Allelic variation and prevalence of serum opacity factor among the *Streptococcus suis* population

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Serum opacity factor of *Streptococcus suis* (OFS) has recently been identified as a virulence determinant of an *S. suis* strain. In this study, we investigated the prevalence and variations of the *ofs* gene among 108 *S. suis* isolates from diseased and healthy pigs, and human patients. PCR screening and sequencing analysis showed that besides the *ofs* gene reported already (designated type 1), there were three allelic variants of *ofs* (designated types 2 to 4). Type-1 and type-2 *ofs* genes were expected to encode functional OFS, and SDS extracts of the isolates with type-1 *ofs* and type-2 *ofs* opacified horse serum. Culture supernatants of the isolates with type-2 *ofs* also showed strong serum opacification activity. In contrast, type-3 *ofs* was interrupted by a point mutation and type-4 *ofs* was disrupted by either insertion of an IS element or genetic rearrangement, and therefore the SDS extracts and culture supernatants of the isolates with type-3 *ofs* and type-4 *ofs* did not show serum opacification activity. Regardless of their origins, approximately 30 % of the isolates possessed functional OFSs, although type-2 *ofs* was found only in three isolates from healthy pigs. Multilocus sequence typing analysis showed that most of the isolates with type-1 *ofs* belonged to the sequence type (ST)1 complex, and most of the isolates with type-3 *ofs* and type-4 *ofs* belonged to the ST27 complex. The isolates with type-2 *ofs* were not assigned to a major ST complex. These results suggest that type-1 OFS contributes to the virulence of a limited number of *S. suis* isolates, i.e. those of the ST1 complex type, whereas other *S. suis* may not possess this category of virulence factor; the importance of type-2 OFS is obscure.

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INTRODUCTION

Streptococcus suis is an important pathogen associated with a wide range of diseases in pigs, including meningitis, septicaemia, arthritis, pneumonia, endocarditis and sudden

Abbreviations: EF, extracellular factor; MLST, multilocus sequence typing; MRP, muramidase-released protein; OFS, opacity factor of *Streptococcus suis*; SOF, serum opacity factor of *Streptococcus pyogenes*; ST, sequence type.

The GenBank/EMBL/DDBJ accession numbers for the sequences of the *ofs* loci of *S. suis* NIAH 11433, DAT299, DAT246, DAT252 and MNCM51 are AB325706, AB325707, AB325708, AB325709 and AB325710, respectively, and for the new allele sequences of *aroA*, *cpn60*, *dpr*, *gki*, *mutS*, *recA* and *thrA* are AB330897–AB330899, AB330900–AB330907, AB330908–AB330909, AB330910–AB330915, AB330916–AB330922, AB330923–AB330927 and AB330928–AB330932, respectively.

A table of isolates and a figure showing Southern blot hybridization analysis are available as supplementary material with the online version of this paper.

death (Staats *et al.*, 1997). *S. suis* is also known as a zoonotic agent affecting people in close contact with diseased pigs or their products (Arends & Zanen, 1988; Chang *et al.*, 2006; Ye *et al.*, 2006; Takamatsu *et al.*, 2008).

Despite increasing research in recent years, knowledge of the virulence determinants of *S. suis* remains limited. The polysaccharide capsule of *S. suis* serotype 2 is proven to be one of the major virulence determinants (Smith *et al.*, 1999). However, not all encapsulated serotype 2 strains are virulent and there is variation in the degrees of virulence among the strains (Vecht *et al.*, 1989, 1992). Published studies have also reported several putative virulence factors, including the muramidase-released protein (MRP), the extracellular factor (EF) and suilysin (Jacobs *et al.*, 1994; Vecht *et al.*, 1991). In contrast to MRP[−] EF[−] serotype 2 strains from Europe, serotype 2 strains expressing the 136 kDa MRP and the 110 kDa EF were highly virulent (Vecht *et al.*, 1991, 1992). Therefore, these factors were thought to contribute to the pathogenesis of *S. suis*. However, because many virulent

isolates lacking these factors have also been isolated from clinical cases in North America (Gottschalk *et al.*, 1998), the significance of these factors in the pathogenesis of *S. suis* is still unclear.

