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An article for Microbiology (Microbial Pathogenicity)

Title: Protective capacities of cell surface-associated proteins of *Streptococcus suis* mutants deficient in divalent-cation-uptake regulators

Running title: Protective capacities of *adcR* for *S. suis* mutants

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ABSTRACT

Many cell surface-associated, divalent cation-regulated proteins are immunogenic, and some of them confer protection against the bacterial species from which they are derived. In this work, two *Streptococcus suis* divalent cation uptake regulator genes controlling zinc/manganese and iron uptake (*adcR* and *fur*, respectively) were inactivated in order to study the protective capacities of their cell surface-associated proteins. The results obtained showed overexpression of a set of immunogenic proteins (including members of the pneumococcal histidine triad family previously reported to confer protection against streptococcal pathogens) in *S. suis* *adcR* mutant cell surface extracts. Likewise, genes encoding zinc transporters, putative virulence factors and a ribosomal protein paralogue related to zinc starvation appeared to be derepressed in this mutant strain. Moreover, protection assays in mice showed that although neither *adcR*- nor *fur*-regulated cell surface-associated proteins were sufficient to confer protection in mice, the combination of both *adcR*- and *fur*-regulated cell surface-associated proteins is able to confer significant protection (50%, $P=0.038$) against a challenge to mice vaccinated with them.

Keywords

ABC, ATP-binding cassette, EMSA, electrophoretic mobility shift assay

INTRODUCTION

Streptococcus suis is an important pathogen that causes significant economic losses in the swine industry worldwide (Higgins et al., 1995). Infection caused by *S. suis* may vary from asymptomatic bacteraemia to fulminant systemic disease, with meningitis as the most outstanding feature (Higgins et al., 1995). It is also an important zoonotic agent for humans, who may acquire the infection through skin wounds upon contact with pigs and/or their by-products. Recently, during an outbreak in China, 215 human cases of *S. suis* infection were documented, and 39 of those patients died (Yu et al., 2006). More than 30 serotypes of *S. suis*, based on its capsular antigens, are currently known (Higgins et al., 1995). Serotype 2 is the most frequently isolated from diseased animals (Staats et al., 1997). Efforts to control infection are hampered by the lack of effective vaccines against *S. suis*. Furthermore, mechanisms involved in pathogenesis of this micro-organism are not yet completely understood (Gottschalk & Segura, 2000).

Iron and zinc are essential components of many bacterial proteins either as structural or catalytic cofactors (Andrews et al., 2003; Babich & Stotzky, 1978). The concentration of both elements in the free state in the body fluids of mammals is extremely low in order to prevent bacterial proliferation. On the other hand, metals at high concentrations are toxic to micro-organisms. Toxicity occurs through the displacement of essential metals from their native binding sites or through ligand interactions (Babich & Stotzky, 1978; Chamnongpol et al., 2002). In addition, iron can be particularly toxic under aerobic conditions, interacting with either oxygen or oxygen-reduced species (Touati, 2000). Consequently, micro-organisms require homeostatic mechanisms to control intracellular metal levels.

Fur (ferric uptake regulator) is a transcriptional regulator of genes involved in iron uptake and storage in many bacterial species (Andrews et al., 2003). In *Escherichia coli* and *Bacillus subtilis*, and in species closely related to them, transcription of the zinc transporter genes is regulated by Zur (zinc uptake regulator), which belongs to the Fur protein family (Patzner & Hantke, 2000). However, in *Streptococcus* species, zinc transporter genes are controlled by AdcR, a transcriptional regulatory factor of the MarR protein family (Loo et al., 2003).

Several cell surface-associated transporters involved in divalent cation uptake, including ATP-binding cassette (ABC) transporters, have been shown to elicit a protective immune response (Garmory & Titball, 2004; Tanabe et al., 2006). It has been shown that in *S. suis*, AdcR controls at least two operons related to Zn²⁺/Mn²⁺ uptake, encoding a complete ABC transporter (SsuiDRAFT 1237–1240) and a divalent cation-binding lipoprotein (SsuiDRAFT 0103) (Aranda et al., 2008). Interestingly, both cation-binding lipoproteins encoded by these operons (SsuiDRAFT 1237 and SsuiDRAFT 0103) are immunogenic when analysed on Western blots using immunologically reactive serum obtained from mice surviving after inoculation with the wild-type *S. suis* 89/1591 strain (Aranda et al., 2008). Furthermore, a high level of protection against *S. suis* challenge is obtained in mice when vaccinated with the product of the ssuiDRAFT 0103 ORF (Aranda et al., 2008).

Different strategies have been developed to produce vaccines against pathogenic bacteria, including overexpression on the bacterial cell surface of divalent cation-uptake transporters which are induced in the presence of chelating agents, or by the construction of strains deficient in cation-uptake repressors (Garrido et al., 2008). In this context, the aim of this work has been to obtain knockout *S. suis* mutants for the regulator genes of either zinc/manganese- or iron-uptake systems to study their possible use as a protective tool against this pathogen.

METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this work are listed in Supplementary Table S1. *E. coli* cells were grown in Luria–Bertani (LB) medium (Miller, 1992). When necessary, ampicillin (50 µg ml⁻¹), kanamycin (50 µg ml⁻¹) or spectinomycin (50 µg ml⁻¹) was added to LB medium. *S. suis* was grown in Todd–Hewitt medium (TH medium; Difco). When required, spectinomycin (100 µg ml⁻¹) was added to TH medium. Unless otherwise indicated, all cultures were incubated at 37 °C, with shaking for *E. coli*. DNA extractions, cloning, and other molecular techniques used were performed as described elsewhere (Sambrook & Russell, 2001). All oligonucleotides (Roche) used in this work are listed in Supplementary Table S2.

Construction of *S. suis* mutants. The *adcR* and *fur* genes were inactivated by precise in-frame deletion. Briefly, upstream and downstream regions of both *adcR* and *fur* genes (including partial coding regions) were amplified by overlap-extension PCR using genomic DNA from *S. suis* P1/7 strain as a template. A PCR product of 892 bp with a deletion of 229 bp was obtained for the *adcR* gene, and a PCR product of 848 bp with a deletion of 222 bp was obtained for the *fur* gene. Both PCR fragments were cloned into the pCR4-TOPO vector following the manufacturer's instructions and propagated in *E. coli* One Shot TOP 10 cells. Inserts were amplified afterwards by PCR using primers M13F and M13R (Supplementary Table S2), amplicons were digested with *EcoRI* and cloned into the *EcoRI*-digested thermosensitive suicide vector pSET4s, which carries the *spc* and *repA* genes conferring spectinomycin resistance and thermosensitivity, respectively (Takamatsu et al., 2001b), giving rise to plasmids pSET4 Δ *adcR* and pSET4 Δ *fur*. Recombinant plasmids were propagated in *E. coli* ABLE-C cells, purified, and used to electroporate *S. suis* electrocompetent P1/7 and UA5000 strains, as described elsewhere (Takamatsu et al., 2001a). Cells were then plated on TH agar containing spectinomycin and incubated for 48 h at 28 °C to select for clones that had incorporated the plasmid. Several clones were then selected and purified by single-colony isolation on TH agar containing spectinomycin and incubated at 37 °C. Integration of the construct into the chromosome as a result of homologous recombination via a single crossover was verified by PCR in temperature- and spectinomycin-resistant colonies. These colonies were then passaged several times onto non-selective media at 28 °C and screened for loss of vector-mediated spectinomycin resistance. Candidate spectinomycin-sensitive, temperature-resistant clones were analysed by PCR, which showed the exchange of the wild-type allele for the genetic segment containing the desired deletion as a consequence of a homologous recombination through a double-crossover event. Allelic replacement in candidate clones was further confirmed by sequencing of the region in the resulting mutants.

Preparation of *S. suis* cell surface-associated proteins. Extraction of these proteins was performed as described elsewhere (Tavares & Sellstedt, 2000), with some modifications. Briefly, cells from 200 ml overnight culture were pelleted at 4 °C by centrifugation at 8000 g for 10 min. The cells were washed with 62.5 mM Tris/HCl (pH 6.8) and the pellet was pipetted up and down for 5 min in 4 ml of the same buffer supplemented with 0.1 % (v/v) Triton X-100 (Roche). The suspension was centrifuged at 8000 g for 10 min at

4 °C. The supernatant was collected and centrifuged for 2 h at 51 030 g at 4 °C in order to concentrate the cell surface-associated protein extract. Finally, the protein pellet was resuspended in 100 µl 62.5 mM Tris/HCl (pH 6.8), visualized by SDS-PAGE (Laemmli, 1970) and stored at 4 °C. The SDS-PAGE gel was stained with Brilliant Blue G Colloidal (Sigma) in order to avoid any stain interference with the subsequent procedures. The identification of overexpressed proteins was done by the Servei de Proteòmica i Bioinformàtica (SepBio) of the Universitat Autònoma de Barcelona (UAB). Thus, electrophoretic bands were in-gel digested with trypsin and analysed by MALDI-TOF MS as previously reported (Abella et al., 2007).

