RESEARCH LETTER

Existence and characterization of allelic variants of Sao, a newly identified surface protein from *Streptococcus suis*

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Abstract

Surface antigen one (Sao) is a newly identified protein from the major zoonotic pathogen, *Streptococcus suis*. In search of functional proteins related to the pathogenesis of Chinese *S. suis* 2 (SS2), unexpectedly, a variant of Sao protein was obtained. To test its prevalence in *S. suis*, PCR assay was adopted to address the coding genes systematically. It was found that there are three allelic variants of *sao* gene, namely *sao-S*, *sao-M*, and *sao-L* based on the different lengths of the genes (~1.5, ~1.7, and ~2.0 kb, respectively). These differences were determined to be caused by heterogeneity within the number of C-terminal repeat sequences (R), which had been seen as a pathogenicity-related domain in the plant pathogen, *Xanthomonas oryzae*. Two variants (*sao-M* and *sao-L*) were only found in SS2. All three variant proteins were prepared *in vitro* and their biochemical and biophysical properties were characterized. A soluble form of Sao-M protein was then used as a capture antigen to develop an enzyme-linked immunosorbent assay method to detect antibodies against SS2 in convalescent pig sera. Taken together, the results exhibit the properties of Sao proteins and provide an efficient Sao-M-based method for monitoring SS2 infection.

Introduction

Streptococcus suis infection of swine is recognized as the leading cause of highly invasive diseases such as meningitis, septicemia, arthritis, and even sudden death, and has spread over 20 countries, resulting in great economic losses in pig-industries each year (Staats *et al.*, 1997; Huang *et al.*, 2005). *Streptococcus suis* is also a notorious zoonotic agent responsible for more than 200 cases of severe infections in humans worldwide since its first discovery in Denmark in 1968 (Staats *et al.*, 1997). Thirty-five serotypes of *S. suis* have been determined based on differences among their capsule antigens (Staats *et al.*, 1997). However, Hill *et al.* (2005) recently suggested that the two serotypes (serotype 32 and serotype 34) may be excluded from *S. suis* species and redesignated as *Streptococcus orisratti*. Virulence varies greatly among the serotypes and can be divided into three groups: highly pathogenic, hypovirulent, and avirulent (Staats *et al.*, 1997). *Streptococcus suis* 2 (SS2) is the most prevalent serotype isolated from both diseased piglets and patients worldwide and is often associated with severe clinical syndrome (Staats *et al.*, 1997). In particular, two large-scale outbreaks of lethal SS2 infections with a hallmark of streptococcal toxic shock syndrome (STSS) emerged in China (one in Jiangsu Province, 1998, and the other in Sichuan Province, 2005), posing a great global concern to the public health (Tang *et al.*, 2006). As it is known that human SS2 infections have been documented for several decades, the molecular mechanism underlying the high pathogenicity of this bacterial agent is still poorly understood (Staats *et al.*, 1997; Gottschalk *et al.*, 1999). Several bacterial components have been previously implicated in *S. suis* virulence, but the exact role remains unclear (Staats *et al.*, 1997; Gottschalk *et al.*, 1999; Gottschalk & Segura, 2000). So far, the virulence-associated factors identified include components of capsule polysaccharide (CPS) (Smith *et al.*, 2000), extracellular factor (EF) (Smith *et al.*, 1993; Staats *et al.*, 1999), muraminidase-released protein (MRP) (Staats *et al.*, 1999), suilysin (Allen *et al.*, 2001; Lun *et al.*, 2003), adhesions (Tikkanen *et al.*, 1996; Brassard *et al.*,

2004), 38 kDa protein (Okwumabua & Chinnapapakkagari, 2005), glutamate dehydrogenase (GDH) (Okwumabua *et al.*, 2001), fibronectin-binding protein (FBP) (de Greeff *et al.*, 2002; Wang, 2006), hayluronate lyase (King *et al.*, 2004), sortase A (*srtA*) (Osaki *et al.*, 2002), arginine deiminase (Gruening *et al.*, 2006), etc. In some instances, the virulence determination of some *S. suis* strains was still ambiguous, indicating that the virulence of *S. suis* is a complex interplay between the pathogen, its host, and the environment (Gottschalk *et al.*, 1999). Considerable efforts to control severe SS2 infections have still been hampered greatly by the limited knowledge of *S. suis* pathogenesis (Staats *et al.*, 1997; Gottschalk *et al.*, 1999; Gottschalk & Segura, 2000; Haesebrouck *et al.*, 2004).