Recently, a serum opacity factor of *S. suis* (OFS) has been identified as a novel virulence determinant of *S. suis* serotype 2 (Baums *et al.*, 2006). This protein has the typical structural features of Gram-positive surface proteins, which consist of a putative N-terminal signal sequence, a large N-terminal domain, repetitive sequence elements and a C-terminal LPXTG anchor motif (Baums *et al.*, 2006). Particularly, the N-terminal domain is homologous to the corresponding region of the serum opacity factor of *Streptococcus pyogenes* (SOF), and the domain has been shown to have serum opacification activity (Baums *et al.*, 2006). Disruption of *ofs* resulted in a severe attenuation of virulence when investigated in porcine experimental models, suggesting the significance of OFS in the pathogenesis of *S. suis* (Baums *et al.*, 2006). However, it is unknown whether this factor is widely present among *S. suis* strains, as only one strain was used for the reported study.

In this study, we investigated the prevalence of the *ofs* gene using 108 *S. suis* isolates from diseased pigs, healthy pigs and human patients. Our results showed that there were at least four distinct *ofs* genotypes in *S. suis*, and one of the genotypes was strongly associated with isolates of a genetically clonal group that is frequently isolated from pigs and humans with invasive disease throughout the world.

METHODS

Bacterial isolates and culture conditions. A total of 108 *S. suis* isolates were used in this study (see Supplementary Table S1 available with the online journal). Among them, 90 Asian isolates (51 from diseased pigs, 18 from healthy pigs and 21 from human patients) were from our laboratories' collection. Seventeen well-characterized North American and European strains (eight from diseased pigs, three from healthy pigs and six from human patients) were supplied by M. Gottschalk (Faculté de Médecine Vétérinaire, Université de Montréal, Montréal, Canada). The reference strain of serotype 2 was purchased from the National Collection of Type Cultures, London, England. Bacteria were cultured in Todd-Hewitt broth or agar supplemented with 2 % yeast extract (THY) (Difco Laboratories, Becton Dickinson), or Columbia agar (Difco Laboratories) with 5 % horse blood in air plus 5 % CO₂.

DNA methods. *S. suis* chromosomal DNA was extracted as described previously (Osaki *et al.*, 2000). *Ex Taq* polymerase (Takara Bio) was used for PCR amplifications. For PCR-based screening of the *ofs* gene, primers Ofs-F (5'-GATGTGACTGTCCGAGAGC-3') and Ofs-R2 (5'-AAAGTACCTGAGCTCCTACA-3') were used to amplify the internal segment of *ofs* (Fig. 1a), and reaction mixtures were prepared according to the manufacturer's instructions, except that the concentration of MgCl₂ used was 2.5 mM. The program for the amplification consisted of an initial denaturation of 2 min at 96 °C, 30 cycles of 20 s at 96 °C, 10 s at 55 °C and 1 min at 72 °C, and a final extension of 1 min at 72 °C. Southern blot hybridization was performed as described previously (Sekizaki *et al.*, 2001) with a DIG (digoxigenin) DNA labelling and detection kit (Roche). Prehybridization and hybridization were carried out at 65 °C for 2 and 16 h, respectively. For preparation of the *ofs* probe, an internal fragment of *ofs* was amplified from the genomic DNA of NIAH 11433 using primers Ofs-pr1 (5'-AAATAAGGAAGGAGAGGTGG-3') and Ofs-pr2 (5'-ACAAGAAGAGAGAGGGCAGT-3'), and labelled with DIG by using the DIG DNA labelling and detection kit. Sequencing of various PCR products was carried out with a BigDye terminator v3.1 cycle sequencing kit using a 3100 Genetic Analyzer (Applied Biosystems).

Sequence determination of *ofs* regions. Nucleotide sequences of chromosomal regions, which included the *ofs* gene fragments of strain 89/1591, were retrieved from the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>) using the BLASTP program with the OFS amino acid sequence of *S. suis* strain 10 (GenBank/EMBL/DDBJ accession no. AAX56334).

