known to be free of sy" mep" epf" cps2" strains were infected experimentally and cared for in accordance with the principles outlined in the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (European Treaty series no. 123 [http://conventions.coe.int/treaty/EN /Menuprincipal.htm]; permit no. 33-42502-05/1003). The piglets were either 4 to 5 (\( n = 18 \)) or 7 to 8 (\( n = 3 \)) weeks old and were referred to as weaning piglets and growers, respectively. After transport to the experimental facility, fasting piglets and growers were randomly divided into two groups. Each day after arrival, the animals were anesthetized with 2 mg azaperone (Stresnil; Jansen, Neuss, Germany)/kg intramuscularly and 10 mg ketamine hydrochloride (Ursotamin; Serumwerk, Bernburg, Germany)/kg intramuscularly and 10 mg ketamine-hydrochloride (Ketalar; Parke-Davis, Hanover, New Jersey)/kg intramuscularly and 10 mg ketamine–hydrochloride (Ketalar; Parke-Davis, Hanover, New Jersey) subcutaneously. All surviving piglets were necropsied 20 days postinfection (dpi).

After euthanasia, every animal went through the same procedure of necropsy and collection of the following samples for histological (H), cytological (C), and bacteriological (B) investigations: cerebrospinal fluid (B); puncture of one tarsal joint (B and C); peritoneal, pleural, and pericardial swab (B); pleura and peritoneum (H); cranial lobe of the lung (B and H); liver (B and H); spleen (B and H); tonsil (B and H); and brain (H). The histological screenings were carried out with blinded experiments. For comparison, a scoring system was set up. The health status of the animals was investigated every 12 h, including measurement of body temperature and behavior. In the case of high fever (\( >40.5°C \)) apathy, and anorexia, animals were euthanized. Seven days after infection, all surviving animals were sacrificed 20 days postinfection (dpi).

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In the case of high fever (\( >40.5°C \)) apathy, and anorexia, animals were euthanized. Seven days after infection, all surviving animals were sacrificed 20 days postinfection (dpi). The respective open reading frame, called OFS, showed the typical structural features of an MSCRAMM, namely, a putative N-terminal signal sequence, a large N-terminal domain including a proline-rich region, repetitive sequence elements, and a C-terminal LPXTG anchor motif (Fig. 1A). The hidden Markov model trained on gram-positive bacteria (SignalP 3.0; http://www.expasy.org) predicted a signal sequence at the N terminus with a probability of 0.982 and a signal sequence cleavage site between positions 24 and 25 (site probability of 0.741). Results of neural networks of SignalP 3.0 suggested cleavage between positions 22 and 23.

RESULTS

Identification, cloning, and sequence analysis of OFS. The sequencing of the S. suis strain 10 afs gene reported in this work was deposited in GenBank under accession no. AY19647. The sequence of the S. suis strain 10 afs gene reported in this work was deposited in GenBank under accession no. AY19647.

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The OFS sequence N terminal to the repeats was found to be homologous to the respective regions of FnBA of *S. dysgalactiae* and SOF of *S. pyogenes* (Fig. 1B) (ClustalW scores for alignments were 40 for OFS at positions 87 to 673 [OFS87–673] with FnBA174–709, 34 for OFS87–673 with SOF181–802, and, for comparison, 47 for FnBA174–709 with SOF181–802). The repeats consisted of two complete repetitive sequences (R1 and R2) and an additional truncated repeat (R3Δ) (Fig. 1C). This region did not show significant homology to the fibronectin-binding region of MSCRAMMs (ClustalW scores for alignments were 13 for OFS674–845 with FnBA790–994, 9 for OFS674–845 with SOF803–935, 9 for OFS674–845 with SfbI422–573, and, for comparison, 43 for FnBA790–994 with SOF803–935 and 38 for SOF803–935 with SfbI422–573). However, the alignment of OFS671–706 with SfbI411–446 demonstrated that the first 8 amino acids of R1 (and R2) of OFS were homologous to the amino acids N terminal to the first repeat of the fibronectin-binding region FNBR of SfbI (Fig. 1D). In conclusion, the alignment of the putative OFS protein with MSCRAMMs suggested a serum opacification activity as in SOF and FnBA but not necessarily a fibronectin-binding activity of the repetitive sequence elements of OFS.

