CLINICAL AND VACCINE IMMUNOLOGY, Aug. 2007, p. 937–943 Vol. 14, No. 8
1556-6811/07/$08.00+0 doi:10.1128/CVI.00046-07

Immunization with Recombinant Sao Protein Confers Protection against *Streptococcus suis* Infection▽

Yuanyi Li,[1] Marcelo Gottschalk,[1] Miriam Esgleas,[1] Sonia Lacouture,[1] J. Daniel Dubreuil,[1] Philip Willson,[2] and Josee Harel[1]*

Groupe de Recherche sur les Maladies Infectieuses du Porc, Faculté de Médecine Vétérinaire, Université de Montréal, C.P. 5000, St.-Hyacinthe, Québec J2S 7C6, Canada,[1] and Vaccine and Infectious Disease Organization, University of Saskatchewan, 120 Veterinary Road, Saskatoon, Saskatchewan S7N 5E3, Canada[2]

Received 23 January 2007/Returned for modification 22 March 2007/Accepted 25 May 2007

Sao is a *Streptococcus suis* surface protein recently identified as a potential vaccine candidate. In this study, recombinant Sao in combination with Quil A provided cross-protection against *S. suis* serotype 2 disease in mouse and pig vaccination protocols. Subcutaneous immunization of mice elicited strong immunoglobulin G (IgG) antibody responses. All four IgG subclasses were induced, with the IgG2a titer being the highest, followed by those of IgG1, IgG2b, and IgG3. Challenge of the mice with *S. suis* strain 31533 resulted in a mortality rate of 80% for the control group, which received Quil A only. In contrast, all of the mice immunized with Sao survived. In a pig vaccination protocol, intramuscular immunization with Sao also elicited significant humoral antibody responses, and both the IgG1 and IgG2 subclasses were induced, with a predominance of IgG2 production. In vitro assay showed that Sao-induced antibodies significantly promoted the ability of porcine neutrophils in opsonophagocytic killing of *S. suis*. An aerosol challenge of the pigs with *S. suis* strain 166 resulted in clinical signs characteristic of *S. suis* infection in diseased pigs. The vaccine group showed significantly better survival, lower clinical scores, and less *S. suis* recovery from postmortem tissue samples than did the control group. Furthermore, this study also revealed that although challenge *S. suis* strains express Sao size variants, recombinant Sao conferred cross-protection. These data demonstrate that recombinant Sao formulated with Quil A triggers strong opsonizing antibody responses which confer efficient immunity against challenge infection with heterologous *S. suis* type 2.

Streptococcus suis is an important pathogen of swine, causing meningitis, septicemia, arthritis, endocarditis, pneumonia, and substantial economical losses in the swine industry worldwide (17, 22, 46). It is also an important zoonotic agent for humans in contact with diseased pigs or their products, causing life-threatening diseases, as reported for a recent outbreak in China (42). Thirty-five serotypes have been described so far (17). Serotype 2 is the most prevalent type in association with diseases in most countries. The pathogenesis and virulence attributes of *S. suis* are not well defined, and attempts to control the infection are hampered by the lack of an effective vaccine (21).

Different types of vaccines have been developed or are presently under investigation. At present, inactivated commercial autogenous vaccines are used in the field, but results have been inconsistent (19, 34). Furthermore, safety data for autogenous vaccines are lacking, which has liability implications for the use of this type of material (18). Attenuated or avirulent live *S. suis* strains have been tested, and the results were also equivocal (6, 25, 52). In addition to bacterins and live vaccines, a number of purified bacterial components have been developed as vaccine candidates. The capsule polysaccharide is a critical virulence factor of *S. suis*. However, a vaccine based on capsular material was unsatisfactory due to its poor immunogenicity (13). Vaccination strategies using purified suilysin (26) or muramidase-released protein and extracellular protein factor (53) from *S. suis* serotype 2 have been shown to protect pigs from homologous and heterologous serotype 2 strains. However, a substantial number of virulent strains in some geographic regions do not express these proteins (14, 16, 44).