For sequence determination of the *ofs* regions of NIAH 11433, MNCM51, DAT246, DAT252 and DAT299, PCR products amplified by primers Ofs-F and Ofs-R2 were sequenced by primer walking, and BLAST searches (<http://www.ncbi.nlm.nih.gov/BLAST>) were performed using the determined sequences. Because the sequence of the PCR product of NIAH 11433 was identical to that of the corresponding *ofs* region of *S. suis* strain 10, primers Ofs-AF (5'-ACTTCTTCGAT-TACACGTCC-3') and Ofs-AR (5'-CCATCACTATTATAGGCATA-ATCTC-3') were constructed on the basis of the sequence of strain 10 *ofs*. The whole *ofs* gene of NIAH 11433 was amplified, and the resultant 2.95 kb PCR product was sequenced by primer walking. For further sequencing of the upstream region of the *ofs* locus of NIAH 11433, an additional primer, Ofs-sq7 (5'-GAAAATACCTAAAGA-AGGGCG-3'), was designed from the upstream sequence of the strain 10 *ofs*. The chromosomal region containing the upstream region of the *ofs* locus of NIAH 11433 was amplified with primers Ofs-sq7 and Ofs-R2, and the resultant PCR product was sequenced with primer Ofs-sq7.

Because BLAST searches showed that the sequence of the PCR product of MNCM51 was almost identical to those of the corresponding *ofs* regions of strain 89/1591, the whole *ofs* gene of MNCM51 was amplified with primers Ofs-AF2 (5'-TCCCAAAAGCTCTACGCTTC-

Fig. 1. (a) The positions of primers used for PCR amplification of *ofs* internal fragments. (b) PCR amplification of *ofs* fragments with primers Ofs-F and Ofs-R2: the results from six representative isolates producing fragments of distinct sizes are shown, molecular sizes (kb) are indicated on the right.

3') and Ofs-AR2 (5'-TTCAGCCTCTGCAGCTATTG-3'), which were designed from the *ofs* sequences of strain 89/1591. The resultant 5.0 kb PCR product was then sequenced by primer walking.

For sequencing the *ofs* regions of DAT246, DAT252 and DAT299, primers Ofs-IV1 (5'-ATGTAGACTCCCCTTGTGAC-3') and Ofs-IV2 (5'-GATTCAGATAACTCTGCTCC-3') were designed for inverse PCR on the basis of the sequences of the PCR products amplified by primers Ofs-F and Ofs-R2. DNA fragments of about 11.0, 9.0 and 9.0 kb were amplified with primers Ofs-IV1 and Ofs-IV2 from *Pst*I-digested and self-ligated genomic DNA of DAT246 and DAT252, and *Stu*I-digested and self-ligated genomic DNA of DAT299, respectively. The products were then sequenced by primer walking. Signal sequences of the Ofs proteins deduced from the determined sequences were predicted by the SignalP program (<http://www.cbs.dtu.dk/services/SignalP>).

Detection of serum opacification activity. The 1% SDS extraction technique, which has been described elsewhere (Baums *et al.*, 2006), was used with the following modifications. A 1 ml aliquot of mid-exponential phase culture ($OD_{600}=0.3-0.4$) was inoculated into 40 ml fresh THY broth. After 4 h of growth at 37 °C, 160 µl protease inhibitor cocktail (Sigma) was added to the cultures. Incubation at 37 °C was continued for an additional 2 h, and the bacterial cells were collected by centrifugation. The pellet was resuspended in 300 µl prewarmed (42 °C) 1% SDS and rotated end-over-end at 37 °C for 1 h. For the detection of serum opacification activity of secreted proteins, the culture supernatants were filtered with a Millex-GV sterilizing filter unit with a 0.22 µm pore size (Millipore) and placed on ice until use. A 25 µl aliquot of the SDS extract or the filtered culture supernatant was added to 100 µl horse serum (Sigma) in the wells of 96-well plates and mixed. As negative controls, 1% SDS or THY broth was added to the respective wells. The sealed plates were incubated with moderate shaking at 37 °C for 40 h. After the addition of 100 µl 0.9% NaCl, the A_{450} value was measured. Differences in serum opacification activity were compared by unpaired *t*-test using Welch's modification.