**Serum opacification activity of rOFS.** Three different His-tagged rOFS mutants were expressed in *E. coli* for functional studies. The complete OFS sequence, lacking the signal sequence and the C-terminal membrane anchor, was included in rOFS, whereas rOFSΔrep lacked the repetitive sequences, and rOFSSrep consisted solely of these repeats. The proteins rOFS, rOFSΔrep, and rOFSSrep had calculated molecular masses...
of 98.4, 74.9, and 19.1 kDa and isoelectric points at 5.1, 6.3, and 4.5, respectively. rOFS and rOFSrep migrated in SDS-PAGE gels slower than the respective molecular weight standards (Fig. 2). The different constructs were investigated for opacification of mammalian serum. Lysates from E. coli that expressed rOFS or rOFS/H9004 rep showed IPTG-inducible serum opacification in SDS overlays (Fig. 2E) and also in the drop test with horse, human, and pig serum agar plates (results not shown). Neither lysates from the control (vector alone) nor lysates from E. coli that expressed rOFSrep opacified horse, human, or pig serum in these assays. The opacifying bands identified in the SDS-PAGE overlays corresponded to bands recognized in Western blots by polyclonal antisera raised against rOFS (Fig. 2C) or rOFS/H9004 rep (C) as hybridization probes is shown. The 2.9-kb and 1.6-kb positions are based on 23S and 16S rRNA bands, respectively, in the ethidium bromide-stained gel.

Mutagenesis and Northern blot analysis of ofs. To study putative functions and expression of OFS in S. suis, different isogenic ofs knockout mutants of strain 10 were constructed. The mutagenesis was controlled by PCR and Southern blot analysis. Pulsed-field gel electrophoresis analysis showed no indication of genomic rearrangements in the ofs mutants. The growth rates of the wild type and all mutant strains in THB medium were found to be identical (results not shown). The cps2F mRNA transcript was confirmed in strain 10, in the insertion mutant 10Δofs::ermK1, and in the two in-frame deletion mutants 10Δofs2/12 and 10Δofs3/5 but was, as expected, undetectable in the capsular mutant 10cps/H9004 EF and in the double mutant 10cps/H9004 EF/ofs::ermK5 (Fig. 3). Northern blot analysis of S. suis strain 10 and 10cps/H9004 EF also revealed ofs-specific mRNA in these strains, in contrast to the ofs mutants 10Δofs2/12 and 10Δofs3/5 (Fig. 3). This was in accordance with a large deletion of ofS sequence including the sequence used for hybridization in these strains. In strain 10Δofs::ermK1 and in the double mutant 10cpsΔEFSofs::ermK5, the ofS probe also did not hybridize with RNA fragments corresponding to ofS mRNAs of strain 10 and 10cpsΔEFS (Fig. 3). However, small RNA fragments of these insertion mutants hybridized with the...
ofs probe, which might be associated with the presence of the 3′-terminal 151 bp of the ofs probe downstream of the erm gene in these two mutants. In conclusion, mRNA analysis demonstrated transcription of ofs in strain 10 (and 10cpsΔEF) and confirmed the knockout of ofs in the isogenic mutants.

Analysis of OFS-mediated surface-associated serum opacification activity. As outlined above, sequence analysis and functional studies with rOFS suggested the expression of a serum opacity factor on the surface of S. suis. In agreement with this, serum opacification was detectable after incubation of horse serum with SDS extracts of strain 10 and its isogenic capsular mutant, 10cps, of horse serum with SDS extracts of strain 10 and its isogenic mutants (Fig. 4). Western blot analysis of the SDS extracts of all the tested S. suis strains with anti-MRP antibodies revealed equal amounts of this surface-associated protein (results not shown), indicating equal extraction efficiencies of surface-associated proteins. Specificity was shown, as the addition of antiserum against rOFSrep inhibited the serum opacification of S. suis extracts completely (Fig. 4). We then addressed the question of whether expression of OFS on the surface of a gram-positive bacterium is sufficient to provide surface-associated serum opacification activity. For this, plasmid-encoded OFS was expressed in L. lactis. L. lactis pOriOFS, which expressed ofs under the control of a constitutive promoter but carried the original ribosomal binding site of ofs, expressed small amounts of OFS on the surface, as shown by Western blots of SDS extracts (Fig. 4B). However, these extracts did not opacify serum (Fig. 4). Overexpression of OFS in L. lactis was achieved through the introduction of an optimal ribosomal binding site (pOriOFSoptRBS). This resulted in serum opacification activities of L. lactis extracts comparable to those of SOF+ S. pyogenes extracts (Fig. 4). Antibodies against rOFSrep inhibited this OFS-mediated serum opacification. In conclusion, a high level of expression of OFS is sufficient to provide surface-associated serum opacification activity comparable to the SOF-mediated serum opacification activity of S. pyogenes.