We recently identified a surface protein (Sao) which is highly conserved among *S. suis* species (36). Convalescent-phase swine sera have high titers of antibody against this protein, suggesting that Sao is a potent immunogen that is expressed during *S. suis* infection. These findings made Sao a candidate for use in a subunit vaccine. However, in a convenient test, immunization of piglets with recombinant Sao mixed with the oil-in-water Emulsigen reagent triggered a predominant production of immunoglobulin G1 (IgG1), and these antibodies lacked opsonophagocytic function and did not confer protection (36). This suggested that the quality of the type 1/type 2 immune response bias was inappropriate to mediate protection against *S. suis*. It is known that host protection against infection caused by *S. suis*, a highly encapsulated microorganism, is mediated primarily by opsonophagocytosis, which is mainly associated with a Th1-type immune response characterized by IgG2a production (5, 17). The vaccine formulation and components, such as adjuvants, can dramatically influence the vaccine-induced antibody response, including bias to type 1 or type 2 responses, which may have a significant effect on the protective efficacy of a vaccine (1, 30, 43). Evidence from vaccination using surface antigens of other gram-positive bacteria indicated that the efficiency of antibody-mediated op-

* Corresponding author. Mailing address: Groupe de Recherche sur les Maladies Infectieuses du Porc, Faculté de Médecine Vétérinaire, Université de Montréal, C.P. 5000, St.-Hyacinthe, Québec J2S 7C6, Canada. Phone: (450) 773-8521, ext. 18233. Fax: (450) 778-8108. E-mail: josee.harel@umontreal.ca.

▽ Published ahead of print on 13 June 2007.

sonophagocytosis and protection can be improved dramatically by using Th1-directing adjuvants to promote a Th1-type immune response (2, 35). We therefore hypothesized that Sao may be protective in a vaccination protocol involving an optimal adjuvant and higher antigen dosage. In this study, the efficacy of recombinant Sao in combination with Quil A was demonstrated by protection against *S. suis* infection and disease in mice as well as pigs, the target species of this vaccine candidate.

MATERIALS AND METHODS

Bacterial strains. Three *S. suis* strains of serotype 2 were used in this study. Strain S735 was used to clone the *sao* gene and to produce the protein (36). This strain is the reference strain and was originally isolated from a diseased pig in The Netherlands (27). Strains 166 and 31533 originated from pigs with meningitis (4, 32). Strain 31533 was chosen for challenging mice, and strain 166 was used for challenging pigs. Bacteria were grown on plates made with Todd-Hewitt broth (Difco, Detroit, MI) containing 2% agar or in liquid cultures of Todd-Hewitt broth.

Immunization and challenge of mice. Recombinant Sao was produced and purified as previously described (36). Six-week-old female CD-1 mice were randomly assigned (according to body weight) to two groups of 10 mice and immunized subcutaneously twice at a 2-week interval with either 20 μg of purified Sao mixed with 20 μg of Quil A (Brenntag Biosector, Frederikssund, Denmark) adjuvant or 20 μg of Quil A only as a control in 100 μl of phosphate-buffered saline (PBS) per mouse. Ten days after the second vaccination, the animals were challenged intraperitoneally with 1×10^8 CFU per mouse of log-phase *S. suis* strain 31533 in 1 ml of Todd-Hewitt broth. This challenge model was confirmed to reproduce septic shock and meningitis similar to those induced by *S. suis* in pigs (unpublished data). Mice were monitored daily for clinical signs, such as abnormal behavior, rough hair coat, ataxia, and mortality, until day 14 after the infection. Blood samples were collected prior to each vaccination and the challenge for determining antibody responses. Guidelines from the *Guide to the Care and Use of Experimental Animals* of the Canadian Council on Animal Care were followed during the experiment, which followed a protocol that had been approved by the University of Montreal Committee on Animal Care.

Immunization and challenge of pigs. Pigs were used to perform the immunization and protection experiment at the Vaccine and Infectious Disease Organization (University of Saskatchewan, Saskatoon, Canada) in accordance with principles outlined in the *Guide to the Care and Use of Experimental Animals* of the Canadian Council on Animal Care, using a protocol that was approved by the University Committee on Animal Care. Four-week-old piglets with an average weight of 7.79 kg from a herd that is free of *S. suis* serotype 2 were randomly assigned to two groups of 12 piglets each. Animals were injected intramuscularly twice at a 2-week interval with 1 ml of either 200 μg purified Sao mixed with 400 μg Quil A or 400 μg Quil A only in physiological saline as a control. Two weeks after the second injection, the immunized and control animals were challenged by aerosol with 1 ml (6.8×10^6 CFU) of a log-phase culture of *S. suis* strain 166 as previously described (38). Blood samples were collected prior to each injection and challenge for determination of antibody responses. Pigs were monitored for body temperature, clinical signs, and mortality for 10 consecutive days after challenge. A daily clinical score (from 0 to 8) was derived as the sum of attitude and locomotion scores for each pig, based upon signs of nervous, musculoskeletal, or respiratory disease. Attitude scores were given as follows: 0 = normal attitude and response to stimuli; 1 = inactive and slow to respond, with oculonasal secretions; 2 = only responsive to repeated stimuli; 3 = recumbent, nonresponsive, and unaware of surroundings; and 4 = dead. Locomotion scores were given as follows: 0 = normal gait and posture; 1 = slight incoordination, lameness, and/or joint swelling but rises without assistance; 2 = clearly uncoordinated or lame but stands without assistance; 3 = severe lameness and/or severe ataxia; and 4 = dead. Pigs having a clinical score of >2 on either scale were euthanized by lethal injection. A postmortem examination procedure was conducted for all pigs. Brain, tracheobronchial lymph node, and grossly affected joint samples from all pigs and blood samples from euthanized pigs were cultured for bacterial recovery.

