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Immunization of Pigs To Prevent Disease in Humans: Construction and Protective Efficacy of a *Salmonella enterica* Serovar Typhimurium Live Negative-Marker Vaccine▽†

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Zoonotic infections caused by *Salmonella enterica* serovar Typhimurium pose a constant threat to consumer health, with the pig being a particularly major source of multidrug-resistant isolates. Vaccination, as a promising approach to reduce colonization and shedding, has been scarcely used, as it interferes with current control programs relying on serology as a means of herd classification. In order to overcome this problem, we set out to develop a negative-marker vaccine allowing the differentiation of infected from vaccinated animals (DIVA). Applying an immunoproteomic approach with two-dimensional gel electrophoresis, Western blot, and quadrupole time-of-flight tandem mass spectrometry, we identified the OmpD protein as a suitable negative marker. Using allelic exchange, we generated an isogenic mutant of the licensed live vaccine strain Salmoporc and showed that virulence of Salmoporc and that of the mutant strain, SalmoporcΔ*ompD*, were indistinguishable in BALB/c mice. In a pig infection experiment including two oral immunizations with SalmoporcΔ*ompD* and challenge with a multiresistant *S. enterica* serovar Typhimurium DT104 clinical isolate, we confirmed the protective efficacy of SalmoporcΔ*ompD* in pigs, showing a significant reduction of both clinical symptoms and colonization of lymph nodes and intestinal tract. OmpD immunogenic epitopes were determined by peptide spot array analyses. Upon testing of several 9-mer peptides, each including an immunogenic epitope, one peptide (positions F_{100} to Y_{108}) that facilitated the detection of infected animals independent of their vaccination status (DIVA function) was identified. The approach described overcomes the problems currently limiting the use of bacterial live vaccines and holds considerable potential for future developments in the field.

Bacterial food-borne pathogens are of increasing concern to consumers and policy makers worldwide, as they frequently cause epidemic intestinal disease outbreaks and thereby are responsible for high economic losses. One of the most important food-borne pathogens is *Salmonella enterica* serovar Typhimurium, which is responsible for 1.9% (Czech Republic) to 64.7% (New Zealand) of the nontyphoid *Salmonella* infections reported worldwide for the years 2000 to 2001 (16). In the United States, nontyphoidal *Salmonella* strains are responsible for an estimated 1.41 million cases of human infections and more than 500 human deaths annually (24). In Europe, the number of officially reported cases amounts to more than 175,000 according to the report of the European Food Safety Authority (EFSA) for the year 2005 (http://www.efsa.europa.eu/etc/medialib/efsa/science/monitoring_zoonoses/reports/zoonoses_report_2005.Par.0001.File.dat/Zoonoses_report_2005.pdf), with the estimated number of unreported cases being several times higher.

The emergence and rapid spread of multidrug resistant *Salmonella* spp. (especially phage type DT104) (1, 3, 30, 34) limit the therapeutic alternatives in cases of invasive infections and have been shown to be associated with an increased burden of illness (16). Thus, in Europe, 9.1% of human *Salmonella* infections are caused by *S. enterica* serovar Typhimurium, with 21.4% of these infections being due to phage type DT104 (EFSA report for 2005 [see above]). Multidrug-resistant *S. enterica* serovar Typhimurium strains are found in pigs with particularly high frequency (15) and can be isolated from pork and pork products (7, 21) (EFSA report for 2005 [see above]). In Germany, for example, 3.2% of samples taken from minced pork meat were *Salmonella* positive: 67% of these isolates were *S. enterica* serovar Typhimurium, and 69% of these were resistant to more than four antibiotics (EFSA report for 2005 [see above]). In addition, non-food-borne animal-to-human (17, 51) and human-to-human (33) transmissions of systemically spreading multidrug-resistant *S. enterica* serovar Typhimurium DT104 strains have been reported, emphasizing the potential risk of this pathogen to human health.

In order to reduce the potential risk to consumers, it is crucial to minimize pathogen intake into the food chain. In the European Union, surveillance systems are required (regulation [EC] 2160/2003; http://europa.eu.int/eur-lex/pri/en/oj/dat/2003/l_325/l_32520031212en00010015.pdf), and a number of countries are specifically monitoring the occurrence of *Salmonella* in the pork production chain (2, 11, 19, 45, 48). In pigs, *Sal-*

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monella monitoring is based on meat juice serology (26). Therefore, immunization with conventional vaccines cannot be used as a means to reduce infection and shedding of the pathogen, but formulations of vaccines facilitating the differentiation of infected and vaccinated animals (DIVA) (4, 9, 46) would be required.

DIVA live vaccines have been used with good success for the elimination of viral infections such as Aujeszky's disease in pigs (42, 43) or bovine herpesvirus infections (46). For bacterial diseases, only experimental DIVA live vaccines have been described (22), and no DIVA vaccines against food-borne pathogens have been constructed to date. This likely is due to the easy use of antibiotics in livestock for therapy and as feed additives until the recent past (5, 47), the costs and experimental difficulties involved in bacterial DIVA live vaccine construction, and, as genetically modified organisms are involved, the necessity to license these vaccines at the European Agency for the Evaluation of Medicinal Products. This licensing procedure requires extensive experimental testing and therefore is profitable only if the market demand is sufficient.