RNA techniques. RNA was isolated as described elsewhere (Fontaine et al., 2004), with slight modifications. A 10 ml sample of an *S. suis* culture in the mid-exponential growth phase (OD₆₀₀ 0.6) was collected by centrifugation at 8000 g for 10 min. Cells were then resuspended by vigorous vortexing at 37 °C for 10 min in 300 µl Tris-EDTA prelysis buffer containing 10 mg lysozyme ml⁻¹. Total RNA was extracted by using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. DNA contamination was removed from the RNA during purification by treatment with RNase-free DNase (Qiagen), followed by digestion with DNase Turbo (Ambion). The concentration and integrity of RNA were determined by A₂₆₀ measurements and 1% agarose gel electrophoresis, respectively. RT-PCR assays were done using a Titan One Tube RT-PCR system (Roche) following the manufacturer's instructions. Real-time RT-PCR analysis of gene expression was performed for all genes as reported previously (Campoy et al., 2002), with specific internal oligonucleotides used for each gene (Supplementary Table S2). In all cases, the absence of DNA in RNA samples was tested by PCR without the addition of reverse transcriptase.

Mobility shift assays. Electrophoretic mobility shift assays (EMSAs) were done as described elsewhere (Bsat & Helmann, 1999) with slight modifications. DNA promoters were PCR-amplified from P1/7 *S. suis* genomic DNA using suitable oligonucleotides (Supplementary Table S2), and the purified PCR fragments were cloned in *E. coli* DH5α cells via the pGEM-T vector. The presence of the desired promoter was confirmed by sequencing the plasmid DNA using the primers M13F and M13R. DNA probes were prepared by PCR amplification, using primers of which one was labelled with DIG at its 5' end (Supplementary Table S2). DNA–protein reaction mixtures (20 µl) containing 25

ng DIG-DNA-labelled probe and either 0 or 10 µg purified *S. suis* AdcR protein were incubated in EMSA buffer [20 mM Tris/HCl (pH 8), 50 mM KCl, 5% (v/v) glycerol, 1 µg bulk carrier salmon sperm DNA, 0.5 mM DTT, and 0.1 mg BSA ml⁻¹] for 10 min at room temperature. DNA–protein complexes were visualized by separation on a 5% non-denaturing polyacrylamide gel (40 mM Tris/acetate) at 150 V for 1.5 h and then transferred to a Biodine B nylon membrane (Pall Gelman Laboratory). DIG-DNA-labelled protein complexes were detected according to the manufacturer's protocol (Roche). AdcR protein was purified as previously described (Aranda et al., 2008).

Protection assays. Female BALB/cAnNHsd mice (3 weeks old) obtained from Harlan Iberica were used for these studies. All animals underwent a quarantine period of 1 week. A maximum of four animals per cage were housed under specific-pathogen-free conditions at 19–21 °C with an artificial 12 h light/12 h dark cycle; the relative humidity was 50–60%. Antibiotic-free pelleted food and autoclaved water were provided ad libitum during the experiments. Animals were monitored daily for morbidity and mortality. All animal experiments were approved by the UAB Animal Ethics Committee. For protection assays, groups of eight female 4-week-old BALB/cAnNHsd mice were given intraperitoneal injections (0.1 ml) of 50 µg cell surface-associated proteins adsorbed with Imject Alum adjuvant (aluminium hydroxide; Pierce) according to the manufacturer's protocol. One additional group of eight mice was given injections of 0.1 ml solvent including Imject Alum adjuvant as a negative control. After 2 weeks, a second immunization was carried out, followed 3 weeks later by a challenge with an intraperitoneal injection of a 0.1 ml suspension of TH broth supplemented with 10% inactivated bovine serum containing 108 c.f.u. *S. suis* 89/1591. The survival of the mice was subsequently monitored for 7 days. Serum for Western blot assays was obtained as previously reported (Aranda et al., 2008).

Statistical analysis. Fisher's exact test was used to analyse the statistical significance of the lethal challenge data. A statistical test with a P value less than 0.05 was considered to be significant.

RESULTS AND DISCUSSION

Analysis of the *S. suis* *adcR* regulon composition

It has been predicted *in silico* and demonstrated *in vitro* that the TTAACNRGTTAA motif is the AdcR DNA-binding sequence (Aranda et al., 2008; Panina et al., 2003). A search in the complete sequenced genome of *S. suis* strain P1/7 (http://www.sanger.ac.uk/Projects/S_suis/) with the EditSeq program (dnastar) revealed four ORFs with a motif identical to the AdcR-binding sequence in their promoter regions.

Two of the ORFs are *ssu1390* and *ssu1352*, whose predicted products are homologous, respectively, to internalin A (InlA) of *Listeria monocytogenes* [which allows this bacterium to invade cells (Wollert et al., 2007)] and to the 30S ribosomal protein S14, which lacks the typical zinc-ribbon motif present in most ribosomal proteins. Furthermore, several EMSAs confirmed that the purified AdcR protein binds specifically to the promoters of both genes (Fig. 1F1).