It is widely accepted that the cell-wall-exposed proteins/outer surfaces of pathogenic bacteria are of great importance to understanding their pathogenesis (Navarre & Schneewind, 1999; Cabanes *et al.*, 2002). Not only are surface-associated components implicated in bacterial defense machineries but also they are involved in virulence-related behaviors (e.g., adhesion) (Navarre & Schneewind, 1999; Maione *et al.*, 2005). Thus, for vaccine development against pathogenic bacteria, current interest has shifted from the CPS antigen to surface proteins with robust immunogenicity (Navarre & Schneewind, 1999; Cabanes *et al.*, 2002; Maione *et al.*, 2005; Li *et al.*, 2006). Whole genome-wide screening was carried out to search for a universal Group B Streptococcus (GBS) vaccine (Maione *et al.*, 2005). These surface proteins containing a C-terminal cell wall anchoring motif (LPXTG) have been found in a variety of pathogenic microorganisms, and suggested to execute key steps during the process of infection, which range from colonization to invasion (Navarre & Schneewind, 1999; Cabanes *et al.*, 2002; Osaki *et al.*, 2002; Maione *et al.*, 2005). In *S. suis*, MRP belongs to this type of surface protein (Smith *et al.*, 1992). More recently, Li *et al.* (2006) reported a novel surface protein, surface antigen one (Sao), from S735 strain of SS2, and evaluated its potential as a vaccine . Although it failed to protect piglets against SS2 infection completely, it was not determined whether or not this protein played any role in *S. suis* infection.

The availability of the whole genomes of SS2 Chinese strains allows to mine the functional proteins related to high virulence (Chen *et al.*, 2007). During a genome-wide *in silico* screening for surface-exposed proteins or cell wall-associated proteins, a mutant of *sao* was accidentally found, in which 270 bp of repeated sequences at the 3′-terminus was deleted. In light of this unexpected observation, it was attempted to examine the prevalence in various serotypes of *S. suis*, and test its possible genetic variation or molecular polymorphism. Thus, PCR screening was undertaken to test more than 50 different *S. suis* strains comprising 34 kinds of serotypes (except for *S. suis* 12). It was found that Sao proteins exhibit obvious polymorphisms with considerable genetic variation. Furthermore, it was possible to classify Sao into three groups and the proteins were also characterized via a range of biochemical techniques. Immunological data from Western blotting and enzyme-linked immunosorbent assay (ELISA) also demonstrated that Sao-M, the most common type of Sao, has strong immunogenicity. Sao-M has been successfully developed into an effective ELISA method for monitoring *S. suis* infection in both pigs and humans. Therefore, Sao may serve as a useful marker for clinical surveillance of *S. suis* infection.

Materials and methods

Strains, plasmids, and culture conditions

The reference strains of *S. suis* (34 kinds of serotypes) were kindly provided by Prof. Marcelo Gottschalk in Canada and Prof. Astrid de Greeff in Holland. The other SS2 isolates were all kept in the authors' laboratory (listed in Table S1). *Streptococcus suis* were cultivated in Todd–Hewitt Broth (THB, code CM189; Oxoid) at 37 °C for preparing chromosomal DNA as a PCR template (Tang *et al.*, 2006). *Escherichia coli* strains DH5α and BL21 (DE3) were maintained in Luria–Bertani (LB) broth or agar medium at 37 °C for recombinant plasmid amplification and protein expression, respectively (Liu *et al.*, 2006). The commercial pMD18-T vector (Takara) and pET28a (Novagen) were utilized to clone PCR fragments for direct sequencing of *sao* genes, and construct recombinant expression plasmids, respectively.

Molecular manipulation

Streptococcus suis genomic DNAs were extracted using the routine CTAB method as described by Tang *et al.* (2006), and their size and quality were evaluated by electrophoresis on a 0.8% agarose gel (Brazil) before they were used as templates of PCR amplification for *sao*. If necessary, two sets of primers specific for *S. suis* house-keeping genes, 16S rRNA gene and *gyrase*, were utilized to further assess the quality of DNA templates.

To amplify *sao* genes, the specific primers (S-F: 5′-ATGAATACTAAGAAATGGAG-3′; S-R: 5′-TTATAATTTAC GTTTACGTGT-3′) were designed, which, according to the available sequence information, cover the entire putative ORF. Subsequently, the standard PCR in a total volume of 50 μL was conducted in a PTC-225 thermocycler (MJ Research) using ExTaq (Takara). All the candidate PCR products were ligated into a pMD18-T vector (Takara) as the recommended protocol to obtain unique clones for direct DNA sequencing by an ABI 3730 DNA sequencer (Perkin-Elmer Applied Biosystems).

To prepare the soluble recombinant Sao proteins, it was attempted to construct three plasmids using PCR products generated with the primers (Sao-F: 5′-CG *GGATCC* CAAG AAGTAAAAAATACCATC-3′; Sao-R: 5′-CCAA GTCGAC TTATTTCTCACCAGTTACTGG-3′). The primers introduced BamHI and SalI sites into the truncated DNA fragments. The truncated DNA fragments from three *sao* variants (88–1938 bp of *sao-L*, 88–1668 bp of *sao-M*, and 88–1395 bp of *sao-S*) equivalent to mature extra-domains (30–646 aa of Sao-L, 30–556 aa of Sao-M, and 30–465 aa of Sao-S) were introduced into the BamHI and XhoI sites of pET28a (Novagen). Finally, the resulting recombinant plasmids, designated pET28∷Sao-L, pET28∷Sao-M, and pET28∷Sao-S, were all verified to be in frame with the initiation codon of the vector and to have the expected sequence by direct DNA sequencing.