Here, we present the development of an *S. enterica* serovar Typhimurium DIVA live vaccine for pigs. We describe a general approach to construct and test a bacterial DIVA live vaccine involving (i) the identification a of nonessential immunogenic antigen by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), Western blotting, and quadrupole time-of-flight (Q-TOF) tandem mass spectrometry (MS/MS), (ii) the construction of an isogenic in-frame mutant lacking foreign DNA by allelic exchange, (iii) the development of a discriminatory enzyme-linked immunosorbent assay (ELISA) upon epitope mapping by peptide spot array analyses, and (iv) an exemplary immunization and challenge experiment.

MATERIALS AND METHODS

Bacterial strains, plasmids, and primers. The bacterial strains, plasmids, and primers used in this work are listed in Table 1.

Preparation of outer membrane-associated proteins. Membrane-associated proteins were prepared as described previously (14). Briefly, *S. enterica* serovar Typhimurium cells were grown with shaking in 150 ml of Luria-Bertani (LB) broth at 37°C to an optical density at 600 nm (OD_{600}) of 0.7. Cells were harvested by centrifugation at 8,000 × *g* for 10 min at 4°C, resuspended in 30 ml of detergent wash buffer (NaCl [150 mM], Tris-HCl [10 mM, pH 8.0], sodium deoxycholate [0.05%], and sodium azide [0.04%]), incubated for 30 min in a shaking incubator at 37°C, and centrifuged for 10 min at 7,000 × *g*. This treatment efficiently solubilizes membrane-associated lipoproteins while leaving the cells mostly intact. The cell-free supernatant containing outer membrane-associated proteins as well as contaminating integral outer membrane proteins was filtered (0.22-μm pore size) and stored at −20°C until further use. Protein concentrations were determined by Micro-BCA (Pierce Biotechnology, Rockford, ILL). For 2D-PAGE, the cell-free supernatant was precipitated overnight with trichloroacetic acid (10% final concentration). After centrifugation at 15,000 × *g* for 15 min at 4°C, pellets were washed twice with pure acetone, resuspended in 500 to 1,000 μl bidistilled water, and stored at −20°C.

Preparation of outer membrane proteins. Bacteria grown and harvested as described above were resuspended in 2 ml Tris-HCl (50 mM, pH 8.0) with sucrose (25%, wt/vol) and frozen at −70°C. After thawing, bacteria were incubated with lysozyme (2 mg/ml) and sarcosyl (2%, wt/vol) for 1 h, followed by sonication (Branson-Sonifier B-30; Heinemann, Schwäbisch Gmünd, Germany) using a microtip at the maximum-strength setting for three cycles of 30 s and a duty cycle of 60%. The lysate was centrifuged (15,000 × *g* for 30 min), followed by ultracentrifugation of the supernatant (100,000 × *g* for 90 min). The sonication in combination with sarcosyl disintegrates the cytoplasmic membrane and removes outer membrane-associated proteins. The resulting pellet containing the outer membrane fraction with integral membrane proteins was resuspended in 100 μl H_2O and stored at −20°C.

Detection of immunogenic outer membrane-associated proteins. Aliquots of 500 μg of surface-associated proteins were separated using 2D-PAGE with ImmobilineDryStrips (24 cm, pH 4 to 7). Separated proteins were transferred onto a nitrocellulose membrane using a semidry-protein transfer system (NovaBlot; Amersham Pharmacia Biotech AB, Uppsala, Sweden) and screened by Western blotting with three porcine sera (a hyperimmune serum from a pig vaccinated three times with Salmoporc, a pool of field sera from pigs positive by the Salmotype PigScreen ELISA, and a pool of sera from pigs experimentally infected with *S. enterica* serovar Derby), each used at a 1:200 dilution. Blots were developed using an alkaline phosphatase-conjugated goat anti-swine immunoglobulin G (Dianova, Hamburg, Germany) and BCIP (5-bromo-4-chloro-3-indolylphosphate) and nitroblue tetrazolium as a chromogenic substrate. Protein spots reacting strongly with all three sera were selected for identification via Q-TOF MS/MS.

Identification of immunogenic proteins. Coomassie blue-stained spots corresponding to immunogenic proteins were cut from a 2D PAGE gel, trypsinated, and eluted from the gel by using a method slightly modified from that described previously by Wilm et al. (50). Briefly, the gel pieces were dehydrated with 100 μl acetonitrile, rehydrated with 30 μl rehydration buffer (100 mM NH_4HCO_3 containing 10 mM dithiothreitol), and then treated with 100 mM iodoacetamide in 30 μl 100 mM NH_4HCO_3. Dehydration and rehydration were repeated, and the dehydrated gel pieces were then rehydrated with 3 to 15 μl rehydration buffer containing 20 ng/μl trypsin (sequencing grade) for 12 to 16 h at 37°C. Peptides were extracted using 50 mM NH_4HCO_3 followed by a solution containing 50% (vol/vol) acetonitrile and 5% (vol/vol) formic acid. After evaporation of all liquid in a vacuum centrifuge, peptides were resuspended in a solution containing 50% (vol/vol) acetonitrile and 0.1% (vol/vol) formic acid. Peptide sequences were determined from MS/MS fragmentation data recorded on an ESI Q-TOF mass spectrometer (Q-Tof Ultima; Waters, Milford, MA). Proteins were identified by using the program ProteinLynx Globals Server (version 2.1; Waters) and by searching the National Centre for Biotechnology Information (NCBI) complete database (ftp://ftp.ncbi.nlm.nih.gov./BLAST/db/FASTA/).