The other two ORFs of strain P1/7 presenting an AdcR-binding sequence correspond to the *S. suis* 89/1591 homologues previously identified as *ssuIDRAFT 1240 ORF (adcR)* and *ssuIDRAFT 0103 ORF* (encoding a putative zinc-binding streptococcal lipoprotein) (Aranda et al., 2008). These ORFs were annotated as *ssu0112* and *ssu0308*, respectively, in the *S. suis* P1/7 strain (Table 1T1). As in strain 89/1591, in P1/7 *adcR* is the first gene of a transcriptional unit, which also contains three components of an ABC transporter (*Ssu0113–0115*) involved in zinc/manganese uptake (Table 1T1). Furthermore, it is worth noting that both *adcR*-regulated *ssuIDRAFT 0103* and *1237* lipoproteins (*Ssu0308* and *Ssu0115* P1/7 homologues) have been reported to be immunogenic and able to confer protection against *S. suis* in mice (Aranda et al., 2008). In this regard, it is known that lipoproteins are the ABC transporter components most exposed outside the Gram-positive cell surface (Hantke, 2001; Sutcliffe & Russell, 1995). Taken together, these data suggest that *adcR* mutant cell surface-associated proteins could be candidates for effective vaccines against *S. suis* infection.

In order to evaluate the role of the AdcR protein in the regulation of genes whose products could present immunogenic and/or protective abilities, the construction and characterization of an *adcR* in-frame deletion mutant was carried out as described in Methods. After electrotransformation with plasmid pSET4 Δ *adcR* to replace the wild-type

adcR gene of *S. suis* strain P1/7 with an adcR-deleted gene, the *S. suis* UA5000 strain (AdcR⁻) was obtained, with the deletion confirmed by both PCR and sequencing (data not shown). The repressor function of the AdcR protein upon these genes was confirmed by the fact that the null-adcR strain showed derepression of all AdcR-regulated ORFs (Fig. 2F2). Moreover, none of the non-adcR regulated ssu0626, ssu1246 and 0118 genes (lacking an AdcR-binding site in their promoter regions) was induced in the adcR mutant strain when compared with the wild-type strain (results not shown).

The AdcR protein regulates an *S. suis* gene that encodes a ribosomal protein paralogue

It has been proposed that genes that encode ribosomal protein paralogues are regulated by AdcR and thus contribute to survival during zinc starvation (Panina et al., 2003). This hypothesis is based on the fact that original copies of ribosome proteins present zinc-ribbon motifs (C-X₂-C-X_n-C-X₂-C) and are thus likely to bind zinc, while paralogous copies lack these motifs. However, the presence of zinc in the ribosomes seems to be required to stabilize them at high temperature only in thermophilic bacteria and Archaea (Makarova et al., 2001). Therefore, it has been suggested that under conditions of zinc starvation, the induction of paralogous genes could lead to the incorporation of these paralogous proteins into the ribosomes in place of the original ribosomal proteins. After degradation of the original zinc-containing ribosomal proteins, the metal becomes available to be used by other zinc-dependent proteins. Scenarios in concordance with this hypothesis have been reported during zinc starvation for ribosomal proteins of *B. subtilis* and *Streptococcus pyogenes* (Brenot et al., 2007; Makarova et al., 2001). In accordance with this, our results showed that expression of ssu1352 ORF in the adcR-knockout strain, encoding ribosomal protein S14, is approximately 100-fold higher than in the wild-type strain (Fig. 2F2). In this respect, an ORF (ssu0083) encoding the putative original 30S ribosomal protein S14 ORF is also present in the complete genome of *S. suis* strain P1/7. As expected, the predicted product of the ssu0083 ORF includes a zinc-ribbon motif (CEKCGRPHSVYRKFKLCRV) which is absent in the AdcR-regulated ssu1352 ORF (data not shown). Moreover, expression of the ssu0083 ORF, which lacks the AdcR-binding site in its promoter region, is not increased in the adcR mutant strain (data not shown). Although further experiments are needed to fully evaluate its validity, our data support the hypothesis that genes encoding ribosomal protein paralogues regulated by AdcR contribute to zinc homeostasis in streptococci.

Two Pht proteins are under AdcR regulation in *S. suis*

SDS-PAGE analysis of cell surface-associated proteins of both the *adcR* mutant and wild-type strains revealed the overexpression of two proteins of approximately 120 and 100 kDa in the *adcR*-knockout strain (Fig. 3F3). To identify them, both protein gel bands were in-gel digested with trypsin and the peptides obtained were identified by MALDI-TOF MS (data not shown). The results indicated that the two proteins corresponded to *ssu0309* and *ssu1103* ORF products, which have both been annotated as Pht (pneumococcal histidine triad) proteins, showing scores of 165 and 73, respectively (using the mascot search engine, version 2.1). The predicted products of these two genes are proteins of 116.5 and 95.4 kDa molecular mass, respectively, in agreement with the apparent molecular mass seen on SDS-PAGE (Fig. 3F3). Pht proteins have only been found in pathogenic streptococcal species and are involved in the invasion process (Adamou et al., 2001; Panina et al., 2003). This family of proteins shows a conserved and repeated motif HxxHxH (histidine triad motif) which is implicated in zinc binding (Adamou et al., 2001; Panina et al., 2003; Riboldi-Tunnicliffe et al., 2005).