ELISA. Titers of Sao-specific total IgG and IgG subclasses in mouse and swine sera were determined by enzyme-linked immunosorbent assay (ELISA) as previously described (36). Briefly, Polysorb immunoplates (Nunc, Rochester, NY) were coated overnight at 4°C with 100 μl per well of purified recombinant Sao at a concentration of 0.3 μg/ml in carbonate buffer. The plates were incubated with serial dilutions of test sera in PBS containing 0.05% Tween 20 for 1.5 h at room temperature. For determination of antibodies in mice, bound antibodies were detected by incubation with peroxidase-conjugated goat anti-mouse IgG, IgG1, IgG2a, IgG2b, or IgG3 antisera (Serotec, Kidlington, Oxford, United Kingdom) for 1 h at room temperature. For determination of swine total IgG, bound antibodies were detected by incubation with peroxidase-conjugated goat anti-swine IgG (heavy plus light chains) antisera (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 h at room temperature. For swine IgG1 and IgG2 detection, mouse anti-porcine IgG1 or IgG2 (Serotec) was used as the primary antibody, and peroxidase-conjugated goat anti-mouse IgG (heavy plus light chains) (Serotec) was used as the secondary antibody. The plates were developed with TMB substrate (Zymed, South San Francisco, CA). Absorbance was measured at 450 nm in an ELISA reader (Power Wave 340; Bio-Tek Instruments, Inc., Winooski, VT). The serum dilution that resulted in an optical density at 450 nm of 0.1 after background subtraction was considered the titer of that serum.

Opsonophagocytosis assay. To investigate the role of Sao-specific antibody in protection, total IgG was purified from serum pools of control or Sao-vaccinated pigs after the second immunization by using a protein A column (Pharmacia, Uppsala, Sweden). Porcine neutrophils were isolated from pigs that belonged to a high-health-status herd. Complete normal serum from the healthy pig was used as a source of complement. An opsonophagocytosis assay was then performed as previously described (7). Briefly, *S. suis* strain 166 was suspended in complete normal porcine serum containing 25 μg/ml of purified IgG from either Sao-vaccinated pigs or control pigs and preopsonized for 30 min at 37°C. Neutrophils at a concentration of 5×10^6 cells/ml were mixed with 1×10^4 CFU/ml of bacteria in microtubes and incubated for 90 min at 37°C with 5% CO_2. The neutrophil cells were lysed with sterile water, and viable bacterial counts were performed on Todd-Hewitt agar plates. Tubes with bacteria alone were treated similarly and used as controls. The tests were performed eight times. Results are expressed as percentages of killed bacteria.

Western blotting. Fifty microliters of *S. suis* culture supernatant was separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in an 8% acrylamide gel. Proteins transferred to a nitrocellulose membrane (Bio-Rad, Mississauga, Ontario, Canada) were detected by incubation overnight at 4°C with a 1/200 dilution of pooled sera from the mice which received two doses of Sao or from the control animals in Tris-buffered saline–0.05% Tween 20 containing 5% skim milk. Sao-specific antibody was detected with peroxidase-conjugated goat anti-mouse IgG (Serotec) and visualized using 4-chloro-1-naphthol (Sigma, St. Louis, MO) as the substrate.

PCR and sequences. The *S. suis* cell pellet from a 5-ml overnight culture was suspended in 1 ml of lysis buffer (50 mM Tris-HCl, 5 mM EDTA, 3% SDS, 1 mg/ml RNase, pH 8.5) and transferred to a 2-ml screw-cap tube containing 0.5 g of 0.1-mm glass beads (BioSpec, OK). The suspension was homogenized for 3 min at maximum speed using a mini-bead beater (BioSpec). The sample was centrifuged for 3 min at $16{,}000 \times g$ in a microcentrifuge, and then the supernatant was used to extract the genomic DNA following standard procedure. The complete *sao* gene was amplified from the genomic DNA by using the pS1F (5′-ATGAATACTAAGAAATGG-3′) and pS1R (5′-AATTTACGTTTACGTG TA-3′) primer pair, and the DNA fragment flanking the repeating region in *sao* was amplified using the pS2F (5′-GAAATATCGAACCCCCTAAAG-3′) and pS2R (5′-CTTCGACTGTACCATTTTGGT-3′) primer pair. The PCR was performed for 5 min at 94°C, followed by 30 cycles of 1 min at 94°C, 30 s at 46°C, and 1 min at 72°C in a thermal cycler (Eppendorf Scientific Inc., Hamburg, Germany). The amplicons were analyzed in a 0.8% agarose gel and sequenced using the same primers.