Identification and synthesis of immunogenic epitopes. Epitope mapping was performed by peptide spot array analysis using overlapping 15-mer peptides initiating at every third amino acid (12, 13). The peptide-coated membrane was wetted with ethanol to enhance the solvation of hydrophobic peptide spots and then washed three times with Tris-buffered saline (TBS) (10 mM Tris [pH 7.0], 154 mM NaCl [pH 7.0]) for 10 min and finally incubated overnight at 4°C in 10 ml of membrane blocking solution (pH 7.0) (80% [vol/vol] TBS–0.05% Tween 20 [T-TBS; pH 8.0], 20% [vol/vol] casein-based blocking buffer concentrate [Genosys Biotechnology, Cambridge, England], 5% [wt/vol] sucrose). After washing with 10 ml T-TBS (pH 7.0), the membrane was incubated with porcine serum raised against the *S. enterica* serovar Typhimurium vaccine strain Salmoporc for 2 h (diluted 1:200 in membrane blocking solution). The blot was developed as described above. The chromogenic reaction was stopped by washing blots twice with phosphate-buffered saline. The membrane was stripped using 20 ml of *N,N*-dimethylformamide twice for 10 min to dissolve the blue color of spot signals, followed by washing three times with water, stripping mix A (8 M urea, 1% sodium dodecyl sulfate, and 0.5% mercaptoethanol in phosphate-buffered saline [pH 7.0]), stripping mix B (10% acetic acid, 50% ethanol, 40% water), and ethanol, respectively. Afterwards, blotting was repeated with porcine negative serum (a pool of Salmotype PigScreen ELISA-negative sera from pigs from a *Salmonella*-free herd) in order to exclude unspecific epitopes.

Putative immunogenic epitopes (nonamers) were synthesized with an amino-terminal biotinylation linked by a 2-aminohexanoic acid linker and purified by high-performance liquid chromatography (Peptide Specialty Laboratories GmbH, Heidelberg, Germany). Lyophilized peptides were resuspended in distilled water to obtain stock solutions of 5 and 10 mg/ml, respectively. Putative immunogenic epitopes and surface-exposed domains were predicted using the algorithms "Antigenic" and "B2TMR-HMM" (18, 20, 23, 29), respectively.

Construction of an isogenic *S. enterica* serovar Typhimurium Salmoporc Δ*ompD* strain. A truncated *ompD* gene with an in-frame deletion was constructed using a class IIs restriction endonuclease approach (38). Two PCR products of 983 bp and 792 bp were generated using primers oDWST5_outa/oOMPDKO1 and oOMPDKO2/oDWST5_outb, respectively (Table 1). Both fragments were restricted with BsmBI, ligated, reamplified, and cloned into pTOPO2.1 (Invitrogen, Heidelberg, Germany). The insert was confirmed by nucleotide sequence analysis, removed by SacI restriction, and ligated into the mutagenesis vector pROKB2 restricted with SacI, resulting in plasmid pSOM14666. The plasmid was transformed into the donor strain *Escherichia coli* β2155 and mobilized into *S. enterica* serovar Typhimurium Salmoporc by filter mating. The donor was grown on LB agar plates supplemented with kanamycin (50 μg/ml) and diaminopimelic acid (10 mM) at 37°C for 16 h, and the recipient was grown on Columbia sheep

TABLE 1. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Characteristic(s)	Source or reference
Strains		
E. coli β2155	*thrB1004 pro thi strA hsdS lacZΔM15* (F′ *lacZΔM15 laqI*^{q} *traD36 proA*$^+$ *proB*$^+$) Δ*dapA*::*erm* (Erm^r) *recA*::*RPA-2-tet* (Tc^r)::Mu-Km (Km^r) λ*pir*	8
E. coli TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 rec A1 deoR araD139* Δ(*ara leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	TOPO TA cloning; Invitrogen
S. enterica serovar Typhimurium 421/125	*S. enterica* subsp. *enterica* serovar Typhimurium live vaccine strain present in the licensed vaccine Salmoporc	IDT, Dessau-Tornau, Germany
S. enterica serovar Typhimurium 421/125Δ*ompD*	Unmarked *ompD*-negative knockout mutant of *S. enterica* serovar Typhimurium 421/125	This work
S. enterica serovar Typhimurium DT104 27/96	*S. enterica* serovar Typhimurium field isolate carrying phage type DT104 with Amp^r Clin^r Ery^r Pen^r Tet^r	44
Plasmids		
pROCKB2	8.9-kb transconjugation vector based on pROKB1 with *oriR6K* and *mobRP4*, Ap^r Km^r, polycloning site, *sacB*	This work
pSOM14666	Transconjugation vector pROCKB2 carrying a truncated version of *ompD* with *oriR6K*, *mobRP4*, Ap^r Km^r, polycloning site, *sacB*	This work
pTOPO 2.1	Topoisomerase I-"enhanced" *E. coli* cloning vector carrying ampicillin and kanamycin resistance determinants as well as a *lacZ* gene for blue-white selection	TOPO TA cloning; Invitrogen
pSOM810	Vector pTOPO 2.1 carrying PCR product of primers pOMPDKOMP1 and pOMPDKOMP2, starting 516 bp upstream of the *ompD* start codon and ending 31 bp downstream of the stop codon, for complementation of *S. enterica* serovar Typhimurium Δ*ompD*	This work
Primers		
oDWST5outa	5′-TGC TCTAGA CCC GGA GAA ATT ATC AGC AA-3′ (forward primer containing an XbaI restriction site on the 5′ end situated 974 bp upstream of the *ompD* start codon)	This work
oDWST5outb	5′-GCC GAGCTC AGA GAT TGC CAG AGC GTC AT-3′ (reverse primer containing a SacI restriction site on the 5′ end situated 753 bp downstream of the *ompD* open reading frame)	This work
oOMPDKO1	5′-CGTCTCGAAGTTTCATTTTAATAATCCTTAT-3′ (reverse primer with BsmBI restriction recognition site on 5′ end situated 9 bp downstream of the *ompD* start codon; reverse primer to oDWST5_outa for amplification of the upstream DNA fragment used to construct a truncated *ompD*)	This work
oOMPDKO2	5′-CGTCTCGACTTCTGAACTACCAGTTCTAATT-3′ (forward primer with BsmBI restriction recognition site on 5′ end situated 2 bp downstream of the *ompD* stop codon; forward primer to oDWST5_outb for amplification of the downstream DNA-fragment used to construct a truncated *ompD*)	This work
oOMPD1	5′-CCATACCAGGATTGCGCTG-3′ (forward primer situated 393 bp upstream of the *ompD* start codon)	This work
oOMPD2	5′-CGGTAAGCCGAAACCACAG-3′ (reverse primer situated 317 bp downstream of the *ompD* open reading frame)	This work
oOMPDKOMP1	5′-GGCGGGCCGATATTGATATT-3′ (forward primer for complementation of *S. enterica* serovar Typhimurium Δ*ompD* situated 516 bp upstream of the *ompD* start codon; the 516-bp nucleotide sequence was also analyzed for promoters using the Web-based program BPROM [www.softberry.com] to ensure that the promoter of the *ompD* gene was included)	This work
oOMPDKOMP2	5′-GGACTGGCTTTGTATTCAGAC-3′ (reverse primer for complementation of *S. enterica* serovar Typhimurium Δ*ompD* situated 31 bp downstream of the *ompD* stop codon)	This work