Multilocus sequence typing (MLST). MLST was performed by sequencing seven housekeeping genes as described by King *et al.* (2002). The allelic numbers and sequence types (STs) of the isolates were determined by comparing their sequences with those in the *S. suis* MLST database (<http://ssuis.mlst.net>). Novel alleles and STs were assigned through submission of the respective data to the database. Analysis of ST complexes was performed with the eBURST program (<http://eburst.mlst.net>) (Feil *et al.*, 2004).

RESULTS AND DISCUSSION

Variation of the *ofs* gene

Oligonucleotide primers Ofs-F and Ofs-R2 were expected to amplify a 1.96 kb fragment from the *ofs* gene of *S. suis* strain 10 (Fig. 1a). From genomic DNA samples of 29 of the 108 isolates, a PCR product of the expected size was amplified, while 3, 41, 9 and 15 isolates generated products with sizes of approximately 2.1 kb, 1.6 kb, 1.8 kb and 3.3 kb, respectively. From the remaining 11 isolates, including strain 89/1591, no specific PCR products were amplified (Fig. 1b). Since the results showed sequence divergence of the *ofs* genes among the *S. suis* isolates, we selected five representative isolates, NIAH 11433, DAT299, DAT246, DAT252 and MNM51, which generated 1.96, 2.1, 1.6, 1.8 and 3.3 kb products, respectively, and

determined the nucleotide sequences of their chromosomal regions corresponding to the *ofs* locus. In addition, BLAST searches were performed using genome sequencing data of strain 89/1591, and the sequences of two distinct chromosomal regions, which were highly homologous to the 5' and 3' regions of the *ofs* sequence of strain 10, were retrieved.

As shown in Fig. 2, the sequence analysis identified several allelic variants of *ofs*. The *ofs* gene of NIAH 11433 (designated type-1 *ofs*) was 2817 bp in length, and the nucleotide sequence was completely identical to that of strain 10 (Baums *et al.*, 2006). The *ofs* variant of DAT299 (designated type-2 *ofs*) was 3177 bp in length and encoded a protein of 1058 amino acids. Similar to type-1 Ofs, type-2 Ofs also consisted of a signal sequence, a large N-terminal domain, repetitive sequence elements and a C-terminal cell wall sorting signal (Fig. 2). The deduced amino acid sequence of type-2 Ofs was 79.0% identical and 84.9% similar to that of type-1 Ofs. However, due to mutations, the position of the start codon of type-2 *ofs* was different from that of type-1 *ofs*, resulting in alteration of the signal sequence (Fig. 3). In addition, type-1 Ofs possessed three repetitive sequence elements, while type-2 Ofs possessed four repetitive sequence elements (Fig. 2).

The *ofs* regions of DAT246 and DAT252, which generated PCR products of 1.6 and 1.8 kb, respectively, showed similar genetic organizations (Fig. 2). In both regions, ORFs were shifted due to a deletion of an adenine residue between nucleotide positions 625 and 630, resulting in the occurrence of a stop codon at nucleotide positions 649–651. The *ofs* variants of the two strains encoded the same proteins of 216 amino acids, and therefore both of the genes were designated type-3 *ofs*. The deduced amino acid sequence of type-3 Ofs was 54.2 and 85.6% identical to those of type-1 and type-2 Ofs, respectively.

In MNM51, the *ofs* gene was disrupted by the insertion of an IS element. The gene (designated type 4) was supposed to produce a protein of 564 amino acids (Fig. 2), and the deduced amino acid sequence was 70.3 and 84.6% identical to those of type-1 and type-2 Ofs, respectively. Strain 89/1591 also has a similar gene whose nucleotide sequence was completely identical to that of type-4 *ofs* of MNM51. However, in 89/1591, the genetic region was separated by an IS element and was translocated (Fig. 2).