Statistics. Comparison between antibody titers and percentages of killed bacteria was done using the *t* test. The clinical scores were transferred by ranking, and the significance of the difference between groups was determined by the *t* test. Survival distributions were evaluated with chi-square analysis using the Kaplan-Meier method, and the significance of the difference was tested using the log rank test. The Fisher exact test was applied to compare the proportions of postmortem tissues from which *S. suis* was recovered. A P value of <0.05 was taken as significant.

RESULTS

Sao-specific IgG and IgG subclasses. Subcutaneous immunization of mice with Sao in combination with Quil A elicited a significant humoral IgG response after primary immunization. The second immunization boosted the specific antibody

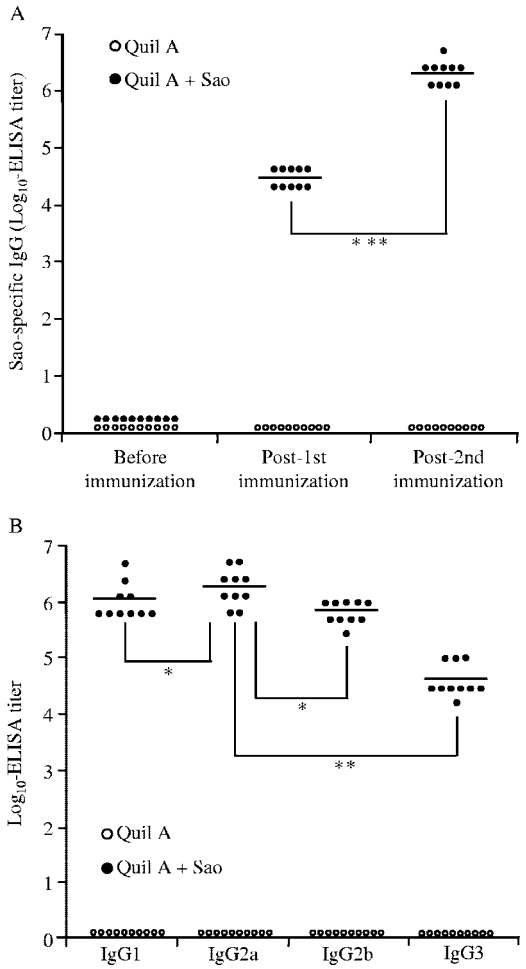

FIG. 1. Serum antibody responses in mice immunized with Quil A (open circles) or Quil A plus recombinant Sao (solid circles). (A) Total Sao-specific serum IgG. (B) IgG subclasses in sera 9 days after the second immunization. Antibody titers for individual mice are shown, with the average titer ($n = 10$) represented as a bar. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

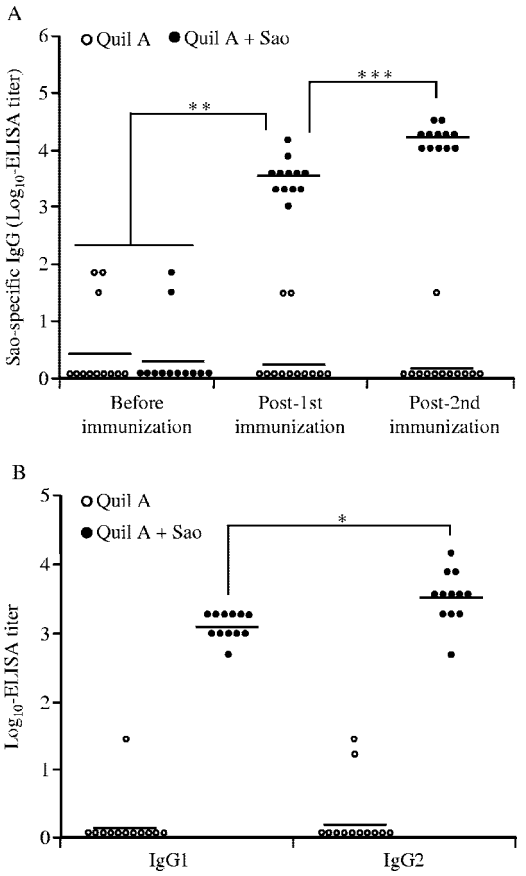

FIG. 2. Serum antibody responses in pigs immunized with Quil A (open circles) or Quil A plus recombinant Sao (solid circles). (A) Total Sao-specific serum IgG. (B) IgG subclasses in sera 13 days after the second immunization. Antibody titers for individual pigs are shown, with the average titer ($n = 12$) represented as a bar. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