blood agar (Oxoid GmbH, Wesel, Germany) at 37°C for 44 h. Bacteria were harvested with sterile cotton swabs and resuspended in TNM buffer (1 mM Tris-HCl [pH 7.2], 100 mM NaCl, 10 mM $MgSO_4$) to an OD_{600} of 1. Filter mating was performed as described previously (28); the conjugation mixture was plated onto LB agar plates supplemented with kanamycin (40 μg/ml) and incubated at 37°C overnight. Colonies were subcultured and screened via PCR with *ompD*-specific primers oOMPD1 and oOMPD2. Colonies containing both the wild type and the truncated gene were considered to be potential genomic cointegrates and were used for counterselection.

Counterselection. A single colony of *S. enterica* serovar Typhimurium Salmoporc cointegrate was inoculated overnight in LB broth. Salt-free LB broth (2.5 ml) containing 10% sucrose was inoculated with 2.5 μl of the culture grown overnight and kept at 37°C in a shaking incubator (200 rpm) for 4 h. In order to improve counterselection efficacy, the culture was subsequently stored at 4°C for 72 h, and 50-μl aliquots were plated onto LB agar. Single colonies were replica plated onto LB agar with (40 μg/ml) and without kanamycin. The genotype of kanamycin-sensitive colonies was determined by PCR with primers oOMPD1 and oOMPD2 and verified by Southern blot analysis, nucleotide sequencing analysis, and pulsed-field gel electrophoresis.

Virulence study in BALB/c mice. Virulence of *S. enterica* serovar Typhimurium SalmoporcΔ*ompD* was assessed in an infection model by determining the 50% lethal dose (LD_{50}) in BALB/c mice in comparison to the parent strain Salmoporc. The animals, 32 female 17- to 20-g BALB/c mice, were infected pairwise with 1×10^2 to 1×10^8 CFU of either the parent or the mutant strain and

observed for 19 days postinfection. Spleens and livers of animals dying during the time of observation were cultured for possible reisolation of the respective strain; isolated *Salmonella* colonies were identified using the IDT *Salmonella* diagnostic kit and a PCR with *ompD*-specific primers, facilitating the differentiation of SalmoporcΔ*ompD* and Salmoporc. The LD_{50} was calculated using Probit analysis software (SPSS Inc., Chicago, IL).

Vaccination trial in pigs. The SalmoporcΔ*ompD* strain constructed in this study was used as an oral vaccine in a vaccination study in pigs (German Landrace) and compared to the parent strain (Salmoporc) and a placebo group. The trial was performed essentially as described previously (41); an *S. enterica* serovar Typhimurium DT104 clinical isolate (strain 27/96) was used as a challenge strain. The animal experiment included three groups of six pigs each, 4 weeks of age, vaccinated orally with placebo, *S. enterica* serovar Typhimurium SalmoporcΔ*ompD* (6×10^8 CFU/ml per vaccination), or *S. enterica* serovar Typhimurium Salmoporc (according to manufacturer's instructions), respectively. Three additional pigs were vaccinated with *S. enterica* serovar Typhimurium SalmoporcΔ*ompD* to investigate the development of antibody titers until 3 weeks after infection. Two rounds of vaccination were performed 3 weeks apart. Blood samples were taken prior to the first (day 0) and second (day 21) immunizations, 1 week before challenge, and at necropsy. For the three pigs kept until 3 weeks after infection, additional blood samples were taken 1, 2, and 3 weeks postchallenge (see Table S4 in the supplemental material). Animals were cared for in accordance with the principles outlined in the *European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes* (European Treaty Series no. 123 [http://conventions.coe.int/treaty/EN/Menuprincipal.htm]; permit no. 06/1066).

