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Streptococcus suis Bacterin and Subunit Vaccine Immunogenicities and Protective Efficacies against Serotypes 2 and 9▽†

Christoph Georg Baums,[1]* Christoph Kock,[1] Andreas Beineke,[2] Katharina Bennecke,[1] Ralph Goethe,[1] Charlotte Schröder,[3] Karl-Heinz Waldmann,[3] and Peter Valentin-Weigand[1]

Institut für Mikrobiologie, Zentrum für Infektionsmedizin, Stiftung Tierärztliche Hochschule Hannover, D-30173 Hannover, Germany[1]; Institut für Pathologie, Stiftung Tierärztliche Hochschule Hannover, D-30173 Hannover, Germany[2]; and Klinik für Kleine Klauentiere und Forensische Medizin und Ambulatorische Klinik, Stiftung Tierärztliche Hochschule Hannover, D-30173 Hannover, Germany[3]

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Streptococcus suis **causes numerous diseases in pigs, most importantly, meningitis, arthritis, septicemia, and bronchopneumonia. One of the major problems in modern swine production is the lack of a vaccine protecting against more than one *S. suis* serotype. The objective of this study was to determine the protective efficacy of a serotype 2 murein-associated protein (MAP) fraction subunit vaccine in comparison to that of a bacterin against experimental challenge with serotype 2 (containing muramidase-released protein [MRP], extracellular factor, and suilysin [SLY]) and serotype 9 (containing MRP variant MRP* and SLY) strains. MAP was shown to include different surface-associated proteins, such as the MRP and surface antigen one (SAO) expressed by both pathotypes used for challenge. The results of this study demonstrated that the serotype 2 bacterin induced protective immunity against homologous challenge. In contrast, the protective efficacy of the MAP subunit vaccine was low, though MAP immunization resulted in high serum immunoglobulin G2 titers against MRP and SAO. Importantly, immunization with bacterin but not with MAP induced opsonizing antibody titers against the serotype 2 strain, and these antibody titers were found to correlate with protection. However, after absorption with a nonencapsulated isogenic mutant, the sera from bacterin-immunized piglets failed to facilitate neutrophil killing, indicating that antibodies directed against capsule may not have been essential for opsonophagocytosis. Furthermore, induction of opsonizing antibodies against serotype 9 was not detectable in the group receiving bacterin or in the group receiving the MAP vaccine. In agreement, protection against the heterologous serotype 9 strain was low in both groups. Thus, identification of an antigen protecting against these two important *S. suis* pathotypes remains an important goal of future studies.**

Streptococcus suis ranks among the five most important health challenges of pigs worldwide (11, 12). It is associated with numerous diseases, such as meningitis, arthritis, serositis, and bronchopneumonia. *S. suis* isolates from diseased animals express a polysaccharide capsule which confers resistance to phagocytosis, as demonstrated for serotype 2 strains (21). Strains of various serotypes have been isolated from affected tissues. In Europe, serotype 2 and 9 strains are the most prevalent types isolated from infections. The 136-kDa muramidase-released protein (MRP) and the 110-kDa extracellular factor (EF) are virulence-associated factors expressed only by virulent serotype 1 and 2 strains (22, 23). The majority of invasive serotype 9 isolates express a larger variant of MRP, termed MRP*, which shares high homology with the 136-kDa MRP protein of serotype 2 strains (20, 23).

A number of *S. suis* proteins have been investigated as vaccine candidates. Wisselink et al. demonstrated that in comparison to immunization with a bacterin, immunization with MRP alone conferred little protection against challenge with serotype 2 strains (24). Combining MRP with EF substantially improved protective efficacy. However, many invasive isolates, including all serotype 9 strains, do not express EF. Furthermore, immunization with a different cell wall-associated protein, surface antigen one (SAO), elicited protective immunity against homologous challenge (16). Jacobs et al. used the hemolysin suilysin (SLY) for immunization of piglets (13). Their results suggested that SLY might be a protective antigen. Importantly, challenge experiments with different serotypes in pigs have not been described for any of these candidates.

The objective of this work was to evaluate the protective efficacy of a subunit vaccine based on murein-associated proteins (MAP) in comparison to a bacterin. The subunit vaccine included major surface-associated immunogens, such as SAO and MRP, expressed by both pathotypes used for challenge. Therefore, MAP was regarded as a promising candidate for induction of cross-protection against these invasive serotype 2 and 9 strains, which are responsible for major economic losses in Europe.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. suis* strain 10 is an MRP$^+$ EF$^+$ SLY$^+$ serotype 2 strain which has been shown to be highly virulent in experimental infections of piglets (2, 21). The isogenic mutant strain 10cpsΔEF is deficient in capsule production and attenuated in virulence (21). A3286/94 is an MRP* SLY$^+$ serotype 9 *S. suis* strain of sequence type 99, which was originally isolated from a pig with meningitis (18, 20). Intranasal experimental infections of growers revealed that A3286/94 is only moderately virulent in comparison to the highly virulent strain 10 (3). *S. suis* was cultured as described previously (2).

* Corresponding author. Mailing address: Stiftung Tierärztliche Hochschule Hannover, Zentrum für Infektionsmedizin, Institut fuer Mikrobiologie, Bischofsholer Damm 15, D-30173 Hannover, Germany. Phone: 49-511 856-7563. Fax: 49-511 856-7697. E-mail: christoph.baums@gmx.de.

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TABLE 1. Evaluation of protection induced by a bacterin and a subunit vaccine in serotype 2 and 9 challenge experiments of growers[a]

S. suis challenge serotype[b] and immunization antigen	No. of piglets	*S. suis* challenge		Morbidity[c]	Mortality[c]	No. of piglets with clinical symptom(s)			No. of piglets with condition/total no. of piglets[d]			
									Max. body temp:		Max. WBC:	
		Application[e]	CFU			CNS[f]	Lameness	Unspecific	<40°C	≥40.5°C	<22 × 10^9/liter	≥22 × 10^9/liter
2												
Bacterin	7	i.n.	10^9	2/7	2/7	1/7	1/7	0/7	5/7	2/7	2/7	5/7
MAP	8	i.n.	10^9	6/8	6/8	2/8	2/8	2/8	3/8	5/8	1/8	7/8
Placebo	8	i.n.	10^9	8/8	7/8	2/8	0/8	6/8	0/8	8/8	1/8	7/8
9												
Bacterin	6	i.v.	10^8	3/6	3/6	1/6	2/6	0/6	3/6	3/6	1/6	5/6
MAP	6	i.v.	10^8	5/6	5/6	0/6	5/6	0/6	3/6	3/6	2/6	4/6
Placebo	6	i.v.	10^8	6/6	5/6	2/6	2/6	2/6	1/6	5/6	3/6	3/6

[a] German landrace piglets from a herd free of *sly*$^+$ *epf*$^+$ *mrp*$^+$ *cps2* and *cps9 S. suis* strains.
[b] The highly virulent serotype 2 strain 10 (*mrp*$^+$ *epf*$^+$ *sly*$^+$ *cps2*) and the moderately virulent serotype 9 strain A3286/94 (*mrp** *sly*$^+$ *cps9*) were used in homologous and heterologous challenge experiments, respectively.
[c] No. of piglets affected/total no. of piglets.
[d] Max., maximum. White blood cell (WBC) counts were performed on days 2, 4, 6, and 10 postinfection. All piglets had WBCs below 20 × 10^9/liter preinfection.
[e] i.n., intranasal; i.v., intravenous.
[f] CNS, central nervous system.