These results demonstrated that in *S. suis* there are at least three allelic variants of *ofs* in addition to the originally reported *ofs* gene. However, because type-3 and type-4 *ofs* were interrupted by mutations, only type-1 and type-2 *ofs* seem to produce intact Ofs proteins. We hereafter refer to the isolates that produced PCR fragments of 1.96, 2.1 and 3.3 kb with primers Ofs-F and Ofs-R2 as isolates with type-1, type-2 and type-4 *ofs*, respectively. The isolates that generated PCR products of either 1.6 or 1.8 kb are referred to as isolates with type-3 *ofs*. In addition, the isolates that did not produce any specific PCR products were further analysed by Southern blot hybridization analysis. Using

Fig. 2. Genetic map of the *ofs* regions of six representative isolates and diagrams of the putative OFS variants. Arrows drawn with grey lines indicate pseudogenes corresponding to residual 3' parts of *ofs*, which are not supposed to be translated. PRR, Proline-rich region; Rn, repetitive sequence element; SS, putative N-terminal signal sequence.

*Cl*I-digested genomic DNA and the *ofs* probe prepared from type-1 *ofs*, they were classified as *ofs*-negative isolates and isolates with type-4 *ofs* (see Supplementary Fig. S1 and Supplementary Table S1 available with the online journal).

type1	-----TTGGACTTGTTCGCTGG
type2	TCATAATGAATCATCCAAATTCACCTTTCGCGAAGCTATCAATTGGACTTGTTCGCTGG
	M N H P K F T L R K L S I G L V S L
type1	GAGCGGGGTAGCTTTATCGGGAAACACTCTATCCTCAAATACAGTGACAGTATATGCAG
type2	GAGCGGGGTAGCTTTATCGGGAAACACTCTATCCTCAAATACAGTGACAGTATATGCAG
	G A G V A L S G T L Y P Q N T V T V Y A
type1	AAACGCTGTTACAGAAAGTACTACTTTAGAAAAAAATGAGGAGATAGATAAATCTCT
type2	AAACACCT-----ACTTTAGAAAAAAC-TGCGGAGACAGATAGCGCTTCT
	E T P T L E K T A E T D S A S
type1	TCGATTACACGTCCTATTGCTGTGAAGACCCCTAAAGTTGTCCAAGCTAATGAATAAAG
type2	CCGATTATGCTACTGCGCGCTGAAAGCTAAAGTTGTCCAAGCTAATGAATAAAG
	P I M P T A A A E D A K V V Q A N E N K
type1	K E R W L T P L L F S A L P K S N A E
type2	GAAGGAGAGGTGGTGTGACTCCTCTCTATTTCTGCCCTTACCAAAATCGAATGCAGAATC
	E G E V V D S S S I S A L P K S N A E

Fig. 3. Comparison of nucleotide sequences between the 5' regions of type-1 *ofs* of NIAH 11433 and type-2 *ofs* of DAT299, and their deduced amino acid sequences.

Serum opacification activity of OFS and its variants

SDS extracts and culture supernatants of eight representative isolates were tested for their ability to opacify a serum. As speculated from the sequence data, only the isolates with type-1 *ofs* and type-2 *ofs* showed surface-associated serum opacification activity (Fig. 4a). The opacification activities of the isolates were significantly higher ($P < 0.05$) than those of the isolates with type-3 *ofs* or type-4 *ofs*, indicating that only type-1 and type-2 *ofs* can express functional OFS on the cell surface.

Baums *et al.* (2006) reported that concentrated culture supernatants of *S. suis* strain 10, which possesses type-1 *ofs*, were negative for serum opacification. In agreement with this, neither P1/7 nor NIAH 11433 showed serum opacification activity in the culture supernatant (Fig. 4b). Interestingly, DAT289 and DAT299, which possess type-2 *ofs*, showed strong serum opacification activity in their culture supernatants (Fig. 4b). Although we do not have any evidence to predict the mechanism, the type-2 OFS specific structures described above may affect the stability of the proteins in the culture supernatant and/or the secretion of the proteins into the supernatant.