Infection of pigs. For oral infection on day 43, a 50-ml culture (LB medium) was inoculated with 5 ml of a liquid culture of *S. enterica* serovar Typhimurium DT104 strain 27/96 (LB medium) grown overnight and was grown with shaking at 37°C to an OD_{600} of 0.9. The culture was placed on ice for 10 min and kept on ice until use for a maximum of 2 h. Piglets were infected twice within 2 hours, and inoculum doses were determined immediately after infection via serial dilution and subsequent plating onto Rambach agar plates supplemented with ampicillin (100 μg/ml). Infections were carried out using a bent-knob cannula and syringe to administer the appropriate dose of *S. enterica* serovar Typhimurium DT 104 strain 27/96 to the piglets 3 weeks after the second immunization via the oral route (5×10^8 CFU/ml per round, resulting in a total dose of 1×10^9 CFU/ml).

Surveillance of animals. Body temperature, feeding behavior, and clinical symptoms were recorded at least daily for each individual pig or as needed. A clinical scoring system was employed to assess the clinical condition of each individual animal as follows. A score of 1 each was given for the occurrence of fever (rectal temperature >40°C), lack of appetite, and diarrhea/vomitus/lethargy, resulting in a minimum clinical score of 0 and a maximum score of 4 per day; the added daily clinical scores for days 1 to 7 were designated the total clinical score. Statistical analysis of the total clinical score was performed using the Wilcoxon test. Animals were bled on days 0, 36, 50, 57, and 65 during the course of the experiment.

Bacteriological examination of organ samples. In order to determine the protective efficacy of the vaccine with respect to colonization, organ samples (ileocecal lymph nodes, ileum [approximately 10 cm cranial of the ileocecal valve], and cecum [apex]) were taken at postmortem examination. Intestinal organ samples were washed, and 0.1 g of mucosa was removed by scraping with a scalpel, taken up to 1 ml with saline, and homogenized using a bead beater (speed of 5.0, 40 s/round; ThermoSavant) with three glass beads (diameter, 3 mm). Tenfold serial dilutions were plated onto Rambach agar containing 100 μg/ml of ampicillin in order to suppress the growth of other *Enterobacteriaceae* and incubated overnight at 37°C. the *Salmonella* colonies grown were counted, the number of CFU/ml was calculated, and individual colonies were confirmed to be exemplary by antibiotic resistance typing and *S. enterica* serovar Typhimurium DT104-specific multiplex PCR (32).

ELISAs. For the OmpD-specific peptide-based ELISA, Nunc Immobilizer Streptavidin F96 microtiter plates were incubated for 1 h with 100 μl of OmpD-derived synthetic, biotinylated peptide (positions F_{100} to Y_{108} of the OmpD protein; 25 μg/ml) as a solid-phase antigen. Porcine sera were preabsorbed with a whole-cell lysate of *S. enterica* serovar Typhimurium SalmoporcΔ*ompD* for 1 h in order to remove possible cross-reactive antibodies directed against similar epitopes of other porins. Serial twofold dilutions of the preabsorbed sera (starting with a dilution of 1:10) were added and incubated for 1 h at room temperature. The ELISA was developed using goat anti-pig peroxidase conjugate (Dianova) and 2,2-azino-di-[3-ethylbenzithiazoline sulfonate] (ABTS; Roche Diagnostics, Mannheim, Germany) as a substrate. To determine the titer of anti-*Salmonella* LPS antibodies, the commercial Salmotype PigScreen ELISA (Labor Diagnostik GmbH, Leipzig, Germany) was carried out according to the manufacturer's instructions.

Nucleotide sequence accession number. The nucleotide sequence for plasmid pRouB2 has been submitted to GenBank under accession number AM180348.

RESULTS

Identification of a suitable marker protein. To identify surface-exposed, immunogenic proteins of the licensed live vaccine Salmoporc, exponentially growing bacteria were harvested and treated with sodium deoxycholate to enrich outer membrane proteins in the supernatant via detergent wash. By separating the enriched outer membrane proteins by 2D-PAGE and screening their immunogenicity by Western blot analyses (see Fig. S1 in the supplemental material), we identified five highly immunogenic proteins (see Table S1 in the supplemental material). They reacted strongly with a pool of sera from animals vaccinated three times with Salmoporc as well as with pools of sera from animals infected with *S. enterica* serovar Typhimurium or *S. enterica* serovar Derby, respectively. The proteins corresponding to the immunoreactive spots were cut from a Coomassie blue-stained 2D-PAGE gel and identified after trypsin digestion using Q-TOF MS/MS. Four proteins could be identified unambiguously as the major porins OmpC and OmpD, a homolog to an outer membrane protein of *Acinetobacter* spp., and elongation factor Tu.