Overexpression of *Ssu1103* and *Ssu0309* proteins in the *adcR* mutant strain suggested that they are under the control of AdcR. In this respect, it must be noted that an AdcR-like motif (TTAACTAGTAAA) is found at position -230 with respect to the *ssu1103* predicted translational starting point. For this reason, the ability of *S. suis* AdcR protein to bind the promoter region of *ssu1103* ORF was analysed by EMSA. Data showed that the AdcR protein specifically binds to the promoter of this gene (Fig. 4F4).

Moreover, and since *ssu0308* and *ssu0309* ORFs were demonstrated to be co-transcribed by RT-PCR analysis (data not shown), a search for putative AdcR boxes upstream of this transcriptional unit was carried out. As expected, one motif with these characteristics (TTAACTGGTTAA) was found at position -26 with respect to the predicted translational starting point of the *ssu0308* ORF, whose product is homologous (99% identity) to the protein encoded by the AdcR-regulated *ssuIDRAFT 0103* ORF of the 89/1591 *S. suis* strain (Table 1T1). Furthermore, EMSA experiments demonstrated that the *S. suis* AdcR protein is able to bind this AdcR-like sequence present upstream of the *ssu0308*–*ssu0309* transcriptional unit (data not shown). In accordance with all these data, derepression of both *ssu1103* and *ssu0309* ORFs was observed in the *adcR* mutant strain (Fig. 5F5).

Furthermore, Pht proteins are involved in the host-cell–pathogen interaction, and it has been hypothesized that they have evolved to become sensitive to AdcR regulation in order to exploit zinc as a compartment-specific regulatory cue (Brenot et al., 2007). It is also noteworthy that Pht proteins present hydrophobic leader sequences, which presumably target these proteins to the bacterial cell surface and induce specific antibodies against pneumococcal sepsis, suggesting that Pht proteins could serve as effective vaccines against streptococcal infections (Adamou et al., 2001; Kunitomo et al., 2008). Our data effectively demonstrate the presence of Pht proteins at the *S. suis* cell surface and that AdcR protein is not only involved in cation homeostasis but also controls putative virulence factors.

Protective capacities of cell surface-associated proteins of *adcR*, *fur* and *adcR fur* *S. suis* mutants

As mentioned above, the *adcR* mutant strain overexpresses genes encoding predicted immunogenic proteins. For that reason, the cell surface-associated proteins of both wild-type and *adcR*-null strains were analysed on Western blots by using immunologically reactive serum obtained from surviving mice after inoculation with the *S. suis* 89/1591 wild-type strain. Results showed that the *adcR* mutant strain extracts presented more immunological proteins than the wild-type strain (Fig. 6F6). However, no significant protective effect was obtained when the cell surface-associated proteins of the *adcR* mutant strain were inoculated into mice prior to a challenge with the wild-type *S. suis* 89/1591 strain (data not shown). This result suggested that *adcR*-regulated proteins associated with the cell surface are not sufficient to prevent the infective process in mice. This could be due to the amount of immunogenic proteins not being sufficient or their exposure to the external medium not being as extensive as necessary to confer good protection. In fact, previous data have demonstrated that a recombinant *adcR*-regulated protein (SsuiDRAFT 0103) is sufficient to confer significant protection against this pathogen in mice (Aranda et al., 2008).

A search in the NCBI database (<http://www.ncbi.nlm.nih.gov/>) allowed the identification of the *Streptococcus thermophilus fur* gene (gene ID: 3166852). Then, this gene was used as a probe against the Sanger blast server (http://www.sanger.ac.uk/Projects/S_suis/), enabling the identification of the *S. suis* P1/7 *fur* gene, which has a high degree of identity (64%) to the *S. thermophilus fur* gene. As mentioned above, the Fur protein binds iron in

the cytoplasm of many bacterial cells and blocks the transcription of iron-regulated genes, several of which express strongly immunogenic proteins (Escobar et al., 1999; Hantke, 1981; Ratledge & Dover, 2000). After electrotransformation with plasmid pSET4Δ*fur* to replace the wild-type *fur* gene of *S. suis* P1/7 strain with a *fur*-deleted gene, a *fur*-defective *S. suis* strain (UA5001) was obtained, with the deletion confirmed by both PCR and sequencing (data not shown). However, no significant protective effect was obtained when the cell surface-associated proteins of the *fur* mutant strain were used to inoculate mice prior to a challenge with the wild-type *S. suis* 89/1591 strain (data not shown). Then, to increase the putative immunogenic capacity of both *S. suis* mutants, the construction of a double mutant strain (UA5002, *AdcR*[−] *Fur*[−]) was carried out. The presence of both the *adcR* and *fur* gene deletions was confirmed by both PCR and sequencing (data not shown).