Preparation of the MAP subunit vaccine. In this study, an *S. suis* subunit vaccine which consisted of MAP was generated. For the preparation of the MAP fraction, an *S. suis* strain 10 culture (100 ml) was grown to an optical density at 600 nm of 0.3 and subsequently incubated at 42°C for two hours. A temperature shift to 42°C was performed to mimic the increase in body temperature associated with *S. suis* infection in piglets. The bacteria were centrifuged and resuspended in 10 ml of buffer containing 30 mM Tris-HCl (pH 7.5), 25% (wt/vol) sucrose, 0.01 M NaEDTA, and 0.2 mg/ml lysozyme. After incubation at 37°C for 45 min, the resulting protoplasts were centrifuged (15 min at 9,270 × *g* and 4°C). The supernatant was recovered, and MAP were precipitated in 10% trichloroacetic acid (vol/vol). The pellet was washed twice with 80% (vol/vol) acetone and subsequently resuspended in 500 μl of phosphate-buffered saline (PBS). The MAP subunit vaccine contained final concentrations of 0.2 mg/ml MAP, 20% (vol/vol) Emulsigen as an adjuvant, and, for comparison with the bacterin, 0.04% (vol/vol) formaldehyde and 40% (vol/vol) Todd-Hewitt broth (THB; Oxoid, Wesel, Germany).

Preparation of the bacterin. The bacterin was generated with *S. suis* strain 10 grown overnight in THB at 37°C and inactivated in 0.1% formaldehyde. Emulsigen was added as an adjuvant (20% [vol/vol]). For comparison with the MAP subunit vaccine, 40% (vol/vol) PBS was also included. Each immunization dose contained approximately 10^9 CFU. The mixtures were stirred for 8 h, screened for sterility, and stored at 8°C.

Cloning of *sao*. Standard DNA manipulations were performed as described previously (19). For the expression of recombinant His-tagged SAO (rSAO), the plasmid pQEsao was constructed as follows. The 1,559-bp PCR amplification product generated with *Pfu* polymerase (Promega, Mannheim, Germany) and the primer pair saopostssBamHI (AAAGGATCCATCATCAGCGCAAAAC AAC) and saopraeanchorPstI (ACACTGCAGATTTGCTTAGCAGTTTCG) was cut with BamHI and PstI (New England Biolabs, Frankfurt, Germany) and cloned into pQE30 (Qiagen, Hilden, Germany). Purified plasmid DNA was verified by restriction analysis.

rSAO and rMRP expression, purification, and generation of polyclonal antisera. IPTG (isopropyl-β-D-thiogalactopyranoside)-induced expression of rMRP and rSAO and subsequent purification by Ni^{2+} affinity chromatography under native conditions were carried out as described previously (3). Purified proteins were controlled by Western blot analysis with monoclonal Penta-His antibodies (Qiagen). Polyclonal antiserum against purified rSao was raised in a New Zealand White rabbit (Charles River Laboratories, Sulzfeld, Germany) by three consecutive immunizations with 0.1 mg of purified protein and Freund's incomplete adjuvant.

SDS-PAGE and Western Blot analysis. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with stacking and separation gels of 4% and 8% acrylamide, respectively (14). Silver staining was performed as described previously (17). For Western blot analysis, proteins were transferred to nitrocellulose membranes (Schleicher & Schuell BioScience, Dassel, Germany). MRP, SAO, and FBPS (fibronectin and fibrinogen-binding protein of *S. suis*) (6) were detected with a 1:1,000 dilution of specific polyclonal sera against respective recombinant proteins and an anti-rabbit immunoglobulin G (IgG)-peroxidase-conjugated antiserum (Amersham Biosciences, Freiburg, Germany) (3). The antiserum against FBPS was kindly provided by Astrid de Greeff (Central Vet. Institute of Wageningen, Lelystad, The Netherlands).

Animal experiments. Forty-two German Landrace piglets from a herd known to be free of *sly*$^+$ *mrp*$^+$ *epf*$^+$ *cps2*$^+$ and *sly*$^+$ *mrp** *cps9*$^+$ strains were infected experimentally and cared for in accordance with the principles outlined in the *European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes* (5a) (permit no. 33-42502-06/1063 and 33-42502-06/1093). The piglets were 3 to 4 weeks old on the day of transport to the experimental facility (weaned on the same day). They were randomly divided into the different groups outlined in Table 1. Piglets were injected intramuscularly twice, at days 7 and 18 after weaning, with 1 ml of the MAP subunit vaccine, the bacterin, or the placebo, which consisted of 40% (vol/vol) PBS, 40% (vol/vol) THB, 20% (vol/vol) Emulsigen, and 0.1% formaldehyde. Based on the comparative evaluation of experimental infections with the serotype 2 (strain 10) and the serotype 9 (A3286/94) reference strains, different routes of challenge were chosen (3). The piglets were challenged 2 weeks after the second immunization either intranasally with 1.8 × 10^9 CFU of strain 10 or intravenously with 1 × 10^8 CFU of strain A3286/94. The health status of the animals was monitored every eight hours. In any case in which a piglet exhibited high fever (≥40.5°C), apathy, and anorexia, as well as in any case in which clinical signs of acute polyarthritis or severe meningitis were observed, the animal was euthanized for reasons of animal welfare. All surviving piglets were sacrificed at 10 days postinfection.

Hematologic analysis. Blood samples were collected before experimental infection (0 days postinfection), on days 2, 4, 6, and 10 postinfection, and prior to euthanasia. White blood cells were counted in a hemocytometer chamber. Leukocytes were differentiated on blood smears stained with the classical Wright stain.

Histopathological and bacteriological screening. The histological screenings were carried out and scored with blind experiments as described previously (2). All tissues screened histologically were also investigated bacteriologically through culture- and PCR-based detection of the challenge strains (2, 3).