Characterization of OmpD as a putative negative marker. In silico analyses of the candidate proteins resulted in the selection of outer membrane porin D (OmpD) as a putative negative marker. By performing a mapping of continuous antigenic epitopes of the OmpD protein by peptide spot array analysis (see Fig. S2 in the supplemental material) with overlapping 15-mers, we identified four highly immunogenic epitopes throughout the protein. These matched only partially with putative immunogenic epitopes and surface-exposed domains predicted using the algorithms "Antigenic" (see Table S2 in the supplemental material) and "B2TMR-HMM" (see Table S3 in the supplemental material), respectively. All four epitopes were synthesized as biotinylated peptides and tested as solid-phase antigens for their applicability in an OmpD-specific ELISA, with peptide 2 facilitating the best discrimination between OmpD antibody-positive and -negative sera (Fig. 1).

Generation of an *ompD*-deficient mutant strain. An isogenic *S. enterica* serovar *Typhimurium* SalmoporcΔ*ompD* mutant was constructed by allelic exchange using the suicide plasmid pSOM14666 (containing an in-frame deletion of *ompD*) (see Fig. S3 in the supplemental material) upon conjugation with *E. coli* β2155 as the donor strain. Plasmid cointegrates were selected on kanamycin, confirmed by PCR, and resolved by sucrose counterselection. Sucrose-resistant and kanamycin-sensitive colonies were confirmed by PCR, nucleotide sequencing, and Southern blot analysis as well as pulsed-field gel electrophoresis (see Fig. S4 in the supplemental material). The phenotype was confirmed by one-dimensional PAGE (Fig. 2) and subsequent Q-TOF MS/MS. The mutant generated is free of foreign DNA and was designated *S. enterica* serovar Typhimurium SalmoporcΔ*ompD*.

Virulence in BALB/c mice. In order to ensure an unchanged virulence of the mutant strain, we challenged 16 groups of two BALB/c mice each with eight different doses of Salmoporc and

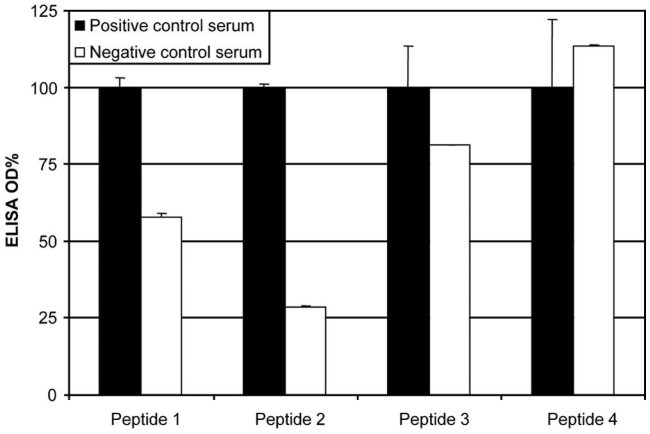

FIG. 1. Discriminatory efficacy of OmpD-derived immunogenic epitopes. Biotinylated epitopes 1 to 4 were used as solid-phase antigens on streptavidin-coated ELISA plates and incubated with internal positive and negative control sera, respectively, obtained from animals in the vaccination trial. The bars show the means and standard deviations of three independent ELISA experiments. OD%, relative optical density of the negative control serum compared to that of the positive control serum (defined as 100 OD%) for each coating peptide.

SalmoporcΔ*ompD* and determined the LD_{50} to be approximately 1.3×10^8 CFU for both strains (see Table S4 in the supplemental material). These results demonstrated that the lack of *ompD* does not additionally attenuate *S. enterica* serovar Typhimurium Salmoporc for BALB/c mice.

Protective efficacy in pigs. Four clinical symptoms (fever [≥40.0°C], lethargy, reduced food uptake, and enteritis [vomitus/diarrhea]) were added to result in a clinical score with a maximum of 4 per animal and day. A significant difference ($P = 0.046$; Wilcoxon test) between either of the vaccinated groups and the placebo group was observed (Fig. 3). All animals in the placebo group developed fever 2 days postinfection and showed reduced feed uptake, whereas none of the vaccinated animals developed fever, and only two vaccinated animals showed hesitant feed uptake. No significant difference between the groups vaccinated with *S. enterica* serovar Typhimurium Salmoporc and *S. enterica* serovar Typhimurium SalmoporcΔ*ompD* was observed.

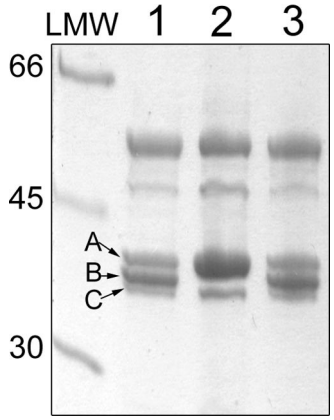

FIG. 2. Characterization of *S. enterica* serovar Typhimurium SalmoporcΔ*ompD*. Phenotypes were characterized using membrane preparations of Salmoporc (lane 1), SalmoporcΔ*ompD* (lane 2), and SalmoporcΔ*ompD* complemented in *trans* with plasmid pSOM810 (lane 3). Protein bands A to C were sequenced using Q-TOF MS/MS and identified as being OmpC (A), OmpD (B), and OmpF (C), respectively. LMW, low molecular weight marker (Amersham Pharmcia Biotech AB, Uppsala, Sweden).