Experimental infections of piglets. In preliminary experiments, an infection model was established, which included the predispersion of piglets through the administration of 1% acetic acid prior to intranasal challenge. Diseased animals developed serositis, meningitis, and arthritis. The 80% lethal dose for weaners was approximately 2 × 10^8 CFU of S. suis strain 10. Mortality was found to be comparably lower in growers (7 to 8 weeks old). Therefore, piglets of two different age classes (nine weaners and four growers each) were infected with 2 × 10^9 CFU of S. suis strain 10 or of the ofs mutant 10Δofs2/12. None of the nine weaners infected with 10Δofs2/12 developed any clinical signs of infection except transient elevation of body temperature up to 40.3°C (Table 2). In contrast, eight of nine weaners infected with S. suis strain 10 showed severe clinical symptoms (high fever with values above 41°C, apathy, and anorexia) and were euthanized for ethical reasons (Table 2). In accordance, many wild-type-infected weaning piglets showed high histological scores (ω = 2.6), whereas no such findings were recorded in the group of weaning piglets infected with the mutant (ω = 0.1) (Table 3). In particular, five cases of serositis (mainly pleuritis) associated with the challenge strain were demonstrated (Tables 3 and 4). In two of these animals, the challenge strain was also detected in the cerebrospinal fluid. One additional sacrificed piglet was positive in the lung and in the spleen. In two of eight moribund

### Table 2. Virulence of wild-type and isogenic ofs mutant strains of S. suis serotype 2 in piglets

<table>
<thead>
<tr>
<th>Age of infected pigs (wk)</th>
<th>Total no. of infected pigs</th>
<th>S. suis strain</th>
<th>Morbidity</th>
<th>Mortality</th>
<th>Severe clinical symptoms</th>
<th>Maximum body temp (°C)</th>
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<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>4–5</td>
<td>9</td>
<td>10</td>
<td>8/9</td>
<td>8/9</td>
<td>8/9</td>
<td>1/9</td>
</tr>
<tr>
<td>4–5</td>
<td>9</td>
<td>10Δofs2/12</td>
<td>0/9</td>
<td>0/9</td>
<td>0/9</td>
<td>5/9</td>
</tr>
<tr>
<td>7–8</td>
<td>4</td>
<td>10</td>
<td>2/4</td>
<td>1/4</td>
<td>2/4</td>
<td>2/4</td>
</tr>
<tr>
<td>7–8</td>
<td>4</td>
<td>10Δofs2/12</td>
<td>1/4</td>
<td>1/4</td>
<td>1/4</td>
<td>3/4</td>
</tr>
</tbody>
</table>

*German Landrace piglets from a herd free of shv + epf + mep + cps2 S. suis strains.

### Table 3. Scoring of pathological findings of piglets infected with wild-type and isogenic ofs mutant strains of S. suis serotype 2

<table>
<thead>
<tr>
<th>Age of infected pigs (wk)</th>
<th>Total no. of infected pigs</th>
<th>S. suis strain</th>
<th>No. of pigs/total no. of pigs per score</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Brain (5 meningitis) 5 (meningitis) 3 (meningitis) 1</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Pleuritis 4^a 2^b</td>
</tr>
<tr>
<td>4–5</td>
<td>9</td>
<td>10</td>
<td>0/9 0/9 0/9</td>
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<tr>
<td>4–5</td>
<td>9</td>
<td>10Δofs2/12</td>
<td>0/9 0/9 0/9</td>
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<tr>
<td>7–8</td>
<td>4</td>
<td>10</td>
<td>1/4 0/4 0/4</td>
</tr>
<tr>
<td>7–8</td>
<td>4</td>
<td>10Δofs2/12</td>
<td>0/4 0/4 0/4</td>
</tr>
</tbody>
</table>

*Scores of 5 and 4 indicate moderate to severe diffuse fibrinous-suppurative inflammations of the brain and serosae, respectively.