Isolation of *S. suis* capsular polysaccharides. Preparation of serotype 2 capsular polysaccharides was essentially done as described by Elliott and Tai (7). Briefly, *S. suis* serotype 2 strain 10 was grown in 1 liter of THB at 37°C until late logarithmic growth phase. Bacteria were centrifuged, washed in PBS, and resuspended in 100 ml 0.1 M glycine buffer (pH 9.2) containing 10 mg lysozyme/ml. After bacteria were incubated overnight at 37°C, cellular debris was eliminated by centrifugation and the supernatant was lyophilized. The lyophilization product was dissolved in 20 ml 0.1 M $CaCl_2$. Nucleic acids were removed by precipitation with 25% ethanol. Capsular polysaccharides were then precipitated by increasing the ethanol concentration to 80%. The pellet was dissolved in 20 ml 50 μM Tris-HCl buffer (pH 7.4). Polysaccharides were further purified by RNase, DNase, and proteinase K treatment and subsequent phenol extraction. After extensive dialysis against H_2O, capsular polysaccharides were lyophilized and resuspended in PBS. Serotype 2-specific capsular polysaccharides were verified as high-molecular-weight smears in silver staining and Western blot analyses with

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polyclonal rabbit antisera raised against *S. suis* serotype 2. The total carbohydrate concentration was determined by the phenol-sulfuric acid method (8).

Conjugation of capsular polysaccharides with BSA. Conjugation of serotype 2 capsular polysaccharide with bovine serum albumin (BSA) was essentially performed as described by Lees et al. (15). Forty-two microliters of 100 mg/ml 1-cyano-4-dimethylaminopyridium tetrafluoroborate in acetonitrile (Sigma, Taufkirchen, Germany) was slowly added to 2.8 mg of serotype 2 capsular polysaccharides and mixed for 30 s. After the addition of 42 μl of 0.2 M triethylamine (Sigma), the solution was mixed for another 2 min and 5.6 mg BSA was included. After end-over-end rotation for 3 h at 37°C, the reaction was quenched by the addition of 1 M glycine buffer (pH 9) to a final concentration of 0.1 M glycine and subsequent end-over-end rotation at 4°C overnight. Finally, conjugates were dialyzed against PBS for two days. The presence of BSA-conjugated serotype 2 capsular polysaccharide was verified by the detection of band shifts in Western blot analysis with polyclonal rabbit anti-BSA antiserum (Sigma) in comparison to BSA and purified capsular polysaccharides. Hyperimmune sera against conjugated serotype 2 capsular polysaccarides were raised in four piglets by two consecutive immunizations with 0.25 mg of BSA-conjugated serotype 2 capsular polysaccharide and 10% Emulsigen.

Detection of antibodies against MRP, SAO, MAP, and serotype 2 capsular polysaccharides. MAP-, MRP-, and serotype 2 capsular polysaccharide-specific total serum IgG antibodies and SAO- and MRP-specific serum antibodies of IgG isotypes (IgG1 and IgG2) of immunized piglets were determined by enzyme-linked immunosorbent assay (ELISA). Maxisorp plates (Nunc, Rochester, NY) were coated overnight at 8°C with 5 μg *S. suis* antigen (MAP, rMRP, rSAO, or serotype 2 capsular polysaccharides) or BSA (background measurement) in carbonate buffer. The plates were washed between the different incubation steps three times with PBS containing 0.05% Tween 20 (PBST). The plates were blocked with 5% skim milk in PBST for 2 h at 37°C. Samples were added, and the plates were placed on a rocker platform for 1 h at 37°C. For the detection of total IgG, plates were incubated with a 1:20,000 dilution of peroxidase-conjugated goat anti-swine IgG (heavy plus light chain) antiserum (Dianova, Hamburg, Germany) for 1 h at room temperature. For the detection of IgG1 and IgG2, mouse anti-porcine IgG1 and IgG2 antisera (Serotec, Kidlington, Oxford, United Kingdom) and peroxidase-conjugated goat anti-mouse IgG antiserum (Dianova) were used as primary and secondary antibodies, respectively, following the recommendations of the manufacturer. The plates were developed with 2,2-azino-di-[3-ethylbenzithiazoline sulfonate] (ABTS, Boehringer, Mannheim, Germany) and 0.002% H_2O_2 as the substrate. Absorbance was measured at 405 nm.

All of the samples and the controls were measured in a duplicate series of four (seven for reference serum) twofold dilutions in PBST (starting with 1:200). A convalescent-phase serum obtained from a piglet 20 days after experimental infection with strain 10 was used as a standard. Sera from this animal obtained before experimental infection served as a negative control. For positive controls for the MRP or MAP ELISA, sera from piglets immunized with rMRP or MAP in previous experiments (3, 4) were used, respectively. Optical densities were converted to antibody concentrations through log linear regression analysis after background subtraction. The ELISA units for each sample were defined as the mean of the calculated units for each of the four dilutions of the two series. Data were considered only if they met the following criteria: a deviation of duplicates of <22% (IgG isotype-specific ELISA, <15%), a slope of the linear portion of the reference standard curve between 0.8 and 1.2, a correlation coefficient between 0.9 and 1.0, and controls within established ranges (e.g., 135 to 185 units for IgG1 specific anti-MRP control).

Opsonophagocytosis assay. Opsonophagocytic killing in the presence of 20% (vol/vol) porcine serum was assayed essentially according to the method of Baltimore et al. (1). Briefly, *S. suis* strains 10 and A3286/94 from a logarithmic-phase broth culture were centrifuged and resuspended in PBS to give optical densities at 600 nm of 0.5 and 1.0, respectively. Ten microliters of each of these suspensions was added to 400 μl of a neutrophil suspension (in RPMI) and 100 μl of serum. Neutrophils had been isolated from freshly drawn porcine blood. Aliquots for quantitative platings were sampled immediately and after 1 h of end-over-end rotation at 37°C. For *S. suis* strain 10, a starting inoculum of approximately 2×10^6 CFU was incubated with 2×10^6 neutrophils (multiplicity of infection, 1:1). In the case of strain A3286/94, a higher bacterium/neutrophil ratio of 5.6:1 was used (4.5×10^6 CFU bacteria to 0.8×10^6 neutrophils). Strain 10cpsΔEF was included as a positive control in all opsonophagocytosis assays (performed as described for strain 10). The survival factor represented the ratio of CFU at 1 h to CFU at time zero.