response, which was significantly higher than the level after the primary immunization ($P < 0.001$) (Fig. 1A). Furthermore, all four tested IgG subclasses were induced in Sao-immunized mice, with the IgG2a titer being the highest, followed by those of IgG1, IgG2b, and IgG3, as measured in the sera 9 days after the second vaccination. The IgG2a titer was significantly higher than the titers of other IgG subclasses, including IgG1 ($P < 0.05$) (Fig. 1B). In contrast, Sao-specific IgG and its subclasses were below the limit of detection in sera of mice before vaccination and the mice in the control group.

Similar patterns of immune responses were revealed for pigs that received 200 μg of Sao intramuscularly in combination with 400 μg of Quil A. Primary immunization of pigs triggered a strong antigen-specific IgG response. The serum IgG titer was significantly higher than that in control pigs receiving only Quil A or in pigs before immunization ($P < 0.01$). After a second dose of the vaccine, a significant increase in IgG level ($P < 0.001$) was seen in Sao-immunized pigs (Fig. 2A). Assessment of IgG subclasses demonstrated that while both IgG1 and IgG2 subclasses were induced in sera of Sao-immunized animals, the IgG2 response significantly dominated the IgG1 response ($P < 0.05$) (Fig. 2B).

Protection of mice against *S. suis* strain 31533. Sixteen hours after administration of the challenge infection with *S. suis* 31533, all mice in the nonimmunized control group exhibited clinical signs, such as a ruffled hair coat, suggesting fever, and a slow response to stimuli. Beginning about 4 days after the challenge, 8 of 10 mice in this group successively developed severe central nervous system signs, such as running in circles and opisthotonos. All eight of the ill mice died or met criteria for humane euthanasia due to the severity of their condition. In contrast, most of the mice in the Sao-vaccinated group showed only mild and transient rough hair after the challenge, and all mice survived the *S. suis* infection, resulting in complete protection from cause-specific mortality ($P < 0.001$) (Fig. 3).

Protection of pigs against *S. suis* strain 166. One pig in the Sao-immunized group was humanely killed because of an unrelated disease, and this pig was excluded from analysis of the effects of vaccination on disease. Aerosol challenge of the pigs with *S. suis* strain 166 resulted in diseases characteristic of *S. suis* infection. The mean accumulated clinical scores of two groups are presented in Fig. 4A, and clinical signs were signif-

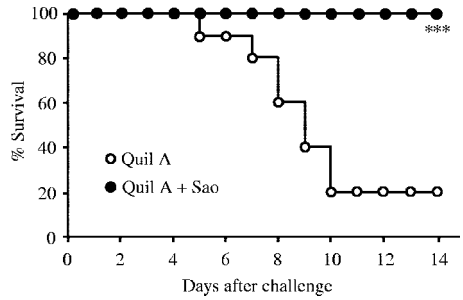

FIG. 3. Survival of mice immunized with Quil A (open circles) or Quil A plus recombinant Sao (solid circles) following challenge with *S. suis* 31533. Each group consists of 10 mice. ***, $P < 0.001$.

TABLE 1. Bacteriological analysis of postmortem samples from pigs immunized with Quil A or Quil A plus recombinant Sao

Sample type	No. of samples with *S. suis* recovery/total no. of samples examined		*P* value[b]
	Quil A	Quil A + Sao	
Blood	4/9[a]	1/11	0.13
Brain	10/12	2/11	0.003**
Lymph node	8/12	6/11	0.68
Joint	4/12	2/11	0.64

[a] Postmortem blood samples could not be obtained from three pigs that died before they were observed.
[b] **, $P < 0.01$.

icantly less severe in the Sao-vaccinated group than in the control group ($P < 0.05$). The body temperature data showed no significant difference between the two groups, although the Sao-vaccinated group tended to have lower temperatures (data not shown). In the control group, three pigs died and four more were euthanized due to high clinical scores prior to the end of the experiment, resulting in a survival rate of 42%. In contrast, only two pigs in the Sao-vaccinated group were euthanized, giving a survival rate of 82%. Comparison of survival curves showed that survival time for the Sao-vaccinated group was significantly longer than that for the control group ($P < 0.05$) (Fig. 4B). Bacterial culture of samples from blood, brain, tracheobronchial lymph nodes, and joints was done to monitor the infection level, and the recovery of bacteria with colonial morphology typical of the challenge strain is summarized in Table 1. Although the number of organs with detectable bacteria for the Sao-vaccinated group was less than that for the control group, only the proportion of positive brain tissue samples from immunized pigs was significantly lower than that from control pigs ($P < 0.01$) (Table 1).