Production of soluble Sao proteins

To obtain soluble versions of the Sao proteins, a general procedure was performed as described earlier in the authors' laboratory with some minor modifications (Liu *et al.*, 2006). First, a single colony from BL21(DE3) transformants was inoculated in LB medium containing 50 mg L^{-1} kanamycin (Sigma) at 37 °C and grown overnight. Then, the culture was diluted 1 : 100 in 2 L of fresh LB medium and incubated at 37 °C. When the culture density ($OD_{600\,nm}$) reached 0.8–1.0, the culture was induced with 0.8 mM isopropyl-β-D-thiogalactopyranoside (Sigma) and kept for about 4–5 h at 37 °C until the bacterial cells were collected by centrifugation at 2400 ***g***. Third, the harvested bacterial pellet was suspended in chilled phosphate-buffered saline (PBS) and lysed by sonication. The extract was centrifuged at 16 200 ***g*** for 20 min at 4 °C, and subsequently filtered through a 0.22 μm membrane for clarification. Finally, the supernatant was loaded onto the nickel-ion (Ni^{+}) affinity column (Qiagen), and the N-terminus 6 × histine-tagged Sao proteins were eluted in elution buffer with 100 mM imidazole after removing the contaminant proteins. The proteins of interest were visualized on a 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and stained with Coomassie brilliant blue R250 (Sigma, St Louis, MO).

Biochemical characterization of Sao proteins

To further characterize Sao variants, FPLC or gel filtration, chemical cross-linking, and circular dichroism (CD) were utilized as described earlier (Ma *et al.*, 2005; Liu *et al.*, 2006).

First, SDS-PAGE was performed according to the standard procedure (Liu *et al.*, 2006). Then, for the analysis by gel filtration, the acquired recombinant Sao proteins were loaded on a Superdex 75 column (Pharmacia) with an AKTA Purifier System (Pharmacia) after they were concentrated by ultra-filtration (10 kDa cutoff) and exchanged from 1 × PBS buffer into the exclusion buffer. The peak fractions were collected and analyzed by 12% SDS-PAGE, and the apparent molecular weights (MW) were estimated by comparison with the standard protein marker (Sangon, Shanghai) run on the same gel.

Second, to discriminate the solution structures of the different Sao proteins *in vitro*, a chemical cross-linking assay was adopted. Briefly, Sao protein purified from gel filtration were dialyzed against a cross-linking buffer (50 mM HEPES, pH8.3; 100 mM NaCl) and concentrated to *c.* 10 mg L^{-1} by ultra-filtration (10 kDa cutoff). Then, the resultant protein was subjected to a chemical cross-linking reaction with ethylene glycol bis-succinimidylsuccinate (EGS) (Pierce). The reactions were incubated for 1 h on ice at different concentrations of EGS, respectively (0, 1.0, and 5.0 mM EGS), and quenched with 50 mM glycine. Eventually, the cross-linked samples were analyzed by 12% SDS-PAGE (Ma *et al.*, 2005; Liu *et al.*, 2006).

Lastly, to acquire information on the secondary structures of these proteins, CD spectra were recorded on a Jasco J-715 spectrophotometer in a 0.1 cm path-length cuvette consisting of a 1 × PBS buffer at 25 °C (Ma *et al.*, 2005; Liu *et al.*, 2006). The records were the mean of triplicate independent experiments.

Immunogenic evaluation of Sao protein

Western blotting and ELISA were used to test the immunogenicity of Sao. Here, negative swine sera were sampled from SPF-pigs and convalescent-phase swine sera were collected from those piglets that had survived infection by *S. suis* ZY05.

For Western blotting, the soluble Sao-M protein was subjected to 10% SDS-PAGE, transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech), and blocked with 5% skim milk in TBST [50 mM Tris (pH 7.3), 150 mM NaCl, 0.1% Tween 20] at room temperature (RT) for 1 h. The membrane was incubated with convalescent-phase swine sera in a 1 : 200 dilution in TBST at RT for 1 h, which was followed by the incubation with HRP-conjugated antiswine antibody (Santa Cruz) in a 1 : 5000 dilution in TBST at RT for 1 h.

To probe whether Sao protein is associated with *S. suis* infection, an Sao-based ELISA was performed. Ninety-six-microtube ELISA plates (Greiner bio-one, Germany) were coated overnight at 4 °C with 100 μL $well^{-1}$ of purified Sao protein at an appropriate concentration in carbonate buffer. After three washes with TBST, the plates were blocked with 5% skim milk in TBST for 1 h at 37 °C. Next, several pig sera diluted in 1 : 1000 in TBST were added to appropriate wells in triplicate at 100 μL $well^{-1}$, incubated for 1 h at 37 °C, and washed three times. Then, the bound antibodies against Sao were detected by incubation for 1 h at 37 °C with HRP-conjugated goat antiswine IgG (Sigma). These plates

were developed with *O*-phenylenediamine as a substrate (Amresco) and H_2O_2 (Sigma) as the oxidation agent; 1 M sulfuric acid was used to stop the reaction. The absorbance score was measured at 490 nm in a micro-plate reader (Model 500; Bio-rad). The results were expressed as means ± standard deviations, and statistical significance was determined by Student's *t* test (Li *et al.*, 2006).