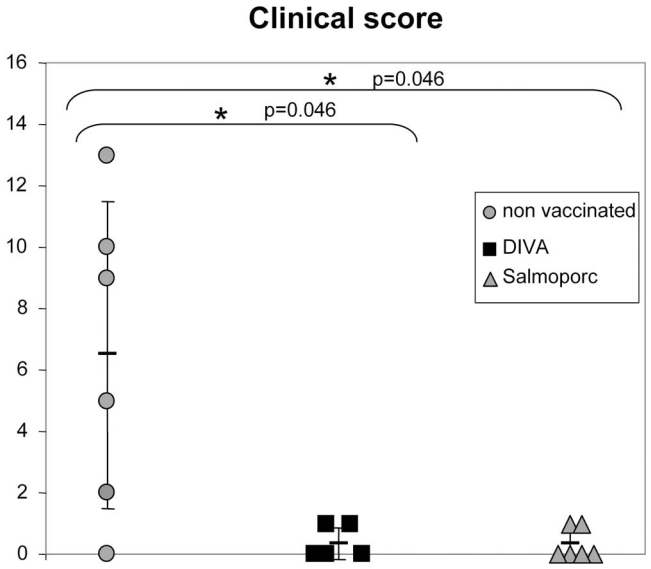

FIG. 3. Clinical scores of pigs in the vaccination and challenge trial. The total scores of individual animals for days 1 to 7 postinfection and the arithmetic means (horizontal bars) calculated for groups 1 to 3 (six animals each) are given. The asterisks indicate statistical significance (Wilcoxon test), and the *P* values are given.

One week postinfection, animals were necropsied. All pigs showed reactive hyperemia in the ileum and cecum, with some of the placebo-vaccinated animals showing highly reactive hyperemia in the cecal mucosa. *Salmonella* colonization was determined for three defined locations (ileal and cecal mucosa and ileocecal lymph node). For the cecum ($P = 0.042$; Friedmann test) and ileocecal lymph nodes ($P = 0.007$; Friedmann test), vaccination with either vaccine significantly reduced colonization (Table 2). However, the reduction of colonization was about 10-fold lower upon vaccination with *S. enterica* serovar Typhimurium SalmoporcΔ*ompD* than upon vaccination with the *S. enterica* serovar Typhimurium Salmoporc parent strain (Table 2).

Marker properties of the OmpD protein. In addition to the protective efficacy, the functionality of the negative marker OmpD was investigated in the vaccination study. In order to function as a serologically detectable marker, antibodies di-

TABLE 2. Reisolation of the challenge strain from ileocecal lymph nodes, cecum, and ileum

Tissue	Reisolation of challenge strain (CFU/g tissue)[a]		
	Nonvaccinated	SalmoporcΔ*ompD*	Salmoporc
Ileocecal lymph nodes	1.21 ± 10^{4}*	5.13 ± 10^{2}*	6.67 ± 10^{1}*
Cecum	6.79 ± 10^{4}*	2.67 ± 10^{2}*	1.67 ± 10^{1}*
Ileum	7.30 ± 10^{4}	1.67 ± 10^{3}	6.67 ± 10^{2}

[a] An * indicates that the values were significantly lower ($P < 0.05$; Friedmann test) than those in the nonvaccinated control group.

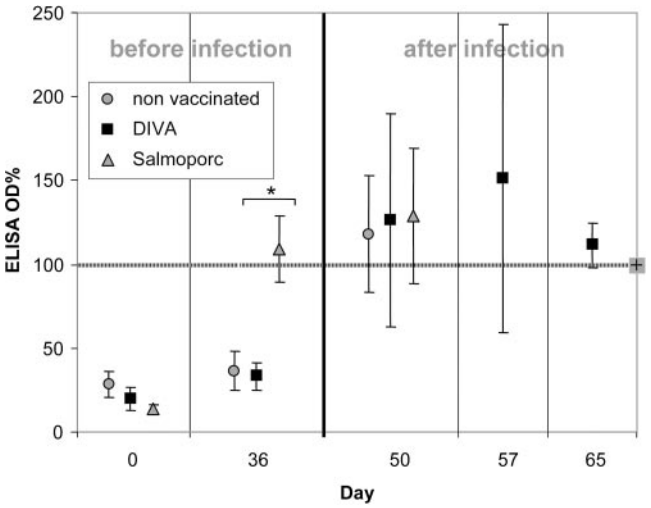

FIG. 4. Serological responses of pigs in a discriminatory ELISA upon immunization and challenge. Animals were immunized on days 1 and 21 and challenged on day 43. Blood was taken on days 0, 36, and 50 (six animals per group) and on days 57 and 65 (three animals). Anti-OmpD antibody titers were determined using an ELISA with OmpD-derived peptide 2 as a solid-phase antigen. The horizontal line marked with "+" (OD% = 100) indicates the position of the internal positive control consisting of the pooled sera from day 50. OD%, relative optical density of the text sera compared to that of the internal positive control serum (defined as 100 OD%).

rected against the OmpD protein have to be developed by the host upon infection with *S. enterica* serovar Typhimurium or vaccination with a commercial *S. enterica* serovar Typhimurium vaccine but not upon vaccination with the marker vaccine strain *S. enterica* serovar Typhimurium Salmoporc-Δ*ompD*. Thus, a serological differentiation of infected and vaccinated pigs is made possible (DIVA principle).