Functional activity of Sao-induced antibodies. To determine the nature of protection, serum antibodies obtained from Quil A-plus-Sao-vaccinated pigs were compared with antibodies from pigs that received Quil A only for the ability to promote opsonization phagocytosis and killing of *S. suis* 166 by porcine neutrophils in vitro. As shown in Fig. 5, antibodies from Sao-vaccinated pigs mediated significantly more efficient opsonophagocytic killing of *S. suis* than antibodies from pigs that received adjuvant alone ($P < 0.001$).

Immune recognition of Sao protein and its variants. The specificity of Sao-induced antibodies was demonstrated by Western blotting with sera pooled from Sao-immunized mice, which recognized the Sao protein of wild-type *S. suis* strains as well as recombinant Sao (Fig. 6). In contrast, sera from non-immunized control mice did not react with the proteins (data not shown). However, size variation of Sao was noted among

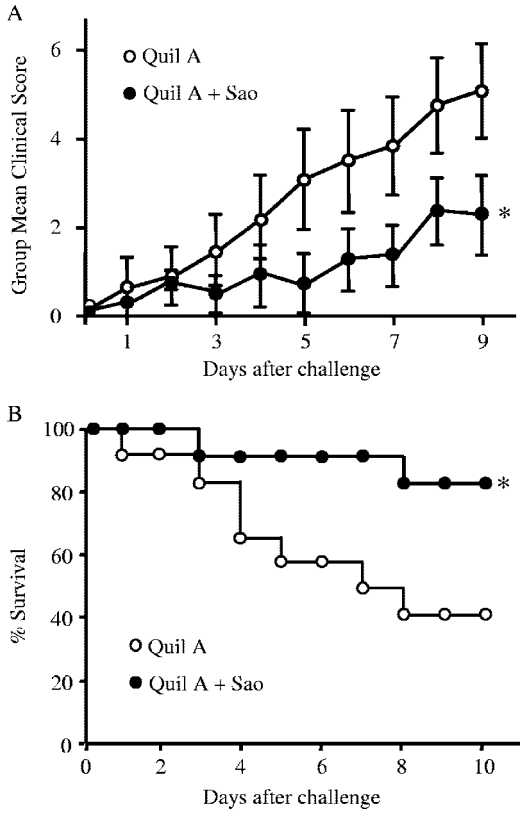

FIG. 4. Protection of pigs immunized with Quil A (open circles) or Quil A plus recombinant Sao (solid circles) following challenge with *S. suis* 166. (A) Clinical scores (daily means and standard deviations) of pigs after challenge. (B) Survival of pigs after challenge. The data are reported for 11 pigs in the Sao-vaccinated group and 12 pigs in the control group. *, $P < 0.05$.

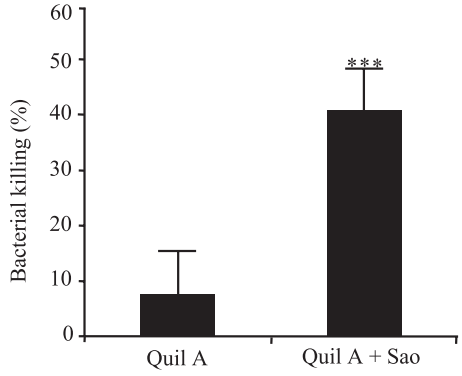

FIG. 5. Effect of antibodies on opsonophagocytic killing of *S. suis* by porcine neutrophils. The antibodies were purified from pooled sera of pigs immunized with Quil A or Quil A plus Sao. Data are expressed as mean percentages ± standard deviations of killed bacteria and are representative of eight independent experiments. ***, $P < 0.001$.

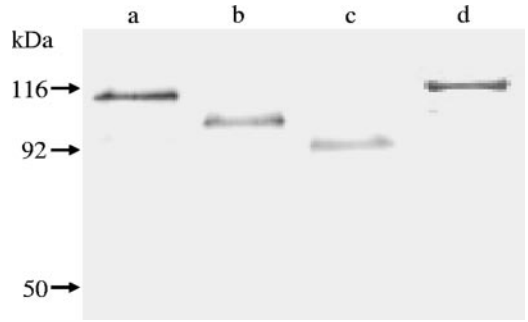

FIG. 6. Western blot showing variation in Sao protein of *S. suis*. Culture supernatants of *S. suis* strain S735 (lane a), strain 166 (lane b), and strain 31533 (lane c) and the purified recombinant Sao protein that was used for immunization (lane d) were separated by SDS-polyacrylamide gel electrophoresis and then transferred to a membrane. The blot was incubated with sera pooled from mice after the second immunization with recombinant Sao. The molecular masses are indicated on the left.