*Scores of 3 and 2 indicate mild focal fibrinous-suppurative inflammations of the respective organs.

*Increased intravascular neutrophilic granulocytes suggestive of early inflammatory changes received a score of 1.

*ω = Σscore_max/n_minimals (see Materials and Methods).
TABLE 4. Reisolation of the challenge strain in pigs inoculated with wild-type and isogenic "ofs" mutant of S. suis serotype 2

<table>
<thead>
<tr>
<th>Age of infected pigs (wk)</th>
<th>Total no. of infected pigs</th>
<th>S. suis strain</th>
<th>No. of pigs in which the S. suis challenge strain* was isolated/total no. of pigs</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tonsile^2 2–7 dpi 20 dpi</td>
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<tr>
<td>4–5</td>
<td>9</td>
<td>10</td>
<td>6/9 1/1</td>
</tr>
<tr>
<td>4–5</td>
<td>9</td>
<td>10Δ&quot;ofs&quot;2/12</td>
<td>5/9 6/9</td>
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<tr>
<td>7–8</td>
<td>4</td>
<td>10</td>
<td>1/4 3/3</td>
</tr>
<tr>
<td>7–8</td>
<td>4</td>
<td>10Δ&quot;ofs&quot;2/12</td>
<td>1/4 1/3</td>
</tr>
</tbody>
</table>

* Challenge strains were identified through PCR assays (see Materials and Methods).
^ Either tonsil swabs or organ samples.
^ One cranial lobe was investigated.
^ Pleural, peritoneal, or pericardial cavity.
^ One tarsal puncture was investigated.
^ Four of the five animals were also positive in the lung.
^ The challenge strain was detectable in multiple organs from these animals.

weaners, no etiological diagnosis was found. Regarding the older piglets (7 to 8 weeks of age), one of four and two of four developed clinical signs of disease after infection with the "ofs" knockout mutant and the wild type, respectively (Table 2). Only one grower showed severe symptoms, namely, neurological deficits in association with a fibrinopurulent meningitis caused by wild-type S. suis. In the other group, one grower with moderate clinical signs was sacrificed. This was the only 1 of all 13 animals infected with the 10Δ"ofs"2/12 mutant for which detection of this genotype was associated with pathohistological findings (Tables 3 and 4). In the other 12 animals, pleural, peritoneal, and pericardial swabs as well as spleen, liver, tarsal joint fluid, and liquor were all negative in bacteriological examinations. However, an investigation of tonsil swabs and tonsils 7 and 20 dpi, respectively, revealed high rates of carriage of the mutant strain 10Δ"ofs"2/12; e.g., this strain was isolated from the tonsils in six of nine weaning piglets 20 dpi (Table 4). Additionally, strain 10Δ"ofs"2/12 was detected in the lungs of three of nine weaners. However, these bacteriological findings were not associated with the detection of pathological changes in this region of the lung (cranial lobe). In conclusion, experimental infection of piglets demonstrated substantial attenuation of virulence but not colonization deficiency of the respiratory tract in an isogenic S. suis serotype 2 "ofs" mutant in comparison with its wild-type parental strain.

DISCUSSION

Although a number of putative virulence factors of S. suis have been described, proof of their function is lacking for most of them. In this study, we identified a gene of S. suis, named "ofs", which encodes a protein homologous to FnBA of S. dysgalactiae and SOF of S. pyogenes. Expression of "ofs" was demonstrated by Northern blot analysis of S. suis serotype 2. Experimental infections of piglets revealed that OFS is a major virulence determinant in S. suis serotype 2. The results are in accordance with the attenuation of virulence in an SOF knockout S. pyogenes mutant described previously by Courtney et al. (5). Those authors studied a mutant with an insertional inactivation of sof. Thus, most likely, the sbfX gene, located downstream of and cotranscribed with sof, was also affected (12). Here, we established an in-frame deletion mutagenesis strategy for S. suis to exclude such effects on genes located downstream of the target gene. Furthermore, this study is the first to demonstrate the importance of a serum opacification factor for pathogenesis in the natural host. Bacteriological investigations suggested that, in contrast to virulence, colonization of the respiratory tract was not affected in the "ofs" mutant.