Analysis of the SDS-PAGE profile of *S. suis* UA5002 (*AdcR*[−] *Fur*[−]) cell surface-associated proteins revealed that the double mutant strain presented a higher number of immunogenic proteins than both *S. suis* single mutants (Fig. 6F6). In order to determine whether the induction of this immunogenic response was enough to protect mice against *S. suis* infection, three groups of eight mice were immunized twice with 50 μg cell surface-associated proteins of the double mutant strain, 50 μg cell surface-associated proteins of the wild-type strain, or the solvent including the adjuvant (as a control). When the animals were challenged with 108 c.f.u. *S. suis* 89/1591, all the mice belonging to either the control group or the group vaccinated with the wild type extract died within 5 days of infection (Fig. 7F7). In contrast, 50% of mice in the group vaccinated with the double mutant extract inoculated with an identical dose of the virulent strain remained alive throughout the experiment (Fig. 7F7). In addition, both wild-type and *adcR fur* double mutant cell surface-associated proteins were analysed on Western blots by using immunologically reactive serum obtained from mice surviving after inoculation with the *S. suis* 89/1591 wild-type strain. The results provided clear evidence that the protective effect of the double mutant extract was correlated with specific antibodies against the overexpressed antigens of this strain (Fig. 6F6). It is worth noting that negative results (no reactivity) were obtained when all the *S. suis* extracts used in this work were tested with serum from non-infected mice (data not shown). All these data indicated that the combination of cell surface-associated proteins under the control of either zinc/manganese- or iron-uptake regulators (*AdcR* and *Fur*, respectively) not only is

immunogenic but also confers a statistically significant level of protection ($P=0.038$) against *S. suis* challenge to mice immunized with these proteins.

To sum up, the data reported in this work demonstrate that the use of cell surface extracts of *S. suis* double mutants defective in genes regulating either Zn^{2+}/Mn^{2+} or Fe^{2+} cations is able to protect mice against challenge with *S. suis*. This double mutant-based strategy has not to our knowledge been used to date and can be applied to other virulent bacterial species. Thus, mutagenesis of this kind of regulator makes it possible to obtain effects in the overexpression of divalent cation-uptake transporters on the bacterial cell surface similar to those induced by chelating agents, overcoming problems associated with the use of these chemicals, such as poor bacterial cell growth, and simplifying the bulk production of this antigen source. In addition, the data reported in this work indicate that the use of purified immunogenic proteins such as Pht or SsuiDRAFT 0103 is a more promising vaccination strategy than the use of protein extracts.

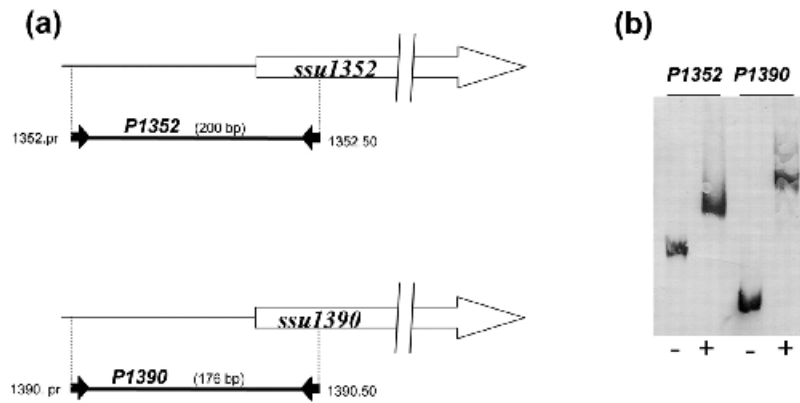


Fig. 1.(a) Schematic representation of *ssu1352* and *ssu1390* ORFs with their promoter regions (P1352 and P1390, respectively). Small arrows indicate the primer pairs used to amplify the promoters for EMSA experiments. (b) Electrophoretic mobility of the DNA fragments containing the indicated *S. suis* promoters in the absence (-) or presence (+) of purified *S. suis* AdcR protein.