For absorption of sera with *S. suis*, 2 ml of strain 10 or strain 10cpsΔEF overnight cultures were centrifuged. Pelleted bacteria were resuspended in 200 μl of serum and rotated for 30 min at 4°C. After centrifugation for 10 min at 10,000 × *g*, 100 μl of the supernatant was used as absorbed serum in opsonophagocytosis experiments.

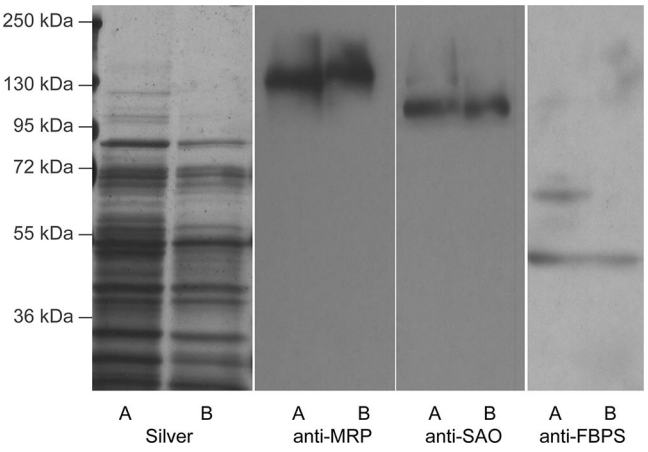

FIG. 1. MAP fraction of *S. suis* serotype 2 strain 10 (A) and serotype 9 strain A3286/94 (B) separated by 8% SDS-PAGE (silver stained, left panel) and analyzed via Western blots probed with anti-rMRP (3), anti-rSAO, or anti-rFBPS antisera as indicated. The sample for strain 10 shown in lanes A was a 1:100 dilution of the MAP fraction used for preparation of the subunit vaccine in the presented study.

Statistical analysis. Statistical analysis with the Mann-Whitney test was performed to analyze differences between the three groups of piglets used in each experiment. The Wilcoxon test was used for comparison of different time point values within the same group. The data in the Kaplan-Meier survival diagrams were analyzed with the log rank test. Probabilities lower than 0.05 were considered significant.

RESULTS

Characterization of a MAP subunit vaccine. MAP fractions of *S. suis* serotype 2 strain 10 and serotype 9 strain A3286/94 were separated by PAGE and analyzed by Western blotting. As demonstrated in Fig. 1, the MAP fractions of both strains showed some similarity in protein band patterns and included MRP/MRP*, SAO, and FBPS. The MAP fraction of *S. suis* strain 10 was used as a subunit vaccine and compared to a bacterin of the same strain in homologous and heterologous challenge experiments.

Challenge of piglets with the homologous serotype 2 strain 10. One piglet in the bacterin-immunized group had to be euthanized prior to challenge because of an unrelated disease, and the data for this piglet were excluded from analysis. High-dose intranasal challenge of piglets with strain 10 resulted in at least two diseased animals in every group. Morbidity and mortality levels for the bacterin-immunized group were significantly lower than those for the placebo group (Table 1 and Fig. 2A). Five of the seven bacterin-immunized piglets showed neither clinical signs of disease nor fibrinosuppurative lesions (Table 2). Elevated numbers of blood leukocytes were, however, temporarily registered in three of these animals (Table 1). In the MAP-immunized piglets, morbidity and mortality levels after serotype 2 challenge were only slightly lower than those for the placebo group (Table 1). Mean times to death were similar for the MAP- and placebo-vaccinated animals (6.1 and 5 days, respectively), but the mean time to death in the bacterin-immunized group was greater (8.9 days) (Fig. 2A).

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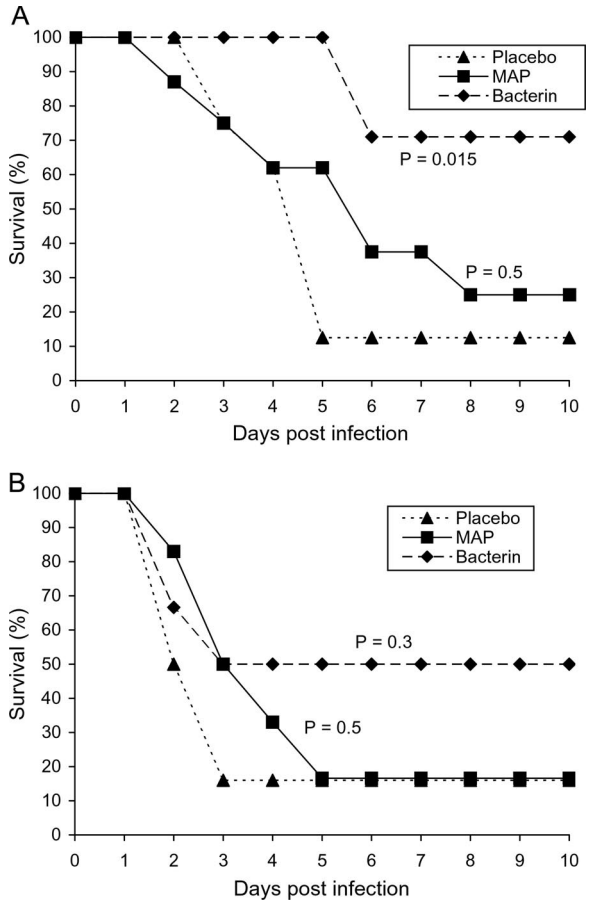

FIG. 2. Kaplan-Meier survival diagrams of piglets challenged with *S. suis* after application of a bacterin, a MAP subunit vaccine, or a placebo on the 7th and 18th days postweaning. Challenge was performed either intranasally with 10^9 CFU of *S. suis* strain 10 (A) or intravenously with 10^8 CFU of MRP* SLY^+ serotype 9 *S. suis* strain A3286/94 (B), both on the 32nd day postweaning. The *P* values of the comparison between the bacterin- and MAP-immunized groups with the placebo group are indicated.

The differences in morbidity and mortality correlated with the results of the histological screening of fibrinosuppurative lesions, which resulted in scores of 1.0, 3.0, and 3.8 for the bacterin-, MAP-, and placebo-immunized groups, respectively (Table 2). The challenge strain was isolated from at least one inner organ of all but one diseased piglet, and the isolation was always associated with fibrinosuppurative inflammations. Isolation of the challenge strain from three or more different tissues was achieved for four of eight animals of the placebo group but none of those from the bacterin-immunized group. For four of five healthy piglets from the bacterin-immunized group, the challenge strain was detected in the tonsils but not in other tissues (Table 3). In conclusion, clinical, pathohistological, and bacteriological screenings demonstrated protective immunity against the homologous serotype 2 strain 10 in the bacterin-vaccinated group but not in the MAP-vaccinated group.