Bioinformatics analysis

All the sequencing results of different *sao* genes (submitted to GenBank) were assembled with Vector NTI Suite 8.0 (Invitrogen). Also, with the aid of Vector NTI Suite 8.0, multiple sequence alignments of these genes were carried out. The phylogenetic tree of Sao proteins was constructed by the neighbor-joining method. A BLASTP search was performed in the protein database of Genbank.

Results

Distribution of different *sao* genes in *S. suis*

Prompted by the unexpected finding of strains carrying *sao* gene variants, it was attempted to determine the range of *sao* variants and correlate variant prevalence with the serotype of *S. suis*. More than 50 different *S. suis* strains covering nearly all of the serotypes were cultivated for isolation of genomic DNA. The results of PCR screening yielded three distinct *sao* amplicons (Fig. 1a). Among those strains tested, the following conclusions could be drawn. First, *sao* is relatively common in different serotypes and/or strains of *S. suis*. Although PCR-negative results were obtained in several isolates such as Denmark strains 6704, 4417, 22083, etc. (Table S1, Fig. 2), this was largely in agreement with the Western blot results described by Li *et al.* (2006). Second, according to various lengths of genes, *sao* could be referred to as *sao-L* (~2.0 kb, L: long), *sao-M* (~1.7 kb, M: middle), and *sao-S* (~1.5 kb, S: small) (Fig. 1a). These data also suggested that *sao-M* is the most prevalent variant comprising about 80%, followed by *sao-L*, and *sao-S* is detected at no more than 6% (Fig. 2).

Molecular characterization of Sao

To further characterize *sao* variation at the molecular level, numerous representative PCR products were cloned into a pMD-T vector, and subjected to DNA direct sequencing on an ABI sequencer 3730. Multiple sequence alignments of these genes indicated that although point mutations are relatively common (not shown), the major cause of the variation could be attributed to deletions within the 3′ end (Fig. 1c and d). Phylogenetic analysis suggested molecular polymorphism as well (not shown). Various numbers of repeated units (27 aa U^{-1}) (Fig. 1c) were identified in a series of Sao proteins. Furthermore, nearly all the deletion events converged in the repeat regions at the C′-terminus of Sao (Fig. 1c and d). For this reason, the repeated regions were used as a query for a BLASTP search of the Genbank database and it was found that repeated regions matched well with the avirulence domain, AvaXa7, in the plant pathogen, *Xanthomonas oryzae* pv. *oryzae*, a principal causative agent of rice blight (not shown). This validated strongly a similar finding given by Li *et al.* (2006).

Soluble expression of Sao proteins *in vitro*

To obtain Sao proteins for further analysis, the pET28a/BL21(DE3) expression system was applied to express the fragment of *sao* lacking the two hydrophobic domains. Fortunately, soluble forms of the three Sao proteins were successfully expressed (Fig. 1b). However, these proteins behaved abnormally in SDS-PAGE. The apparent MW of the Sao proteins purified by affinity chromatography were considerably larger than those estimated by deduction from the DNA sequences (Fig. 1b and Table 1). Meanwhile, it was observed that the size error of Sao-S was much less than that of Sao-L in the SDS-PAGE profile (Fig. 1b and Table 1).

Biochemical properties of Sao proteins

To better understand the biochemical properties of three Sao proteins, each protein was analyzed by gel filtration, chemical cross-linking, and CD. The three Sao proteins (Sao-L, Sao-M, and Sao-S) were eluted from a Superdex-200 column at ~11.8 mL (Fig. 3a), ~12.8 mL (Fig. 3b), and ~13.1 mL (Fig. 3c), respectively, and were resolved from one another almost completely (95%). Also, the elution volumes of the Sao proteins suggested that they are monomeric in solution, which was supported by the chemical cross-linking experiments (Fig. 3d). CD experiments presented preliminary information on the protein secondary structure for Sao proteins (not shown).

Development of a Sao-M-based ELISA method and its application

Because it had been determined that Sao-M was the most common form of Sao in *S. suis*, this variant was subjected to immunological evaluation. Western blotting demonstrated that Sao-M reacts strongly with convalescent-phase sera from pigs clinically infected by SS2 (Fig. 4a). As a further confirmation, sera were sampled from SPF-pigs (negative control) and piglets after SS2 infection. Then, ELISA was performed and the specificity of convalescent-phase sera for Sao-M was confirmed (Fig. 4b).

