Journal of Bacteriology

TroA of Streptococcus suis Is Required for Manganese Acquisition and Full Virulence

Paul J. Wichgers Schreur, Johanna M. J. Rebel, Mari A. Smits, Jos P. M. van Putten and Hilde E. Smith

J. Bacteriol. **2011, 193(19):5073. DOI: 10.1128/JB.05305-11. Published Ahead of Print 22 July 2011.**

JOURNAL OF BACTERIOLOGY, Oct. 2011, p. 5073–5080 Vol. 193, No. 19
0021-9193/11/$12.00 doi:10.1128/JB.05305-11

TroA of *Streptococcus suis* Is Required for Manganese Acquisition and Full Virulence▽

Paul J. Wichgers Schreur,[1,3]* Johanna M. J. Rebel,[1] Mari A. Smits,[1,2] Jos P. M. van Putten,[3] and Hilde E. Smith[1]

Central Veterinary Institute, Wageningen UR, Lelystad, Netherlands[1]; Wageningen Livestock Research, Wageningen UR, Lelystad, Netherlands[2]; and Department of Infectious Diseases and Immunology, Utrecht University, Utrecht, Netherlands[3]

Received 16 May 2011/Accepted 11 July 2011

Streptococcus suis **causes infections in pigs and occasionally in humans, resulting in manifestations as meningitis, sepsis, arthritis, and septic shock. For survival within the host, *S. suis* requires numerous nutrients including trace metals. Little is known about the specific proteins involved in metal scavenging in *S. suis*. In this study we evaluated the role of the putative high-affinity metal binding lipoprotein TroA in metal acquisition and virulence. A mutant strain deficient in the expression of TroA (Δ*troA* mutant) was constructed. Growth of the Δ*troA* mutant in Todd-Hewitt broth was similar to wild-type growth; however, growth of the Δ*troA* mutant in cation-deprived Todd-Hewitt broth and in porcine serum was strongly reduced compared to growth of wild-type bacteria. Supplementing the medium with extra manganese but not with magnesium, zinc, copper, nickel, or iron restored growth to wild-type levels, indicating that TroA is specifically required for growth in environments low in manganese. The Δ*troA* mutant also showed increased susceptibility to H_2O_2, suggesting that TroA is involved in counteracting oxidative stress. Furthermore, the expression of the *troA* gene was subject to environmental regulation at the transcript level. In a murine *S. suis* infection model, the Δ*troA* mutant displayed a nonvirulent phenotype. These data indicate that *S. suis* TroA is involved in manganese acquisition and is required for full virulence in mice.**

Streptococcus suis is an important pathogen of pigs and may cause meningitis, sepsis, arthritis, and septic shock. Occasionally, *S. suis* is able to infect humans. Infected humans may show symptoms similar to those in pigs (1, 5, 11, 32). Although human infections are exceptional, a large outbreak in humans was reported in 2005 in China, with 215 cases and 39 deaths (52). Of the 33 known *S. suis* serotypes, serotype 2 is most frequently isolated from diseased pigs and humans. However, serotype 9 infections are emerging in pigs, especially in Europe (7, 35, 48). Current control measures are insufficient and mainly rely on antibiotic treatment and vaccination with homologous bacterins. Increased antibiotic resistance has been reported for *S. suis* (17, 49), and bacterin-based vaccines do not provide protection against multiple serotypes (6).

For growth and function, bacteria have to acquire numerous nutrients from their surrounding environment. For pathogenic bacteria, an important group of essential nutrients are the trace metals. Metals such as iron, zinc, and manganese have been shown to be essential structural and catalytic cofactors for several bacterial proteins (2). However, the concentration of free available trace metals within an infected host is relatively low compared to the metal concentrations in medium usually applied for *in vitro* growth. Within the host, several trace metals are sequestered; for instance, iron binds to hemoglobin, and zinc and manganese bind to the S100 family of proteins produced by neutrophils (14, 15). This recruitment of trace metals by host proteins has recently been regarded as a mechanism of "nutritional immunity" (46). To counteract nutritional immunity, bacteria have evolved several mechanisms to efficiently scavenge trace metals from protein-metal complexes. An important group of bacterial proteins able to scavenge metals with high affinity are the metal binding lipoproteins. The presence of these proteins on the bacterial surface allows bacteria to acquire metals and to sustain growth in environments with limited amounts of free trace metals (22).