The identification of this novel virulence factor allows us to reveal important aspects of the pathogenesis of S. suis. For this, it is essential to functionally characterize OFS. As OFS carries the typical structural elements ofMSCRAMMs, it might be speculated that OFS functions as an adhesin and in particular that the C-terminal repeats of OFS bind fibronectin. We were not able to detect fibroectin binding of rOFS, rOFSrep, and rOFSp in ligand Western blots, in contrast to the recombinant FNBR of SfbI, which was used as a positive control (data not shown). The lack of detectable binding might be explained by significant differences between the repetitive sequences of OFS and the fibroectin-binding repeats of FnBA of S. dysgalactiae and SOF of S. pyogenes. Interestingly, two adjacent G residues in the MSCRAMM consensus sequence, which were found to be essential for fibroectin binding in the FnBA repeats (18), are lacking in the respective sequences of OFS (Fig. 1). However, similar to MSCRAMMs, the repeats of OFS have a high content of acidic amino acids. This might also explain the discrepancy of calculated molecular weight and migration in SDS-PAGE gels, which has also been described for the repetitive sequences of MSCRAMMs (13, 23). Whatever the precise function of the OFS repeats might be, it is rather unlikely that the investigated "ofs" mutant is completely deficient in fibroectin binding, as the fibroectin- and fibroectin-binding protein FBPS was identified in the same parental strain. Furthermore, attenuation of virulence of the FBPS knockout mutant suggests that FBPS’s function in the pathogenesis of S. suis is not redundant (9).

In this study, a major question was whether the high level of homology of OFS to SOF and FnBA at the N terminus is actually related to a serum opacification factor for pathogenesis in the natural host. Bacteriological investigations demonstrated the importance of a serum opacification factor for pathogenesis in the natural host. Bacteriological investigations suggested that, in contrast to virulence, colonization of the respiratory tract was not affected in the "ofs" mutant.

The identification of this novel virulence factor allows us to reveal important aspects of the pathogenesis of S. suis. For this, it is essential to functionally characterize OFS. As OFS carries the typical structural elements of MSCRAMMs, it might be speculated that OFS functions as an adhesin and in particular that the C-terminal repeats of OFS bind fibronectin. We were not able to detect fibroectin binding of rOFS, rOFSrep, and rOFSp in ligand Western blots, in contrast to the recombinant FNBR of SfbI, which was used as a positive control (data not shown). The lack of detectable binding might be explained by significant differences between the repetitive sequences of OFS and the fibroectin-binding repeats of FnBA of S. dysgalactiae and SOF of S. pyogenes. Interestingly, two adjacent G residues in the MSCRAMM consensus sequence, which were found to be essential for fibroectin binding in the FnBA repeats (18), are lacking in the respective sequences of OFS (Fig. 1). However, similar to MSCRAMMs, the repeats of OFS have a high content of acidic amino acids. This might also explain the discrepancy of calculated molecular weight and migration in SDS-PAGE gels, which has also been described for the repetitive sequences of MSCRAMMs (13, 23). Whatever the precise function of the OFS repeats might be, it is rather unlikely that the investigated "ofs" mutant is completely deficient in fibroectin binding, as the fibroectin- and fibroectin-binding protein FBPS was identified in the same parental strain. Furthermore, attenuation of virulence of the FBPS knockout mutant suggests that FBPS’s function in the pathogenesis of S. suis is not redundant (9).
antiserum against ROFS\textsubscript{rep} inhibited this activity of \textit{S. suis} extracts, which supports the conclusion that this opacification activity is OFS mediated. Finally, overexpression of OFS in \textit{L. lactis} resulted in surface-associated serum opacification activity as high as that in SOF\textsuperscript{−} \textit{S. pyogenes} strains.