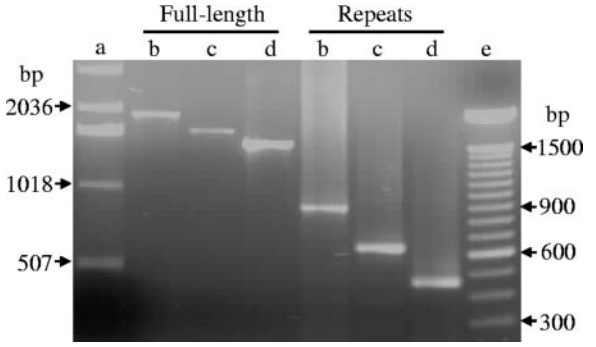

FIG. 7. PCR amplification products of the full-length *sao* gene of *S. suis* and the DNA fragments flanking the repeats of *sao*. The variations of complete *sao* were correspondingly revealed in the differences in the DNA fragments spanning the repeats. Lanes: a, 1-kb DNA ladder (Life Technologies); b, strain S735; c, strain 166; d, strain 31533; and e, 100-bp DNA ladder (Invitrogen).

the *S. suis* strains. While *S. suis* strain S735, from which the Sao protein antigen was produced, expressed an apparent 110-kDa Sao protein similar in size to the antigen, strains 166 and 31533 expressed Sao variants of approximately 100 kDa and 93 kDa, respectively (Fig. 6).

Genetic analysis of Sao variants. The genes encoding Sao variants were analyzed by PCR. As shown in Fig. 7, the size of *sao* varied among *S. suis* strains S735, 166, and 31533 and corresponded to the size of the Sao variants observed in the Western blot. One of the features of the Sao protein is the presence of 10 repeats of a 27-amino-acid sequence separated by 3-amino-acid spacers near the C-terminal end (36). Analysis of the PCR fragments spanning the repeating region of *sao* suggested that a variable number of repeats accounted for the size differences of *sao* (Fig. 7). Indeed, DNA sequence analysis revealed a deletion of a 270-bp nucleotide sequence, corresponding to three repeats plus the spacers, in strain 166 and a deletion of a 450-bp sequence, corresponding to five repeats plus the spacers, in strain 31533 compared with the sequence of *sao* in strain S735.

DISCUSSION

In our continued effort to search for an *S. suis* protein(s) useful in the development of a vaccine, a highly immunogenic surface protein, Sao, was identified from a virulent strain (S735) of *S. suis* serotype 2. In a convenient pig immunization protocol, Sao in combination with Emulsigen triggered a predominant production of IgG1 that did not confer protection against *S. suis* challenge infection (36). In this study, we first used a mouse model to test the protective efficacy of recombinant Sao in combination with Quil A and found that the experimental vaccine induced a predominant generation of IgG2a which confers protection against *S. suis* infection. This prompted us to further evaluate the protective capacity of Sao in combination with Quil A in pigs, the target host of this potential vaccine. Indeed, it significantly protected the pigs against *S. suis* infection and disease.

Induction of the appropriate type of antigen-specific immune responses is crucial for the success of vaccines. The IgG subclass produced as a consequence of immunization reflects the type of immune responses. In mice, serum IgG1 is associated with a Th2-type response, whereas serum IgG2a is associated with a Th1-type response, which is particularly effective at mediating bacterial opsonophagocytosis (48). Of the mouse IgG subclasses, IgG2a is the most effective at binding to FcγRI on phagocytic cells (41, 48). Thus, it is likely that predominant IgG2a production in the current mouse vaccination protocol contributed most to the observed protection. However, it is also possible that IgG2a is not the only effector of protection induced by vaccination with Sao. Some studies have shown that IgG2b and IgG3 are also associated with Th1-type immune responses and are critically involved in bacterial opsonophagocytosis and protection against infection with gram-positive pathogens (9, 31, 40, 47). In this pig immunization and challenge protocol, the Sao-induced immune response was characterized by predominant IgG2 production. Although the concept of "Th1/Th2" balance is not yet well documented for pigs, recent evidence showed that porcine IgG2 had greater complement-activating ability than did IgG1 (11).