Challenge of piglets with the heterologous serotype 9 strain A3286/94. Intravenous challenge of piglets with strain A3286/94 resulted in at least 50% mortality in each group. Morbidity and mortality levels for the bacterin-immunized group were slightly lower than those for the placebo group (*P* values of 0.0426 and 0.28, respectively), and the morbidities and mortalities of the MAP- and placebo-immunized groups were similar (Table 1). Mean times to death were 6.2, 4.5, and 3.5 days in bacterin-, MAP-, and placebo-vaccinated animals, respectively (Fig. 2B). The pathohistological screening revealed only mild fibrinosuppurative lesions in animals of the bacterin group, whereas three animals of the placebo group and two animals of the MAP-vaccinated group showed moderate (MAP-vaccinated group) or severe (placebo group) fibrinosuppurative synovialitis and/or meningitis. The differences in pathohistological findings resulted in substantially different pathology scores for the three groups (Table 2). However, some pathohistological findings might have not been detected in the bacterin-immunized group, as synovialitis was not diagnosed in two piglets that showed severe lameness prior to euthanasia. Bacteriological screening of A3286/94-infected animals revealed a high detection rate of the challenge

TABLE 2. Scoring of fibrinosuppurative lesions of piglets infected with *S. suis* strain 10 or A3286/94 after immunization with a bacterin or a MAP-based subunit vaccine

S. suis challenge serotype and immunization antigen	No. of piglets	No. of piglets with condition and score[a]/total no. of piglets															ω[c]
		Meningitis and/or chorioiditis			Pleuritis or peritonitis			Synovialitis			Splenitis[b] or hepatitis			Pneumonia			
		5	3	1	4	2	1	4	2	1	4	2	1	4	2	1	
2																	
Bacterin	7	1/7	0/7	0/7	0/7	1/7	0/7	0/7	0/7	0/7	0/7	1/7	1/7	0/7	0/7	0/7	1.0
MAP	8	2/8	0/8	2/8	4/8	1/8	0/8	0/8	0/8	1/8	1/8	3/8	0/8	0/8	3/8	0/8	3.0
Placebo	8	1/8	1/8	3/8	5/8	3/8	0/8	0/8	0/8	0/8	0/8	3/8	2/8	0/8	3/8	0/8	3.8
9																	
Bacterin	6	0/6	0/6	0/6	0/6	2/6	0/6	1/6[d]	0/6	0/6	0/6	1/6	1/6	0/6	0/6	0/6	1.2[e]
MAP	6	0/6	0/6	1/6	0/6	0/6	0/6	3/6[d]	0/6	0/6	0/6	0/6	2/6	1/6	0/6	0/6	2.3[e]
Placebo	6	2/6	0/6	1/6	0/6	0/6	0/6	2/6	0/6	1/6	1/6	2/6	0/6	0/6	0/6	0/6	3.0

[a] Scores of 4 and 5 indicates moderate to severe diffuse or multifocal fibrinosuppurative inflammations. Scores of 2 and 3 indicates mild focal fibrinosuppurative inflammation.
[b] Neutrophilic accumulation of the splenic red pulp.
[c] The sum of the highest scores of each animal for any of the investigated organs was divided by the number of animals ($\omega = \Sigma score_{max}/n_{animals}$) (2).
[d] For one animal of each group, the score of 4 was assigned due to high numbers of neutrophils in the smear of the joint fluid.
[e] Synovialitis was not diagnosed in one animal with acute lameness in the bacterin and in two animals with acute lameness in the MAP-vaccinated group.

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TABLE 3. Reisolation of the challenge strain from pigs immunized with a bacterin or MAP-based subunit vaccine and then reinfected

S. suis challenge serotype and immunization antigen	No. of piglets	*S. suis* challenge		No. of piglets with indicated site of *S. suis* challenge strain[a] isolation/total no. of piglets						
		Application[b]	CFU	Tonsils	Lung[c]	Serosa[d]	Spleen	Liver	CSF[e]	Joint fluid[f]
2										
Bacterin	7	i.n.	10^9	3/8	1/7	0/7	0/7	0/7	1/7	1/7
MAP	8	i.n.	10^9	2/8	3/8	2/8	0/8	1/8	2/8	0/8
Placebo	8	i.n.	10^9	2/8	4/8	6/8	3/8	2/8	1/8	0/8
9										
Bacterin	6	i.v.	10^8	0/6	0/6	1/6	1/6	0/6	0/6	0/6
MAP	6	i.v.	10^8	0/6	1/6	1/6	0/6	0/6	0/6	0/6
Placebo	6	i.v.	10^8	0/6	1/6	1/6	1/6	1/6	2/6	0/6

[a] Challenge strains were identified through PCR as described in Materials and Methods.
[b] i.n., intranasal; i.v., intravenous.
[c] One cranial lobe was investigated.
[d] Pleural, peritoneal, or pericardial cavity.
[e] Cerebrospinal fluid.
[f] One tarsal puncture was investigated in each animal. In cases of lameness, additional joint punctures were screened.

strain in the placebo group in comparison to those of the bacterin- and MAP-immunized groups (Table 3). In conclusion, neither the serotype 2 bacterin nor the respective MAP subunit vaccine conferred significant protection against mortality after heterologous intravenous challenge with a serotype 9 strain. However, clinical, histological, and bacteriological differences suggested partial protection in the bacterin-immunized group.

Seroconversion of piglets immunized with bacterin and MAP. Immunization of piglets with either the bacterin or the MAP subunit vaccine elicited IgG titers against MAP and MRP that were significantly higher than those in the placebo group and comparable to or higher than those in the convalescent-phase reference serum, as shown for anti-MRP in Fig. 3A and B and for anti-MAP in Fig. S1A and B. Antibody titers against the MAP fraction and against MRP were similar in bacterin- and MAP-vaccinated animals (anti-MRP; Fig. 3A and B). Differentiation of MRP-specific IgG1 and IgG2 antibodies revealed that immunization with both antigens induced an increase of both IgG isotypes, resulting in mean IgG1/IgG2 ratios of 0.6 in bacterin-vaccinated piglets and 0.4 in MAP-vaccinated piglets (Fig. S2).

In all three groups of the homologous challenge experiment, piglets showed seroconversion against SAO prior to challenge. IgG1 and IgG2 titers against SAO were similar in the bacterin-vaccinated group and the placebo group ($P = 0.9$; Fig. 3C). In contrast, MAP-immunized piglets developed significantly higher IgG titers against SAO than did piglets of the placebo group (P values of 0.027 for IgG1 and 0.021 for IgG2; Fig. 3C). The mean ratios of IgG1 to IgG2 were 0.64 (standard deviation, 0.3) in bacterin-immunized piglets and 0.56 (standard deviation, 0.4) in MAP-immunized piglets.