With the increase in bacterial genome sequencing efforts, increasing numbers of genes encoding putative high-affinity metal binding lipoproteins have been identified. Within each *S. suis* isolate sequenced so far, at least four potential high-affinity metal binding lipoproteins have been annotated (12, 18). Three (SSU0115, SSU0308, and SSU0606 of *S. suis* isolate P1/7) have been partially characterized in *S. suis* isolate 89/1591 and were shown to be immunogenic and inducible under divalent cation deprivation (3). The fourth putative metal binding lipoprotein, designated TroA and corresponding to SSU1869 in *S. suis* isolate P1/7, was found to be present in an *S. suis* cell wall fraction which was very effective in inducing proinflammatory cytokine and chemokine transcription of porcine peripheral blood mononucleated cells (47). The objective of the present study was to investigate the ability of TroA to scavenge specific metals and to investigate the importance of this scavenger function for growth under cation-deprived conditions, for the oxidative stress response, and for virulence in a murine infection model.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A virulent *S. suis* serotype 9 pig isolate (H. E. Smith et al., unpublished results), strain 8067, and its isogenic mutants as well as complemented mutant strains were routinely grown on Co-

* Corresponding author. Mailing address: Central Veterinary Institute, Wageningen UR, Edelhertweg 15, 8219 PH Lelystad, Netherlands. Phone: 31320238423. Fax: 31320238153. E-mail: paul.wichgersschreur@wur.nl.

▽ Published ahead of print on 22 July 2011.

TABLE 1. Primer sequences

Primer no.	Application	Primer name	Sequence (5′–3′)[a]
1	Δ*troA* mutant	TroA-partB-2-F_ApaI	**GGGCCC**TGGAGAAGATTGGCTGGAGTGG
2	Δ*troA* mutant	TroA-partB-2B-R_SalI	CC**GTCGAC**GGACAATAGCAGAGCAAAACTGAAC
3	Δ*troA* mutant	TroA-partA-2-F_XmaI	TC**CCCGGG**GATTGTCGATCCGCACTTCTGG
4	Δ*troA* mutant	TroA-partA-2-R_SacII	**CCGCGG**CAACAATAGCCGTCCGACTCCC
5	Δ*troA* mutant	Over TroA heen F	AAATAGTCGGAATGACAGCACTGG
6	Δ*troA* mutant	Over TroA heen R	CTATGCCGTAGGTCGCCTGATAA
7	Δ*troA* mutant	Falling out TroA 2 F	GGCTCTAGTAAGCCAAGAGTGG
8	Δ*troA* mutant	Falling out TroA 2 R	CATCCTCATCCATCGTATTCAAGTC
9	Spectinomycin	SpecF_SalI	GC**GTCGAC**GCAGGTCGATTTTCGTTCGT
10	Spectinomycin	SpecR_XmaI	AT**CCCGGG**ATGCAAGGGTTTATTGTTTTCTAA
11	pSET5 vector	RepA-F	GGGCGTATCTATGGCTGTCA
12	pSET5 vector	RepA-R	CTCCCCTAAGGCGAATAAAAG
13	Expression of *troA*	Expr-TroA-F-BamHI	AG**GGATCC**CTTCTCTGCGATGAGTTTATTGGTAA
14	Expression of *troA*	Expr-TroA-R-SalI	AT**GTCGAC**ATTTTCGCAGAGCACCAATGC
15	Expression of *troA*	Chloramp-F-SalI	AG**GTCGAC**CTTGGTCTGACAGTTACCAATGC
16	Expression of *troA*	Chloramp-R-Sall	GG**GTCGAC**CCGAGGCTCAACGTCAATAAAGC
17	Real-time PCR	SSU0934-Fabi	GCTTATGACGCCTATACACCTGAA
18	Real-time PCR	SSU0934-Rabi	AAGGCAAAACCAATACCGAACA
19	Real-time PCR	SSU1078-Fabi	GCTTATGACGCCTATACACCTGAA
20	Real-time PCR	SSU1078-Rabi	TCGCCAATTGAGCAAAATCTG
21	Real-time PCR	SSU1869-Fabi	CCTTGTTCTCTATCACGGTTTGC
22	Real-time PCR	SSU1869-Rabi	CCATCCTCATCCATCGTATTCA

[a] Sequences in boldface correspond to restriction sites.

lombia agar plates (Oxoid Ltd., London, United Kingdom) supplemented with 6% horse blood and incubated at 37°C with 5% CO_2. Suspension cultures were grown in Todd-Hewitt broth ([THB] Oxoid Ltd., London, United Kingdom) for 18 h at 37°C without agitation. *Escherichia coli* was grown on Luria-Bertani (LB) agar plates or in LB broth. When required, antibiotics were added to the growth medium at the following concentrations: for *E. coli*, ampicillin at 100 μg/ml, chloramphenicol at 8 μg/ml, and spectinomycin at 100 μg/ml; for *S. suis*, chloramphenicol at 5 μg/ml and spectinomycin at 100 μg/ml.