In contrast to SOF\textsuperscript{−} \textit{S. pyogenes} strains, concentrated supernatants of wild-type \textit{S. suis} were negative for serum opacification in different assays (data not shown). It has previously been reported that surface-associated proteins other than SOF of \textit{S. pyogenes} are not secreted and are difficult to remove from the cell surface with chaotropic agents (23). At present, we can only speculate that these differences are related to the variations in the LPXTG motifs (LPSTG for OFS versus LPASG for SOF).

Interestingly, the addition of protease inhibitors 2 h before SDS extraction was crucial for the demonstration of surface-associated serum opacification in \textit{S. suis}. This observation suggests the proteolytic degradation of OFS under culture conditions, which has been described previously for other surface-associated virulence factors (20, 34). Based on the results of the animal experiments, we speculate that in the host, OFS expression might be upregulated and/or the half-life of OFS might be longer than that in vitro. Heterologous expression of OFS in \textit{L. lactis} clearly demonstrated that a high level of expression of OFS is sufficient to provide serum opacification activity on the surface of the bacterium, similar to SOF\textsuperscript{−} \textit{S. pyogenes} strains. Future studies will have to show whether \textit{S. suis} actually upregulates OFS expression during infection.

The finding that the repeats of OFS were not required for serum opacification of rOFS is in agreement with mapping experiments performed with SOF (5, 23). The serum opacification activity was located between amino acids 148 and 843 in SOF of \textit{S. pyogenes} M2 (5) and N terminal to residue 812 in SOF of M22 (23). Amino acids 148 to 843 of SOF were identified to be homologous to amino acids 56 to 723 of OFS in this study. As rOFS\textsubscript{rep} opacified horse serum, the serum opacification function is apparently located N terminal to P672 in OFS, which corresponds to P802 in SOF.

Taken together, our results allow us to conclude that the virulence factor OFS confers surface-associated serum opacification activity. This activity has not been proven to be essential for virulence, and other as-yet-unknown functions of OFS might be involved in pathogenesis. However, as the C termini of OFS and SOF differ significantly and fibronec tin binding was undetectable for rOFS, it is plausible that the shared activity of the N-terminal domain of SOF and OFS contributes to virulence. Opacification of serum by SOF is related to the disruption of HDL (7). HDL has anti-inflammatory functions; e.g., it inhibits C-reactive protein-induced upregulation of adhesion molecules such as vascular cell adhesion molecule 1, intercellular adhesion molecule 1, and E-selectin (33). Recently, Courtney et al. (7) showed that SOF-mediated disruption of HDL correlated with the binding of apolipoprotein A-I (ApoA-I). Interestingly, ApoA-I has been described as a negative acute-phase protein in pigs. In experimental \textit{S. suis} infections, this decrease starts as early as the first day postinfection, and the ApoA-I concentration is maintained at low levels (4). Future studies will have to show whether this is related to the expression of OFS and how putative targeting of ApoA-I by OFS might modulate the host response favoring the survival of this pathogen.

ACKNOWLEDGMENTS

Kerstin Thies (Tierarztliche Hochschule Hannover) is acknowledged for excellent help in establishing the infection model. We thank H. Smith (DLO-Lelystad, The Netherlands) for \textit{S. suis} strains 10 and 10cpsLEF as well as for the anti-MRP antibody. Daisuke Takamatsu (National Institute of Animal Health, Japan), Bernd Kreikemeyer (Hospital of the Rostock University), and Dieter Reinscheid (Fachhochschule Bonn-Rhein-Sieg, Germany) kindly provided pSETs and \textit{S. pyogenes} and the \textit{L. lactis} pORi23 strains, respectively. We thank the company Valoix for the actuators and Frank Matanic (Valoix) as well as Phillip Willson (Veterinary Infectious Disease Organization, Canada) for technical support. Furthermore, Christina Neis, Laurentiu Benga, and numerous collaborators from the Institut fuer Virologie (Tierarztliche Hochschule Hannover) were involved in the animal experiments. Andreas Mietze is acknowledged for excellent work during his practical.

This study was supported by a grant from the Akademie fuer Tiergesundheit, Bonn, Germany, to Christoph Baums and a grant of the Deutsche Forschungsgemeinschaft (DFG), Bonn, Germany (SFB507), to Ralph Goethe and Peter Valentin-Weigand.

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