Salmonella antibody titers as assessed in the Salmotype PigScreen ELISA steadily rose during the study in both vaccinated groups, whereas titers in the placebo group stayed negative (see Fig. S5 in the supplemental material). Antibody titers in the OmpD-specific peptide-based ELISA developed in the *S. enterica* serovar Typhimurium Salmoporc-vaccinated group but remained negative before challenge in the *S. enterica* serovar Typhimurium SalmoporcΔ*ompD*- and placebo-vaccinated groups (Fig. 4) (day 0 and day 36). Shortly after challenge, OmpD-specific seroconversion was observed in the *S. enterica* serovar Typhimurium SalmoporcΔ*ompD*- and placebo-vaccinated groups (Fig. 4) (day 50). Three animals vaccinated with *S. enterica* serovar Typhimurium Salmoporc Δ*ompD* before infection were monitored serologically up to 3 weeks postchallenge. OmpD-specific antibody titers remained positive until the end of the experiment (Fig. 4) (days 57 and 65).

DISCUSSION

In this report, we describe the identification of a DIVA marker, its introduction into the conventionally attenuated live vaccine strain Salmoporc, and the development of a discriminatory ELISA system. Salmoporc was chosen for this work because it has been shown to reduce both shedding and colonization of the porcine intestinal tract (41). The approach developed is straightforward and might be generally applicable to the construction of live bacterial DIVA vaccines.

The adaptation of a widely used allelic exchange strategy with *sacB* as the counterselectable marker allowed the construction of a *Salmonella* vaccine strain carrying an in-frame deletion of the *ompD* gene not containing foreign DNA. Although this method is more laborious than the commonly used PCR-based allelic exchange described previously by Datsenko and Wanner (6), this approach might be advantageous for vaccine development and subsequent licensing. Thus, the resulting strain is indistinguishable from a spontaneously occurring deletion mutant and therefore is not considered a genetically modified organism according to European regulations on genetic engineering (directive [EC] 2001/18; http://europa.eu/eur-lex/pri/en/oj/dat/2001/l_106/l_10620010417en00010038.pdf).

The protein OmpD, chosen as selectable marker, is one of the most abundant proteins in the outer membrane of *Salmonella*, representing about half of the 1×10^5 to 2×10^5 porin molecules per cell under favorable growth conditions (27, 36). It is present in all *S. enterica* serovars with the exception of *S. enterica* serovar Typhi (35, 37). In contrast to the major porin OmpC, however, OmpD is not found in other gram-negative bacteria (40). The presence of OmpD and other porins in the detergent wash fraction is likely due to the presence of membrane vesicles (blebs) commonly formed by gram-negative bacteria (31, 52). On the one hand, its high abundance makes OmpD a highly attractive choice as a negative marker. Thus, seroconversion is likely to occur upon infection of pigs with any *Salmonella* serovar (except *S. enterica* serovar Typhi), thereby activating the DIVA function. This broad-spectrum DIVA function is mandatory for a porcine *Salmonella* vaccine, as legislation is not limited to certain serovars. Therefore, conventional marker strains, such as rough mutants (39, 49), cannot be used. On the other hand, OmpD is homologous to other porins common among *Enterobacteriaceae*. Therefore, in order to obtain a sufficiently discriminatory ELISA efficacy, not the entire OmpD protein but an OmpD-specific peptide selected by peptide spot array analyses had to be used as a solid-phase antigen.

In addition to the more difficult setup of a discriminatory serological test, the use of a major immunogenic protein as a DIVA antigen has two other possible drawbacks. Thus, the deletion might (i) cause an additional attenuation of the mutant strain and (ii) diminish protective efficacy. For the deletion of OmpD from *S. enterica* serovar Typhimurium, reports with respect to attenuation are controversial (10, 25). Both studies, however, have been carried out using an *ompD* mutant obtained by Tn*10*-based mutagenesis (10, 25). In order to ensure unchanged virulence of the *S. enterica* serovar Typhimurium SalmoporcΔ*ompD* strain, we carried out an LD_{50} determination in BALB/c mice. This experiment unambiguously showed equal virulence for both strains.

The vaccination study performed demonstrated that pigs vaccinated with either vaccine were protected equally well from clinical symptoms, thereby confirming the results from the mouse experiment. On the other hand, the DIVA vaccine showed a reduced efficacy in comparison to the Salmoporc parent strain with respect to reducing organ colonization. Thus, the number of *S. enterica* serovar Typhimurium DT104 cells to be isolated from ileocecal lymph node, cecum, and

ileum was 10-fold higher in pigs immunized with SalmoporcΔ*ompD* than in those immunized with the Salmoporc parent strain. These results imply that a deletion of the OmpD protein, although of no consequence with respect to causing septicemia in mice upon intraperitoneal application, might reduce colonization and survival upon oral application in the porcine gastrointestinal tract. However, the DIVA vaccine strain still reduces *S. enterica* serovar Typhimurium reisolation rates significantly (10- to 100-fold).

As an infection occurring after vaccination is not masked, the *S. enterica* serovar Typhimurium SalmoporcΔ*ompD* vaccine strain, despite its reduced efficacy in reducing colonization, might be a valuable tool in serosurveillance-based *Salmonella* control programs aimed at reducing the risk of human infection. The fact that *S. enterica* serovar Typhimurium SalmoporcΔ*ompD* does not contain foreign DNA and that its parent strain, *S. enterica* serovar Typhimurium Salmoporc, has been used extensively in the field should facilitate the realization of future field studies and subsequent licensing procedures. Furthermore, the *S. enterica* serovar Typhimurium SalmoporcΔ*ompD* vaccine strain might be usable as a carrier of foreign antigens and might therefore open new ways for the construction of multivalent live vaccines for livestock.

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