Protein sequence analysis. Protein alignments were performed using the MegAlign program of DNASTAR and were visualized with the Jalview program (13).

Growth evaluation. (i) THB growth. Overnight THB cultures of wild-type and mutant bacteria were diluted 1:100 in fresh THB supplemented with or without 0.5 mM EDTA. Subsequently, the optical density at 600 nm of 400-μl samples was followed in time using a Bioscreen C instrument (Thermo Scientific, Breda, The Netherlands) at 37°C.

(ii) Plate assay. Overnight *S. suis* THB cultures were diluted to 10^5 CFU/ml in Dulbecco's phosphate-buffered saline (D-PBS). Subsequently, 3 μl of bacterial suspension was spotted onto Colombia agar plates supplemented with 6% horse blood and EDTA (2 or 0.5 mM) and various trace metals (0.5 mM). Bacterial growth was evaluated after 24 h of incubation at 37°C and 5% CO_2.

(iii) Growth in porcine serum. Overnight THB cultures were diluted to 10^9 CFU/ml in D-PBS. Subsequently, 100% normal porcine serum (Sigma-Aldrich, Zwijndrecht, The Netherlands) with or without additional $MnCl_2$ (0.5 mM) was inoculated with 10^4 CFU of *S. suis* per ml. At the start and after 3 h of incubation at 37°C (without agitation), bacteria were serially diluted and plated onto Colombia agar plates containing 6% horse blood.

DNA techniques. Chromosomal *S. suis* DNA was isolated as previously described (34). PCRs were used to amplify specific fragments. PCR mixtures consisted of 1× Phusion High-Fidelity DNA polymerase master mix (Bioke, Leiden, The Netherlands) containing specific primers at a final concentration of 0.25 μM and containing 0.1 to 0.5 ng of DNA template/μl. PCR conditions were as follows: denaturation for 1 min at 98°C, followed by 35 cycles of 15 s of denaturation at 98°C, 15 s of annealing at 55°C, and 15 s/kb of elongation. Plasmid DNA was isolated with a Plasmid DNA Purification System (Promega, Leiden, The Netherlands). DNA purifications were performed with zymogen cleanup kits (BaseClear, Leiden, The Netherlands). Ligations were performed with T4 DNA ligase (Promega), and ligation mixtures were used to transform *E. coli*. Plasmids were introduced into *S. suis* via electroporation (37).

Generation of the *troA* mutant (Δ*troA*). Primers used in this study are listed in Table 1, and PCRs were performed under the conditions described above. Primers 1 and 4 were used to amplify a fragment of the chromosomal DNA of strain 8067 containing the intact *troA* gene flanked on both sides by 1.5-kb regions. This fragment was ligated to the blunt cloning vector pJET1.2 (Fermentas, St. Leon-Rot, Germany) according the manufacturer's instructions, and ligation mixtures were transformed to *E. coli.* Plasmid DNA (designated pJET-*troA*) obtained from transformants was then used to replace an internal fragment (about 300 bp) of *troA* by the spectinomycin (Spc) resistance cassette. To do this, we used an inverse PCR strategy on pJET-*troA* using primers 2 and 3. In addition, the Spc cassette was amplified from pGA14-*spc* (22) using primers 9 and 10. The amplified fragments were purified, digested with XmaI and SalI, and ligated. Ligation mixtures were introduced into *E. coli* to generate pJET-*troA-spc.* The entire insert fragment of pJET-*troA-spc* was subsequently amplified using primers 1 and 4 and ligated to the thermosensitive shuttle vector pSET5 (41), which was linearized with the SmaI restriction enzyme, generating pSET5-*troA-spc*. The pSET5-*troA-spc* plasmid was then introduced into *S. suis* strain 8067 by electroporation, and transformants were selected on Columbia agar plates at 30°C in the presence of spectinomycin. Several individual colonies were grown overnight in THB (10 ml) containing spectinomycin at 30°C. The overnight cultures were then diluted 1:100 in THB without antibiotics and incubated for 4 h at 38°C. Cultures were serially diluted on Columbia agar plates containing spectinomycin at 38°C to select for chromosomal integration. Individual colonies that had lost the vector-mediated chloramphenicol resistance were confirmed to have the expected mutant genotype by PCR using primer pairs 5/6, 7/8, and 11/12.