Opsonophagocytic killing mediated by bacterin- and MAP-induced antibodies. Pre- and postimmunization sera of all piglets were investigated for their ability to promote opsonization of *S. suis* and subsequent killing by neutrophils in vitro. The survival factors determined for *S. suis* strain 10 in neutrophil killing assays were significantly lower with sera from bacterin-immunized animals than with sera from MAP- or placebo-immunized animals. In contrast to sera from MAP-immunized piglets, sera of bacterin-immunized piglets induced a significant increase in the neutrophil killing of *S. suis* strain 10 in comparison to the respective preimmunization sera (Fig. 4A and C). This indicated that opsonizing antibodies against strain 10 were elicited by the homologous bacterin but not by the MAP subunit vaccine. Except for in one animal, the induction of opsonizing antibodies was found to correlate with the survival of piglets in the *S. suis* strain 10 challenge experiment (Fig. 4B).

In contrast, the survival factors of *S. suis* serotype 9 strain A3286/94 were similar in neutrophil killing assays with post- and preimmunization sera from piglets of all three groups (as indicated in Fig. 4C, by a ratio of 1). In conclusion, neither immunization with the serotype 2 bacterin nor immunization with the MAP subunit vaccine induced opsonizing antibodies against the heterologous serotype 9 strain A3286/94 (Fig. 4C).

To determine whether antibodies against the capsule were involved in bacterin-induced opsonization of strain 10, postimmunization sera were absorbed with either strain 10 or the isogenic nonencapsulated mutant strain 10cpsΔEF and subsequently tested in the neutrophil killing assay. As demonstrated in Fig. 5B, postimmunization sera which had been absorbed with the isogenic nonencapsulated mutant failed to facilitate killing of strain 10. Comparable survival rates were observed when sera were absorbed with strain 10 or with its isogenic encapsulated mutant. Therefore, antibodies directed against the capsule may not have been essential for opsonophagocytosis in bacterin-immunized piglets. This was in agreement with the finding that serum IgG titers against serotype 2 capsule were very low in all piglets and that there was no difference among the three groups (Fig. 5A).

DISCUSSION

In this study, we designed experiments to evaluate *S. suis* vaccine candidates for use in porcine practice. Experiments included challenges with the two most important *S. suis* pathotypes in Europe, a highly virulent homologous serotype 2 strain and a heterologous serotype 9 strain. Both have been characterized previously via experimental infections with pigs (3). A

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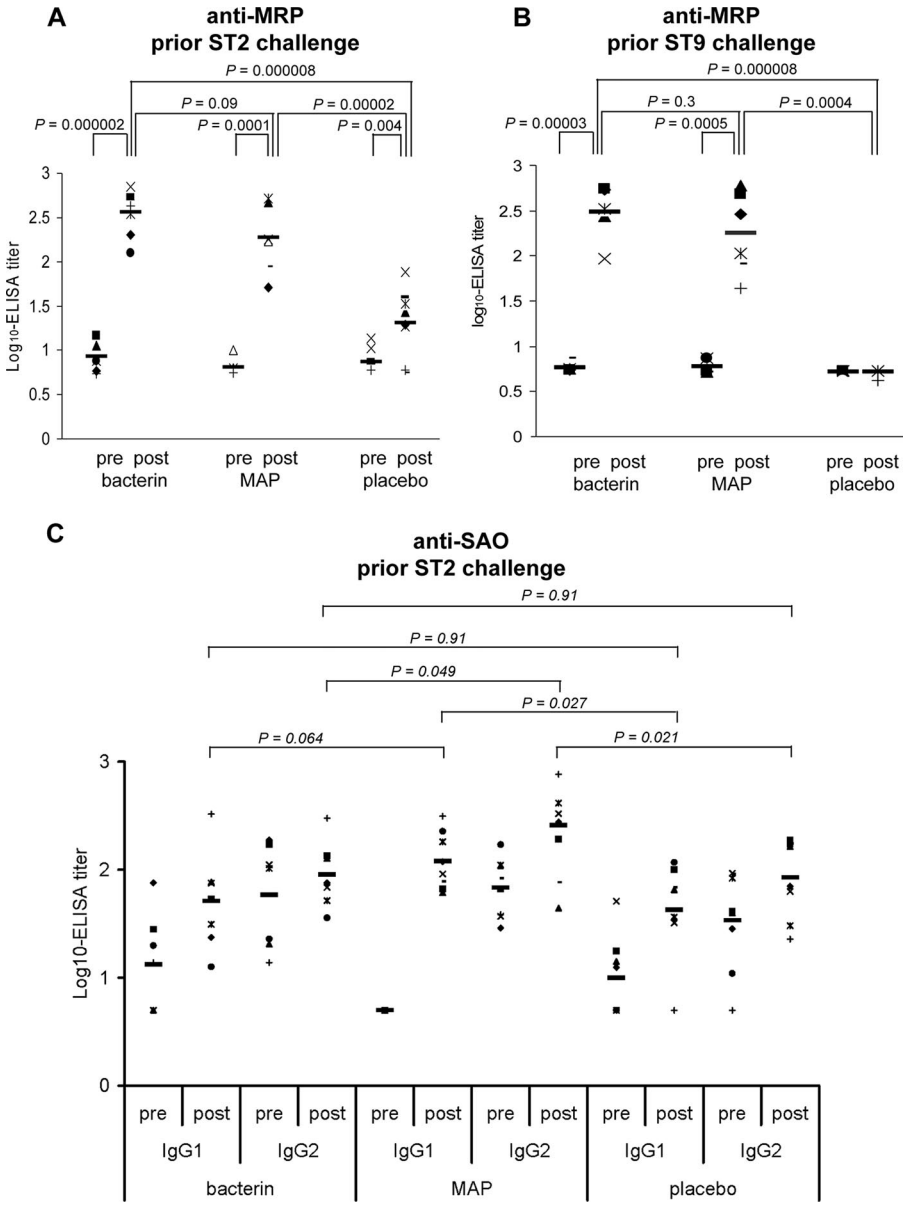

FIG. 3. Serum IgG responses against surface-associated proteins MRP (A and B) and SAO (C) in pigs immunized with a bacterin or a MAP subunit vaccine. The serum samples were drawn before immunization and 14 days after the second immunization, prior to challenge either with serotype 2 (ST2) strain 10 (A and C) or with serotype 9 (ST9) strain A3286/94 (B). (See also Fig. S1 and S2 in the supplemental material.)

comparison with a *S. suis* bacterin was included because that type of vaccines is commonly used today (10).