In *S. pyogenes*, expression of *sof* is positively regulated by the multiple-gene regulator Mga (formerly designated VirR), which also co-regulates the expression of *emm*,

Fig. 4. Serum opacification activity of *S. suis* isolates with distinct *ofs* genotypes. The 1% SDS extracts (a) or culture supernatants (b) of the isolates were tested for serum opacification activity with a microwell plate assay as described in Methods. The absorbance of serum incubated with 1% SDS alone (a) or THY broth alone (b) was subtracted from each value (corrected absorbance). Mean values and SDs of three or four independent experiments, each performed in triplicate, are shown.

encoding M protein, and *scpA*, encoding the C5a peptidase (Podbielski *et al.*, 1992; McLandsborough & Cleary, 1995). However, it is unknown how *S. suis* regulates the expression of *ofs*. Intriguingly, even though the deduced amino acid sequences of type-1 OFS in P1/7 and NIAH 11433 are identical to each other (D. Takamatsu, M. Osaki & T. Sekizaki, data not shown), the serum opacification activity of the SDS extract of P1/7 was significantly lower than that of NIAH 11433 ($P=0.0280$) (Fig. 4a). Although the difference in the serum opacification activities could be caused by differences in the extraction efficiency of surface-associated proteins between the two strains, *S. suis* may have some regulatory mechanisms for the expression of *ofs*.

Correlation of the *ofs* genotypes with MLST results

As shown in Table 1, regardless of their origin, approximately 30% of the isolates tested possessed either type-1 or type-2 *ofs*. However, isolates with type-2 *ofs* were recovered only from three healthy pigs. By means of MLST analysis using representative isolates, a strong association between the ST1 complex and isolates with type-1 *ofs* was observed. In addition, the majority of isolates with type-3 *ofs* and type-4 *ofs* were assigned to the ST27 complex. On the other hand, isolates with type-2 *ofs* were not related to known ST complexes (Table 2).

In *S. suis*, more than 400 isolates have been analysed by MLST, and 3 major clonal complexes have been identified so far (the ST1, ST27 and ST87 complexes) (King *et al.*, 2002; Chang *et al.*, 2006; Ye *et al.*, 2006; Rehm *et al.*, 2007). Among the three complexes, the ST1 complex was strongly associated with isolates from pigs with classical invasive diseases, including septicaemia and meningitis (King *et al.*, 2002). In addition, the majority of human clinical isolates, including those of the outbreak in Sichuan, China, in 2005, belonged to the ST1 complex (King *et al.*, 2002; Chang *et al.*, 2006; Ye *et al.*, 2006). The ST27 and ST87 complexes contained significantly higher numbers of swine lung isolates (King *et al.*, 2002).

In our previous report, we superimposed the distribution of the virulence-associated genes *mrp* (encoding MRP), *epf* (encoding EF) and *sly* (encoding suilysin) on the MLST data, and found that all the isolates assigned to the ST1 complex were positive for *mrp* (or its variant), *epf* (or its variant) and *sly*. However, the isolates assigned to the ST27 complex did not possess one or more of these genes (Takamatsu *et al.*, 2008). Similar data were also reported by Rehm *et al.* (2007). Among the isolates they analysed, all the isolates assigned to ST1 were positive for *mrp*, *epf* and *sly*, while the other isolates, except for one, lacked one or more of these genes. These results suggest that *mrp*, *epf* and *sly* can be used as epidemiological markers for ST1 complex members. However, because *mrp*, *epf* or *sly* mutants have been shown

Table 1. Prevalence of type-1 and type-2 *ofs* genes among the isolates used in this study

Bold text indicates results of particular interest.