Given the robust immunogenicity of Sao-M, next, it was of interest to evaluate the potential of Sao-M as a diagnostic antigen for investigation of *S. suis* infection. A Sao-M

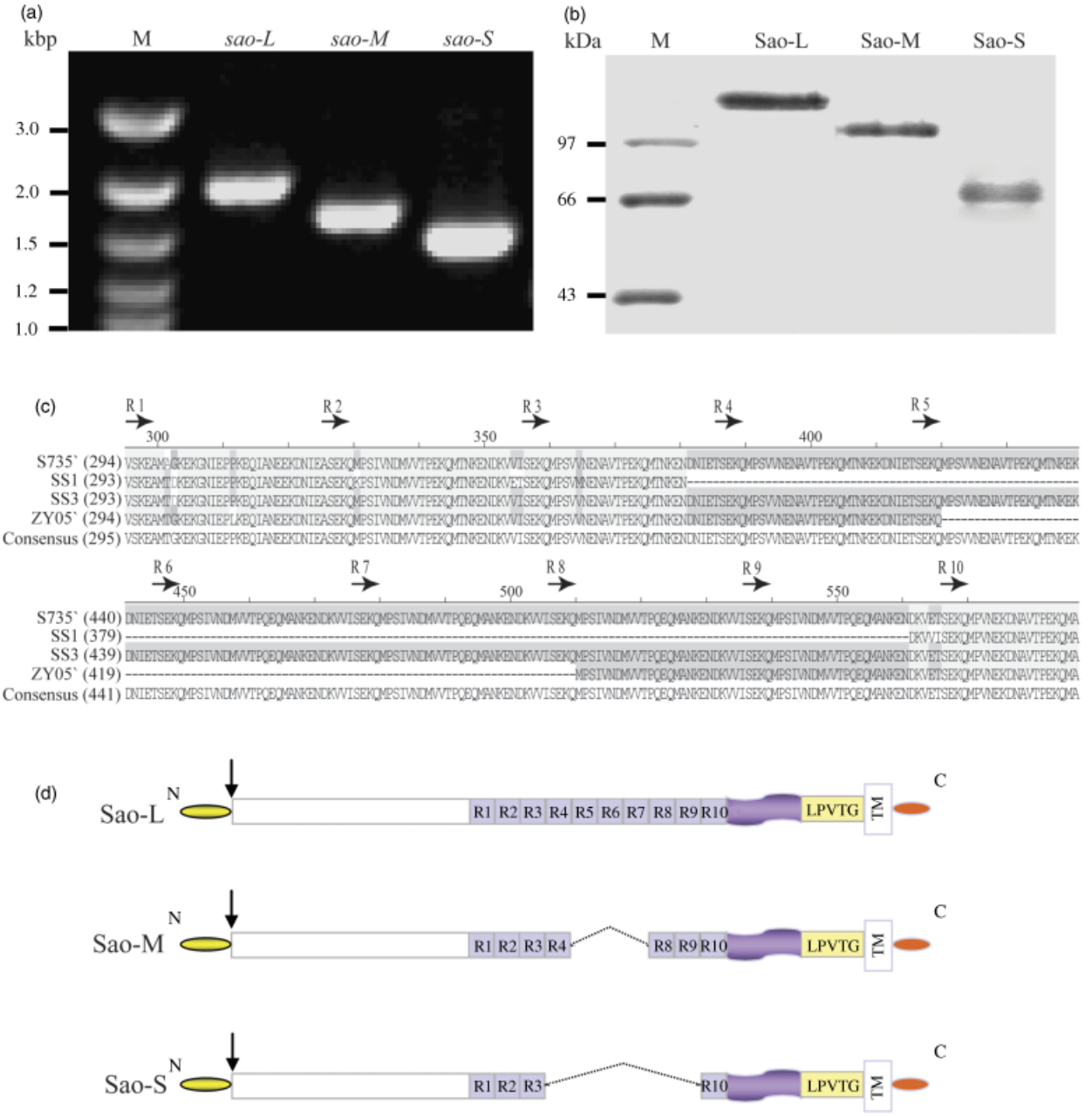

Fig. 1. Three types of Sao proteins from *Streptococcus suis*. (a) PCR identification of *sao* variants. M: 100 bp DNA Ladder (Fermentas, Vilnius, Lithuania). *sao-L*: *sao* in ~2.0 kb; *sao-M*: *sao* in ~1.7 kb; *sao-S*: *sao* in ~1.5 kb. (b) *In vitro* expression analysis of the three Sao proteins. M: protein MW standard marker (Sangon, Shanghai, China). (c) Multiple-sequence alignment of the repeated regions from different Sao proteins. Three types of Sao proteins are aligned using the software of Vector NTI Suite 8.0. The N-terminal sequences were found to be nearly identical, while the C-terminal regions were discontinuously well matched. Here, the focus is on the variations in the C-terminus and this information is presented as described by Li *et al*. (2006). R1, R2, R3 . . . R10: Repeated region1, 2, 3 . . . 10 in the C-terminus of Sao. S735: Holland isolate of *S. suis* 2 (Sao-L); SS1: Holland strain 5428 of *S. suis* 1 (Sao-S); SS3: Holland strain 4961 of *S. suis* 3 (Sao-L); ZY05: a highly virulent strain of SS2 from Ziyang County of Sichuan Province in China, 2005 (Sao-M). (d) Linear representation of three types of Sao proteins. The vertical arrow indicates the predicted signal peptidase cleavage site. The ellipses in yellow and red correspond to the hydrophobic region at the N-terminus and the charged amino acids of the C-terminal tail. The curved blue boxes refer to the possible cell wall-associated regions. The dashed lines represent the deletion of repeated regions. TM refers to the possible transmembrane region at the C-terminus.