The results of this study demonstrate that a MAP subunit vaccine conferred less protection than a serotype 2 bacterin. This was surprising, as MAP was shown to include important surface-associated antigens, such as SAO and MRP. Li et al. demonstrated partial protection in pigs immunized with rSAO (16). In contrast, we did not observe protection against *S. suis* strain 10 in MAP-immunized piglets, though these piglets had high humoral antibody titers against SAO. In this study, low antibody titers against SAO were already detected prior to immunization and in the placebo group. This finding is most likely related to other SAO-expressing *S. suis* strains colonizing the upper respiratory tract of all piglets used for challenge. We cannot exclude the possibility that the immune response against SAO after MAP and bacterin immunization, in particular the domination of IgG2 over IgG1, was influenced by the preimmunization status of the piglets. However, as high IgG2-dominated antibody titers against SAO were observed in both studies prior to challenge, the preimmunization status is unlikely to explain the different outcome of challenge. One reasonable explanation may be that we applied the 90% lethal dose for challenge, which is 26 times higher than the dose used by Li et al. (16). Since the serotype 2 bacterin conferred significant protection against this challenge, it seems at least questionable whether a vaccine based on the antigen SAO might induce better protection than an *S. suis* bacterin.

In agreement with our results, Wisselink et al. found that

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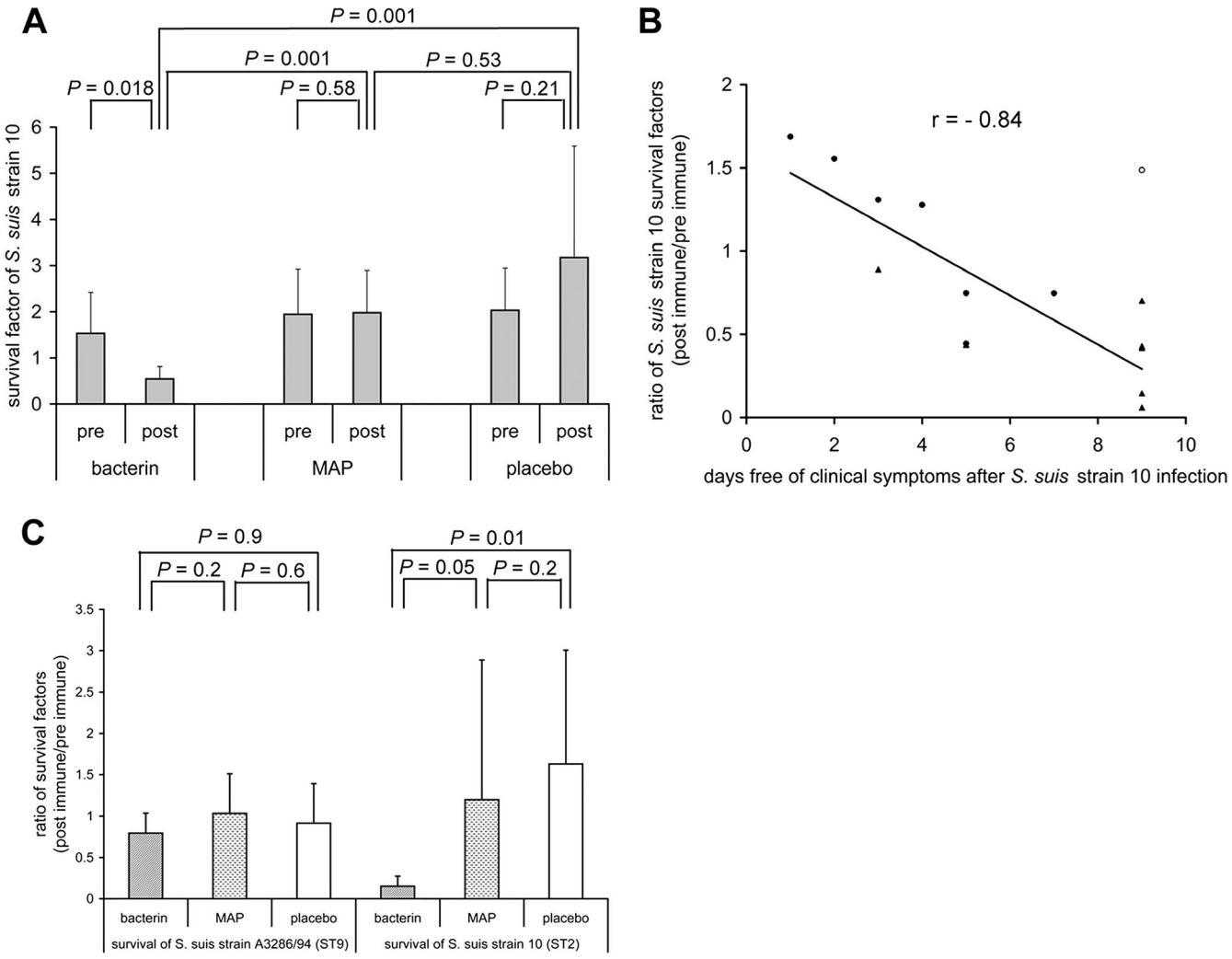

FIG. 4. Opsonophagocytic killing by porcine neutrophils in the presence of serum of piglets immunized with a bacterin or a MAP subunit vaccine. (A) The survival factors of strain 10 were determined in neutrophil killing assays with sera taken either prior to immunization (pre) or two weeks after the second immunization (post). (B) Correlation of the induction of opsonizing antibodies as determined by the ratio of postimmunization survival factors to preimmunization survival factors as shown in panel A and development of disease after homologous strain 10 challenge. Triangles and circles represent bacterin- and MAP-immunized piglets, respectively. Data for one MAP-immunized piglet (open circle) were not included in the calculation of the regression coefficient. (C) Comparison of the induction of opsonizing antibodies against serotype 9 (ST9) strain A3286/94 and serotype 2 (ST2) strain 10 as determined by the ratio of respective survival factors in the presence of postimmunization and preimmunization sera. The sera were from the piglets challenged with *S. suis* A3286/94.

immunization with MRP was less protective than immunization with a bacterin (24). As the authors performed only serotype 2 challenge, it was, however, not clear if MRP might induce cross-protection against invasive serotype 9 strains. It has to be noted that these serotype 9 strains express an MRP variant with high homology to the serotype 2 MRP (20). On the other hand, the results of our study demonstrate that high-level antibody responses against MRP, as developed by the MAP-vaccinated animals, did not correlate with protection against serotype 2 or 9 challenge.