Adjuvants play an important role in the efficacy of vaccines. The type of adjuvant used can direct the type of immune response generated to an administered antigen (43). It has been shown previously that an appropriate adjuvant is essential in determining the outcome of vaccination and that protection following vaccination is obtained only after switching immune responses to a predominantly Th1 type, such as the case with vaccines against *Streptococcus pneumoniae* (2, 39), *Mycobacterium tuberculosis* (37), *Chlamydia pneumoniae* (3), and *Brucella abortus* (20). The adjuvant Quil A has been shown to enhance antibody levels and, more importantly, to shift the response towards type 1, thus resulting in the induction of both bactericidal and opsonophagocytic antibodies (12, 29, 30, 50). In our modified pig protocol, it appears that Sao combined with Quil A triggers an adequate immune response bias which consequently leads to protection. To determine the nature of protection, Sao-induced antibodies were analyzed for the ability to promote opsonophagocytic killing of *S. suis* in the presence of white blood cells, an important immunological correlate of protective immunity against *S. suis* (7). We found that antibodies purified from the sera of pigs that received two doses of Sao

vaccine in combination with Quil A exhibited strong opsonic capacity. Given our previous study showing that Sao combined with Emulsigen triggered a predominant production of IgG1 and that these antibodies lacked opsonophagocytic function (36), this result indicated that Sao in the present formulation may more adequately induce protective antibodies that are capable of triggering leukocyte effector. The enhanced level of opsonizing antibodies is likely related to the predominant generation of IgG2, directed by using Quil A adjuvant. However, it should be emphasized that the type of immune response induced could also be affected by the antigen dose (10, 15, 51). In contrast with our previous study, in which 100 μg of Sao/per pig was used, a dosage of 200 μg was applied in this trial. Although the exact factor(s) crucial in directing the immune response toward the adequate bias was not defined, this study did provide the basis of a suitable formulation for further clinical evaluation of the Sao protein as a vaccine candidate for control of *S. suis* disease in pigs.

S. suis strain S735, from which the *sao* gene was originally cloned, was not used for challenging the animals due to a controversial report about its virulence in experimental infection models (8, 49). We previously confirmed that a Sao-specific antibody raised in rabbits cross-reacted with cell lysates of *S. suis* strain 31533 (unpublished data) and strain 166 (36). Thus, these strains were chosen for challenging animals to investigate the cross-protection of recombinant Sao against heterologous *S. suis* field strains. The sera from the animals immunized with recombinant Sao recognized size variants of Sao expressed by *S. suis* field strains, suggesting that differences in Sao among the *S. suis* strains used in this study do not alter the immune recognition of the recombinant Sao-elicited antibody. One of the features of the Sao protein is the presence of a region of 10 repeats near the C-terminal end (36). Variation of repeat numbers has commonly been observed in bacterial proteins, such as EF of *S. suis* (45), the M protein of *Streptococcus pyogenes* (24, 28), and the alpha-like protein of group B streptococcus (33). Therefore, we assumed that the size difference of Sao occurred due to variation in the number of repeats. This was confirmed by DNA sequencing. It has been proposed that the size variation of gram-positive bacterial proteins, such as the M protein, is a mechanism by which organisms can escape from the host immune system (23, 24, 28). However, our study showed that Sao-specific antibody cross-reacted with Sao variants, and moreover, the Sao vaccine offers cross-protection against *S. suis* strains expressing Sao variants. This discrepancy may result from the structural difference between Sao and the M protein. In the M protein, the highly variable repeat region is present in the N-terminal half and the highly conserved region is present in the C-terminal half (27). Since the M protein is a C-terminally anchored protein, the N terminus extends outwards from the cell and epitopes close to the C terminus may be masked by other cell wall components. As a result, variation in the N terminus alters the ability of certain antibodies, originally produced in response to the parent protein, to bind to the mutant molecules or opsonize the mutant organisms (27). In contrast to the case for the M protein, the variable repeat region in Sao is located in the C-terminal half and the conserved region is located in the N-terminal half (36). Therefore, deletion of some repeats does not render them inaccessible to antibody binding.

In summary, we have shown that recombinant Sao in a vaccine formulation with Quil A triggers strong opsonizing antibody responses which confer protection against experimental *S. suis* infection. In addition, Sao protects against challenging strains expressing Sao size variants. These findings suggest that Sao is a potential candidate for development of a subunit vaccine against *S. suis* infection. However, an optimum vaccine formulation remains to be studied.

ACKNOWLEDGMENTS

We thank Mariela Segura (McGill University) for scientific exchanges and Marylene Kobisch (AFSSA, Ploufragan, France) for providing strains 31533 and 166.

This work was supported by Valorisation Recherche Quebec (VRQ 2201-141) and the NSERC Canadian Research Network on Bacterial Pathogens of Swine (225155-00).

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