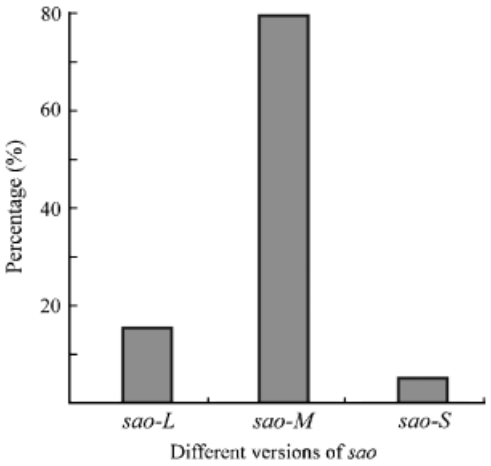

Fig. 2. Distribution of different *sao* genes in *Streptococcus suis*. The PCR technique is utilized to test the prevalence of the *sao* gene among various strains of *S. suis*. About 75% of all the strains tested here can be confirmed to be PCR positive (also seen in Table S1). Three types of *sao* genes (*sao-L*, *sao-M*, and *sao-S*) have been drawn according to the different sizes of the PCR fragments. The proportion of the above three *sao* types is measured based on the samples collected in this study.

Table 1. Biochemical properties of different versions of Sao

Sao types	Strains	Serotype	Length (aa)	Predicted IP	Estimated MW (kDa)	Apparent MW* (kDa)
Sao-L	4961	3	670	4.8	74.8	~130
Sao-M	ZY05	2	580	4.8	64.6	~110
Sao-S	5428	1	489	5.0	54.5	~60

*The apparent MW was estimated on the basis of migratory rates of the equivalent Sao proteins in 12% SDS-PAGE.
IP:,isoelectric point; MM, molecular weight.

protein-based ELISA method was used successfully to monitor sera from *S. suis*-infected animals. Based on the results obtained from numerous samples, it was determined that the optimal conditions for the assay consisted of $1\,\mu g\,mL^{-1}$ of Sao-M, sera diluted 1 : 1000, and 1 : 1000 diluted HRP-conjugated goat anti-swine secondary antibody. The ELISA results demonstrated that the assay could unambiguously distinguish between the positive, suspicious, and negative samples (Fig. 4c). Therefore, this method shows promise for application in laboratory, clinical, and even field monitoring of *S. suis* infection in the swine-industry. This could help establish prevention and alarm systems for dealing with outbreaks of zoonotic diseases (such as meningitis, septicemia, arthritis, etc.) caused by *S. suis* infections of humans.

Discussion

Streptococcus suis causes a series of severe invasive diseases in piglets, with substantial economic impact upon the swine-industry (Staats *et al.*, 1997; Huang *et al.*, 2005). Recently, *S. suis* infection garnered great attention worldwide due to public health concerns over its potential to cause serious zoonotic infections with associated clinical syndromes such as septicemia, meningitis, STSS, etc. (Gottschalk & Segura, 2000; Huang *et al.*, 2005; Tang *et al.*, 2006). The conventional strategy to control *S. suis* infections is overly dependent on prophylactic and therapeutic antibiotics, which has led to the isolation of antibiotic-resistant strains in recent years (Huang *et al.*, 2005; Tang *et al.*, 2006). Also, a lack of data describing the virulence factors contributing to the pathogenesis of *S. suis* has greatly hampered the development of novel vaccines and pharmaceutics against *S. suis* infections (Staats *et al.*, 1997; Gottschalk *et al.*, 1999). Thus, there is currently considerable interest in identifying and characterizing surface-exposed virulence factors of *S. suis*. Recently, a promising candidate called Sao has been described. Sao is a novel C-terminally anchored surface protein identified from S735, a virulent Holland strain (Li *et al.*, 2006).