Complementation of the *troA* mutant. To complement the *troA* mutant with an intact *troA* gene, we constructed an expression plasmid containing the wild-type *troA* gene including its putative promoter region. Primers 13 and 14 were used to amplify the *troA* fragment, using the PCR conditions described above. The fragment was cloned into pJET1.2, generating pJET1.2-*troA*-expr. Subsequently, pJET1.2-*troA*-expr was digested with SalI and BamHI, and the *troA* fragment was purified and cloned into pGA14 (33) digested with SalI and BamHI, generating pGA14-*troA*-expr. Finally, the chloramphenicol resistance gene (*cat*) of pSET5, amplified with primers 15 and 16 and digested with SalI, was introduced at the SalI sites of pGA14-*troA*-expr to yield pGA14-*troA*-expr-*cat*. The plasmid was subsequently introduced into the Δ*troA* mutant generating Δ*troA*::pGA14-*troA*. RNA expression of the *troA* gene in the Δ*troA*::pGA14-*troA* mutant was confirmed by quantitative real-time PCR.

TroA expression analysis. Porcine serum (100 ml) with or without additional $MnCl_2$ (0.5 mM) was inoculated with 5×10^5 CFU of *S. suis*/ml and allowed to grow for 6 h. Bacteria were collected by centrifugation (4,500 × *g* for 30 min), and RNA was isolated using 1 ml of TRIzol reagent (Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions. Subsequently, the samples were DNase treated and further purified using a High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany). RNA quantity and quality were checked with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA) and a Bioanalyzer system (Agilent, Amstelveen, The

Netherlands). Fifty ng of RNA (RNA integrity number [RIN] of >7) was used to prepare cDNA using random hexamers (Promega) and Superscript III (Invitrogen) according the manufacturers' instructions. *troA* expression levels were subsequently measured using *troA*-specific primers (Table 1). In the PCR, 20-times-diluted cDNA was added to 1× Power SYBR green master mix (Applied Biosystems, Nieuwe Kerk aan de IJssel, The Netherlands) containing 0.625 μM (each) forward and reverse primer. The PCR was performed on a 7500 Fast Real-Time PCR system (Applied Biosystems). The PCR program consisted of a denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 59°C for 30 s, and elongation at 72°C for 36 s. As a control, mRNA transcription levels of two unrelated lipoproteins (corresponding to SSU934 and SSU1078 in *S. suis* strain P1/7) were assessed. Expression levels were expressed as threshold cycle (C_T) values.

Oxidative stress assay. To measure the susceptibility of *S. suis* toward oxidative stress, an adaptation of the method of Johnson et al. (19) was used. Briefly, overnight-grown bacteria were diluted to 1×10^8 CFU/ml in 10 ml of THB. Subsequently, cultures were subdivided into two cultures of 5 ml, and H_2O_2 was added to one culture to a concentration of 25 mM. After 30 min of incubation at 37°C (with agitation at 200 rpm), samples were serially diluted in D-PBS containing 1 mg/ml catalase (Sigma-Aldrich) and plated onto Colombia agar plates to determine the viable counts.

CD1 murine infection model. A total of 25 female 5-week-old CD1 mice (Charles River Laboratorium, Maastricht, The Netherlands) were randomly divided into two groups of 10 mice and one control group of 5 mice. After 1 week, mice were inoculated intraperitoneally with 1×10^9 CFU in 0.5 ml of D-PBS of either wild-type or Δ*troA* mutant bacteria (day 0). Control mice were sham inoculated with D-PBS. Mice were subsequently monitored twice a day for clinical signs over a period of 10 days. Mice showing irreversible disease symptoms (including apathy, lethargy, and/or nerve disorders) were euthanized. Surviving animals were euthanized on day 10 postinfection. After euthanization, liver, spleen, and brain were collected, homogenized in D-PBS, and plated for bacterial counts. The experiment was approved by the Animal Experiments Committee of the Central Veterinary Institute (Lelystad, The Netherlands), in accordance with the Dutch Experiments on Animals Act.

Statistical analysis. Statistical analyses were performed in GraphPad Prism. Normal distribution of data was evaluated using a Kolmogorov-Smirnov test. Subsequently, normally distributed data were analyzed using an unpaired Student's *t* test, and non-normally distributed data were analyzed using a Mann-Whitney test. *P* values of <0.05 were taken as significant.