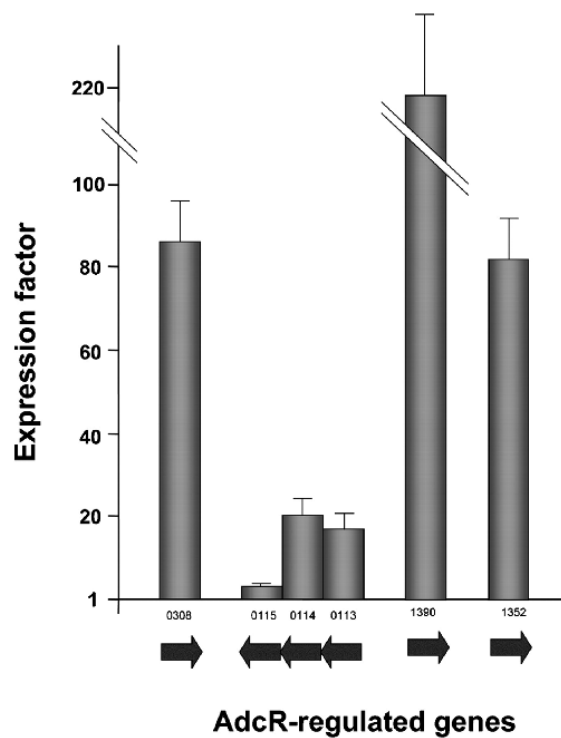


Fig. 2.Expression factors of several ORFs (Table 1T1) in the *S. suis* *adcR* mutant (UA5000). The expression factor is the ratio of the mRNA concentration of each gene from the UA5000 strain with respect to the wild-type strain (P1/7). The amount of mRNA of each gene was determined by using a standard curve generated by the amplification of an internal fragment of the *S. suis* P1/7 ORF encoding cysteine synthase (see

Supplementary Table S2 for primer sequences). The results are the mean of two independent experiments, each carried out in duplicate. Error bars, sd.

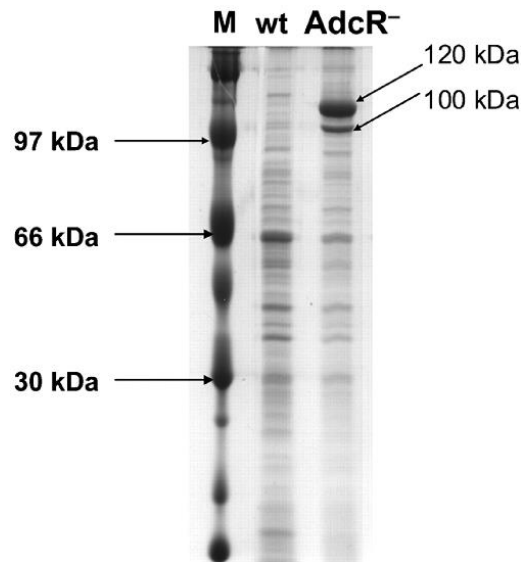


Fig. 3. SDS-PAGE profile of cell wall-associated proteins of both *S. suis* P1/7 (wt) and UA5000 (*AdcR*⁻) strains. Two overexpressed proteins of UA5000 cell extracts are indicated by arrows with their apparent molecular masses. The molecular mass marker (M) and its values are shown on the left of the figure.

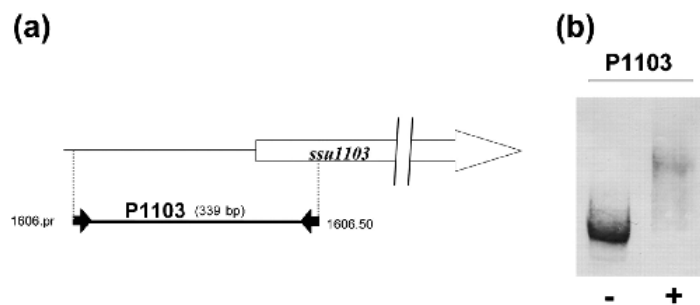


Fig. 4.(a) Schematic representation of the *ssu1103* ORF with its promoter region (P1103). Small arrows indicate the primer pairs used to amplify the promoters for EMSA experiments. (b) Electrophoretic mobility of the DNA fragment containing the indicated *S. suis* promoter in the absence (-) or presence (+) of purified *S. suis* *AdcR* protein.

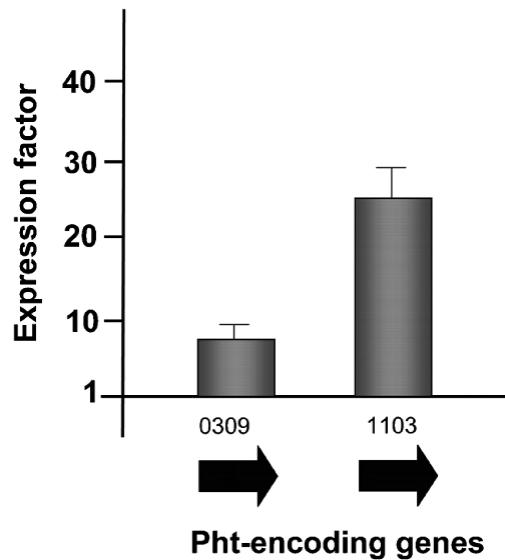


Fig. 5. Expression factors of Pht-encoding ssu0309 and ssu1103 ORFs in the *S. suis* *adcR* mutant (UA5000). The expression factor is the ratio of the mRNA concentration of each gene from the UA5000 strain with respect to the wild-type strain (P1/7). The amount of mRNA of each gene was determined by using a standard curve generated by the amplification of an internal fragment of the *S. suis* P1/7 ORF encoding cysteine synthase (see Supplementary Table S2 for primer sequences). The results are the means of two independent experiments, each carried out in duplicate. Error bars, sd.