Sao was found from an *in silico* search of surface-exposed proteins in the *S. suis* genome. Based on the search, a deletion mutant of *sao* could be identified that had 270 bp of repeated sequences missing from its 3′-terminus . Then, 34 serotypes of *S. suis* were examined and three forms of *sao* genes were found among the different strains. Although sao genes could not be amplified from several *S. suis* strains (e.g., 10581, 2726, 865191, etc.), the absence of *sao* genes might have been caused by the considerable variation during the evolution of these serotypes. This was also validated by the result of a Western blot described by Li *et al.* (2006). Although the existence of *sao* genes in a few strains (e.g., 4417) could not be detected despite positive results at the protein level (Li *et al.*, 2006), other approaches will be attempted to determine the reasons for this discrepancy. The most frequently observed form of the *sao* gene was *sao-M*, which is the medium-sized version at ~1.7 kb. The results resemble those reported by Smith *et al.* (1993). In that study, the authors also found EF, a derivative of EF* lacking the repeat units at its C-terminus, and was correlated with increased virulence (Smith *et al.*, 1993). Therefore, it would be of interest to determine whether or not the present deletion variants in Sao proteins have any correlation with their pathogenicity of the organisms. The aberrantly larger apparent MW of Sao-L and Sao-M proteins, can be surmised to be due to the poor binding of SDS, which gives less charge density to the fragments than to the standard proteins and hence a higher apparent MW.

The homology of the Sao protein to the avirulence domain of a prominent plant pathogen (AvrXa7) (not shown) also hints at a role of the high invasiveness and/or virulence of *S. suis* (Yang *et al.*, 2000). In the case of *avr*

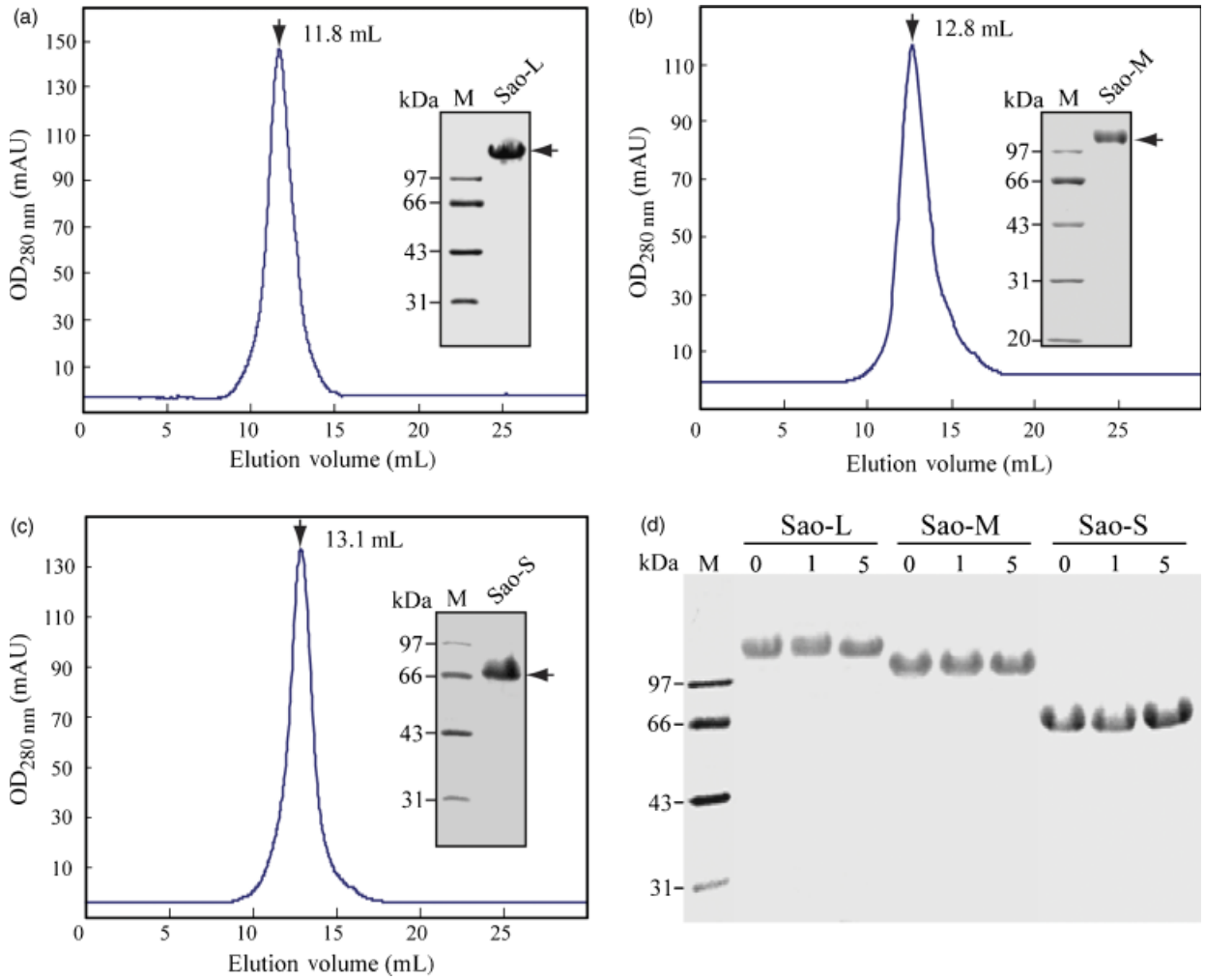

Fig. 3. Biochemical characterization of Sao proteins. (a), (b), and (c) FPLC profiles of three types of Sao proteins (Sao-L, Sao-M, and Sao-S). They are run on a Superdex 200 column, and the arrows indicate their respective elution volumes. (d) Chemical cross-linking of Sao proteins. 0, 1.0, and 5.0 correspond to the mM concentration of EGS. M: protein MW standard marker (Sangon, Shanghai, China).