Origin of isolates	No. of isolates (%)			Total
	Type 1	Type 2	Other	
Diseased pigs	17 (28.3%)	0 (0%)	43 (71.7%)	60 (100%)
Healthy pigs	3 (14.3%)	3 (14.3%)	15 (71.4%)	21 (100%)
Human patients	9 (33.3%)	0 (0%)	18 (66.7%)	27 (100%)

Table 2. Association of MLST typing results with *ofs* genotypes

<i>ofs</i> genotype	Isolate/strain	Origin	ST (ST complex)*
Type 1	P1/7	Diseased pig	1 (1)
	NIAH 11433	Diseased pig	1 (1)
	D282	Diseased pig	1 (1)
	DAT294	Healthy pig	112 (unrelated)
	DAT300	Healthy pig	115 (unrelated)
	H11/1	Human	1 (1)
	MNCM01	Human	1 (1)
	MNCM06	Human	1 (1)
	MNCM07	Human	11 (1)
	MNCM16	Human	1 (1)
Type 2	DAT289	Healthy pig	110 (unrelated)
	DAT291	Healthy pig	111 (unrelated)
	DAT299	Healthy pig	114 (unrelated)
Type 3	DAT246	Diseased pig	28 (27)
	DAT252	Diseased pig	108 (64)
	MNCM21	Human	101 (27†)
	MNCM43	Human	28 (27)
	MNCM50	Human	104 (27†)
Type 4	TD10	Healthy pig	25 (27†)
	MNCM04	Human	25 (27†)
	MNCM10	Human	25 (27†)
	MNCM24	Human	25 (27†)
	MNCM25	Human	102 (27†)
	MNCM26	Human	25 (27†)
	MNCM33	Human	103 (27†)
	MNCM51	Human	25 (27†)
	MNCM54	Human	102 (27†)
	MNCM55	Human	25 (27†)
	LPH3	Human	103 (27†)
	LPH4	Human	25 (27†)
	LPH5	Human	103 (27†)
	LPH12	Human	25 (27†)
	98-3634	Human	25 (27†)
<i>ofs</i> negative	DAT288	Healthy pig	109 (unrelated)
	DAT297	Healthy pig	113 (unrelated)
	DAT301	Healthy pig	116 (unrelated)

*The STs of NIAH 11433, DAT246, DAT252, DAT288, DAT289, DAT291, DAT294, DAT297, DAT299, DAT300 and DAT301 were determined in this study. MLST data for the other isolates were retrieved from the *S. suis* MLST database (<http://ssuis.mlst.net>).

†ST25, ST101, ST102, ST103 and ST104 belong to the ST27 complex, only with a less-stringent approach that defines an ST complex by the sharing of alleles at five or more of the seven loci.

to be as virulent as the respective wild-type strains after experimental infection of newborn or young pigs (Smith *et al.*, 1996; Lun *et al.*, 2003), it is unclear whether or not these markers have some role in the pathogenesis of the ST1 complex members. On the contrary, severe attenuation in virulence of an *ofs* mutant was demonstrated by an experimental infection model in piglets, and *ofs* was suggested to be an important virulence determinant (Baums *et al.*, 2006). These results, together with the present findings, suggest that type-1 OFS is one of the important virulence factors that may confer upon the ST1 complex members an ability to cause invasive diseases.

SOF of *S. pyogenes* has been established as a virulence determinant (Courtney *et al.*, 1999). Disruption of high-density lipoprotein (HDL), which is an anti-inflammatory and anti-atherogenic factor, by SOF is suggested to contribute to the pathogenesis of *S. pyogenes* infection (Courtney *et al.*, 2006). Although it is unknown how OFS is involved in the pathogenesis of *S. suis*, a similar mechanism may contribute to the development of invasive diseases by this organism. However, in our data, approximately 70 % of the *S. suis* isolates from diseased pigs and human patients did not possess functional *ofs* genes (Table 1). In addition, genes encoding type-2 OFS showing

higher opacification activities than type-1 OFS were found only in isolates from healthy pigs. These results indicate that OFS is not an essential nor a sufficient factor for *S. suis* isolates to cause invasive diseases. For further comprehensive understanding of the pathogenesis of *S. suis*, identification of additional virulence determinants and comparison of their distribution with the MLST dataset will be needed.

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