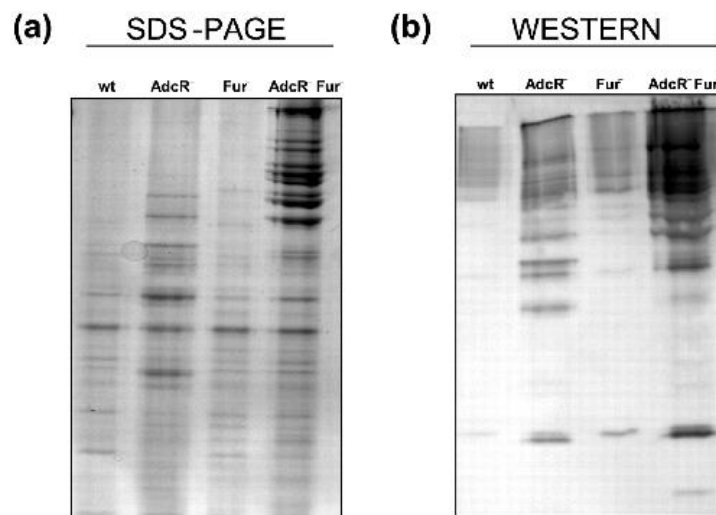


Fig. 6.(a) SDS-PAGE profile of *S. suis* P1/7 wild-type (wt), UA5000 (*AdcR*⁻), UA5001 (*Fur*⁻) and UA5002 (*AdcR*⁻ *Fur*⁻) strains. (b) Western blot analysis with serum recovered from mice infected with *S. suis* strain 89/1591. The analysis was carried out with cell surface-associated proteins obtained from *S. suis* P1/7 wild-type (wt), UA5000 (*AdcR*⁻), UA5001 (*Fur*⁻) and UA5002 (*AdcR*⁻ *Fur*⁻) strains.

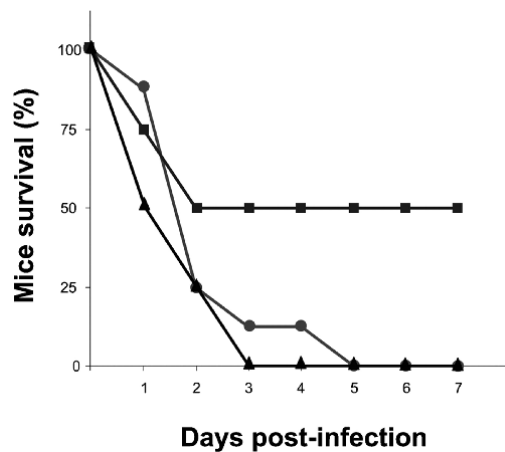


Fig. 7. Survival curve of mice vaccinated with cell surface-associated proteins of wild-type P1/7 strain (●), cell surface-associated proteins of adcR for UA5002 strain (■), or solvent including the adjuvant as a negative control (▲). After the last immunization, an intraperitoneal challenge with 10⁸ c.f.u. wild-type *S. suis* 89/1591 cells was administered to each animal (n=8 mice per group).

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Table 1. Nomenclature of *adcR*-regulated genes in P1/7 and 89/1591 *S. suis* strains

ORF (P1/7)*	ORF (89/1591)†	Putative function	AdcR-binding sequence and position‡
ssu0112§	ssuiDRAFT 1240	Regulatory protein (AdcR)	TTAACAAGTTAA (–28)
ssu0113§	ssuiDRAFT 1239	ABC transporter component; ATPase	
ssu0114§	ssuiDRAFT 1238	ABC transporter component; permease	

ssu0115§	ssuiDRAFT 1237	ABC transporter component; zinc-binding lipoprotein	
ssu0308			ssuiDRAFT 0103
ssu0309			ssuiDRAFT 1195
ssu1390	ssuiDRAFT 0326	Internalin A	TTAACTGGTTAA (–31)
ssu1352	ssuiDRAFT 0240	30S ribosomal protein S14	TTAACTGGTTAA (–36)
ssu1103	ssuiDRAFT 1606	Pht protein	TTAACTAGTAAA (–230)

Table Footnotes:

- *ORF name in *S. suis* strain P1/7 (sanger.ac.uk).
- †ORF name in *S. suis* strain 89/1591 (<http://www.ncbi.nlm.nih.gov>).
- ‡Position with respect to its predicted translational starting point.
- §||Genes constituting a single transcriptional unit. The AdcR motif is located upstream of the first gene listed.