in plant pathogenesis, this gene is associated with a hypersensitive response of the plant towards the pathogens (Yang *et al.*, 2000). As such, several questions regarding Sao variants require further investigation. These are (1) is there an analogous host R protein binding to the Avr-like repeats of Sao? (2) Is a type III secretion system involved? (3) Does Sao function as a superantigen similar to what occurs with *Staphylococcus aureus* (Banks *et al.*, 2003) and *Streptococcus pyogenes* (GAS) (Alouf & Muller-Alouf, 2003)?

Sao also shows great promise as a diagnostic antigen. Based on the ELISA results with Sao-M, the protein was found to be very immunogenic and very discriminatory between uninfected and convalescent animals. This should be of great utility for monitoring samples in the laboratory, clinic, and field, which may prevent the further spread of this emerging pathogen.

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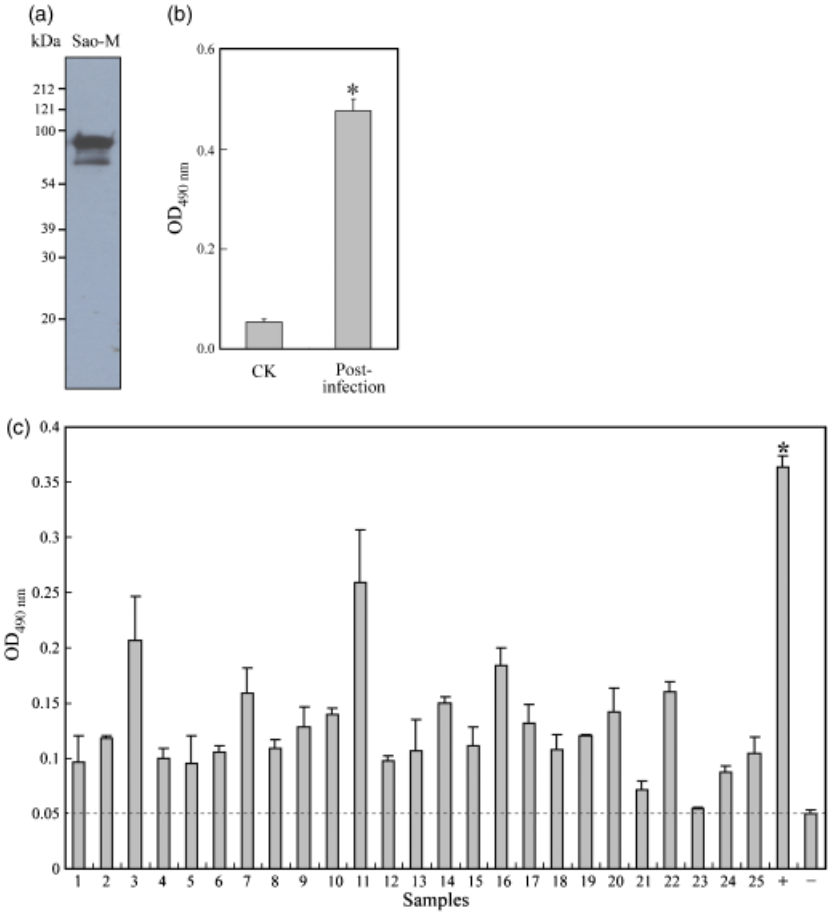

Fig. 4. Development of a Sao-M-based ELISA method for monitoring of *Streptococcus suis* infection. (a) Western blotting of recombinant Sao-M with the convalescent-phase sera cl inically infected by *S. suis* ZY05. (b) ELISA analysis of recombinant Sao-M with negative swine serum and swine serum postinfection. The results are expressed as means of absorbance values with standard errors (SE). $P \leq 0.01$. (c) Sao-based ELISA assay of swine serological samples. +: positive serum confirmed in laboratory. −: negative serum confirmed in laboratory. Samples from 1 to 25 are those sera collected from field pig farms or slaughterhouses. The dashed line is used to indicate the background of the sera samples. The results are expressed as means of absorbance values with SEs. $^{*}P \leq 0.01$.

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Authors' contribution

Y.F., F.Z., and X.P. contributed equally to this work.

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Supplementary material

The following supplementary material is available for this article online:

Table S1. PCR investigation of *sao* distributed in *S. suis*.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1574-6968.2007.00859.x (This link will take you to the article abstract).

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