As antibody titers against SAO, MRP, and MAP were very similar among the MAP- and bacterin-immunized piglets, we tried to identify qualitative differences in the antibody responses which might explain the differences in protective efficacy. Opsonophagocytosis experiments with porcine neutrophils and sera from the immunized piglets demonstrated a significant increase in neutrophilic killing efficacy in the presence of sera from bacterin- but not MAP-immunized piglets. The induced opsonizing activities of the different sera as determined in the neutrophil killing assay were found to be a good prognostic indicator for survival. This is in agreement with the important role of neutrophils in the pathogenesis of *S. suis* (5). Therefore, it is reasonable to hypothesize that the induction of opsonizing antibodies in bacterin- but not MAP-immunized piglets was responsible for the different protective efficacy of these two vaccines.

Interestingly, high IgG2 antibody titers against SAO induced by MAP immunization did not correlate with opsonization, in contrast to the results from an earlier study (16). This might be explained by the fact that we used a much lower neutrophil/

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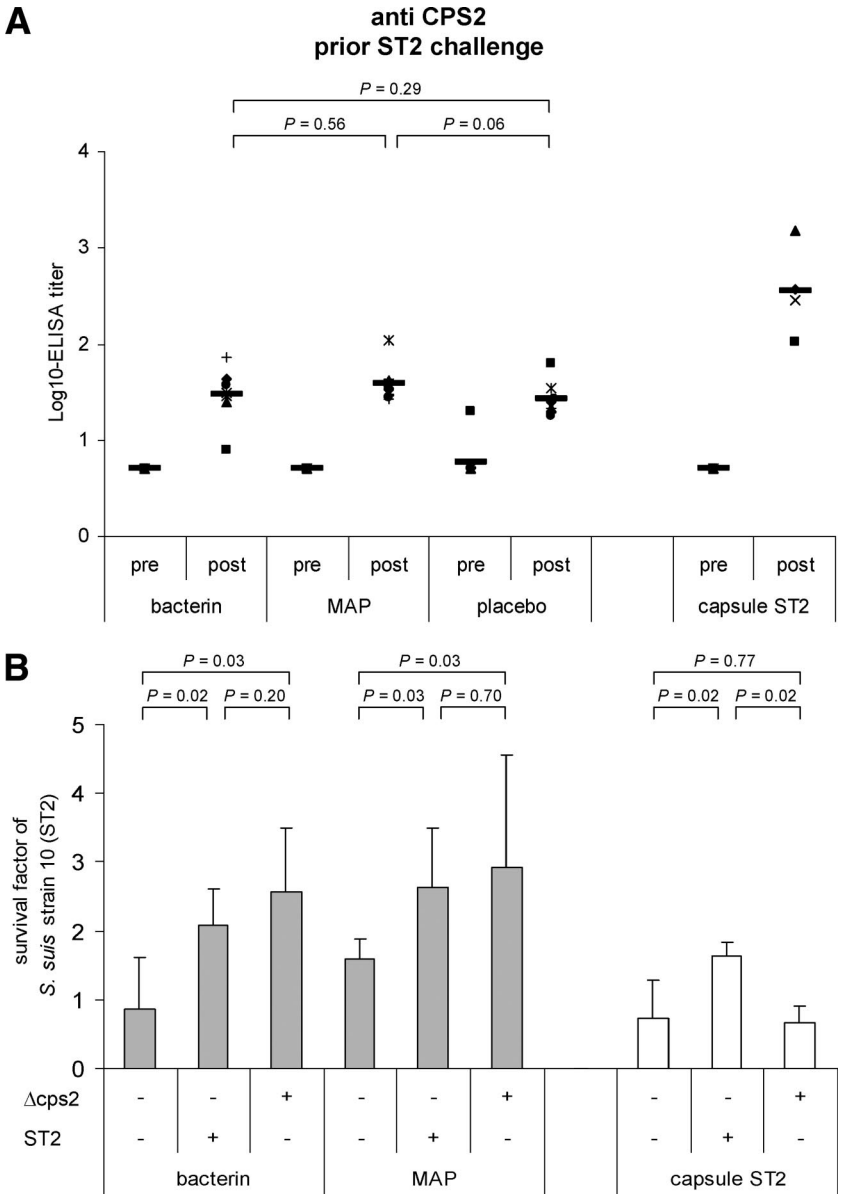

FIG. 5. Antibody responses against serotype 2 (ST2) capsular polysaccharides in piglets immunized with a bacterin, a MAP subunit vaccine, or, for comparison, BSA-conjugated ST2 capsular polysaccharides. (A) Serum IgG responses against ST2 capsular polysaccharides. (B) Opsonophagocytic killing by porcine neutrophils in the presence of absorbed sera from immunized piglets. Sera had been absorbed with *S. suis* strain 10 (ST2) or the isogenic capsule mutant 10cpsΔEF (Δcps2) as indicated below the *x* axis and were then tested in neutrophil killing experiments with *S. suis* strain 10.

bacterium ratio (1:1) than Li et al. (500:1; 16). The lower ratio was chosen because it is more likely to represent the in vivo situation, at least at an early stage of inflammation.

The lack of protection in MAP-immunized piglets might be explained by the hypothesis that important antigens represented by the MAP subunit vaccine, in particular MRP and SAO, are in the course of *S. suis* infection not accessible to antibodies due to encapsulation. Gor et al. (9) found that only one of three investigated MAP of *S. pneumoniae* induced protection. Their results suggested that antibody accessibility of a MAP is a critical feature for its putative function as a protective antigen. Interestingly, a nonencapsulated *S. suis* mutant bacterin induced less protection than the respective wild-type bacterin, though it elicited identical levels of antibody against MRP (25). The authors of the latter study discussed the enhanced protection in wild-type bacterin-immunized piglets in relation to induction of antibodies directed against capsule. However, in accordance with our findings, antibody titers against the capsule were rather low. Furthermore, we demonstrated that absorption of sera with the same nonencapsulated mutant eliminated opsonizing activity of sera from wild-type bacterin-immunized piglets. Therefore, we concluded that induction of opsonizing antibodies in wild-type bacterin-immunized piglets was directed against surface components other

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than the capsule. As the results of this study suggest that induction of opsonizing antibodies is critical for protective immunity against *S. suis*, identification of antigens inducing such antibodies is an important objective of our future studies. Furthermore, it is important to include a *S. suis* MRP* serotype 9 bacterin in future studies to investigate whether our results obtained for MRP^+ EF^+ serotype 2 are similar for both pathotypes.

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C. G. Baums and C. Kock contributed equally to this work.

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