RESULTS

Structural analysis of *S. suis* TroA. In the NCBI database, SSU1869 of *S. suis* serotype 2 isolate P1/7 is predicted to be part of the TroA-like superfamily, and we therefore designated the protein TroA. The protein is 307 amino acids long and contains a signal peptide of 19 amino acids. At the COOH end of the signal peptide, a lipobox motif is present, suggesting that TroA is a lipoprotein (18). The P1/7 TroA protein shows a high level of amino acid sequence identity (>99%) to corresponding proteins in other serotype 2 isolates (12, 18) and to a serotype 1 (isolate 6388), a serotype 7 (isolate 7919), and a serotype 9 (isolate 8067) (unpublished data) isolate (Fig. 1). Orthologous proteins in other streptococcal species share protein sequence identity levels of less than 30%; however, total alignment scores are >100 (1) (Fig. 1). In *Streptococcus pneumoniae* the closest TroA orthologue is known as the pneumococcal surface adhesin A protein (PsaA) (23), and in *Streptococcus uberis* and *Streptococcus agalactiae*, the closest TroA orthologues are known as MtuA (45) and MtsA (42), respectively. TroA of *S suis* is predicted to be part of an ABC transport system, expressing a permease protein (SSU1865; TroD), a membrane protein (SSU1866; TroC), and an ATP-binding protein (SSU1867; TroB) (Kyoto Encyclopedia of Genes and Genomes). The histidine residues at amino acid positions 66, 129, and 195 and the aspartic acid at amino acid position 279 of the *S. suis* TroA protein correspond to the metal binding residues in PsaA (24).

TroA is involved in metal scavenging. To provide experimental evidence for the proposed biological function of the *S. suis* TroA lipoprotein in metal scavenging, we constructed a *troA* isogenic mutant (Δ*troA*) and a *troA* complemented Δ*troA* mutant (Δ*troA*::pGA14-*troA*) in the *S. suis* serotype 9 strain 8067. Growth of the wild-type, mutant, and complemented mutant bacteria was evaluated by measuring optical densities in nutrient-rich THB and in cation-deprived THB. Cationic deprivation of THB was obtained by supplementing THB with the cation binding compound EDTA. In nutrient-rich THB, growth rates of Δ*troA* and Δ*troA*::pGA14-*troA* mutant bacteria were similar to growth of wild-type bacteria (Fig. 2A). However, in contrast to wild-type and complemented bacteria, growth of the Δ*troA* mutant bacteria was strongly reduced in cation-deprived THB (Fig. 2B). The differences in growth rates between wild-type and mutant bacteria were most apparent at a concentration of 0.5 mM EDTA (data not shown). The reduced growth of the Δ*troA* mutant in cation-deprived medium provides evidence that TroA is required for growth in environments low in trace metal concentrations.

TroA is involved in manganese acquisition. To identify which specific metal(s) limits *S. suis* growth in cation-deprived medium and which require TroA expression, we evaluated growth of the Δ*troA*, Δ*troA*::pGA14-*troA*, and wild-type bacteria on cation-deprived Colombia agar plates (0.5 mM EDTA) supplemented with specific trace metals. The Δ*troA* mutant bacteria were unable to grow on plates containing 0.5 mM EDTA in contrast to wild-type and the complemented Δ*troA*::pGA14-*troA* mutant bacteria (Fig. 3). Addition of 0.5 mM free magnesium chloride ($MgCl_2$), zinc chloride ($ZnCl_2$), copper chloride ($CuCl_2$), nickel sulfate ($NiSO_4$), or iron sulfate ($FeSO_4$) did not restore growth of the Δ*troA* mutant bacteria; however, growth was restored by the addition of 0.5 mM manganese chloride ($MnCl_2$). We next examined the minimal concentration of manganese chloride required to restore growth in cation-deprived THB. As shown in Fig. 4, growth of the Δ*troA* mutant could be restored by the addition of manganese chloride at concentrations of ≥20 μM. These results indicate that the TroA protein is required for growth in medium containing <20 μM free manganese chloride.

TroA is required for manganese acquisition in porcine serum. To test whether TroA-mediated manganese acquisition is also important for bacterial growth in a more biologically relevant environment, we evaluated growth of the wild-type, Δ*troA*, and Δ*troA*::pGA14-*troA* mutant bacteria in porcine serum. Wild-type and Δ*troA*::pGA14-*troA* mutant bacteria were able to grow efficiently in porcine serum at levels similar to growth in THB (Fig. 5). Growth of Δ*troA* mutant bacteria in porcine serum was strongly reduced compared to growth of the wild-type and Δ*troA*::pGA14-*troA* mutant bacteria, whereas addition of manganese to the porcine serum restored growth of the Δ*troA* mutant bacteria toward wild-type levels (Fig. 5). These results indicate that manganese levels in porcine serum are insufficient to sustain efficient growth of Δ*troA* mutant bacteria. The results also indicate that *S. suis* TroA has an important role in manganese acquisition in environments, like porcine serum, low in free available manganese (27).

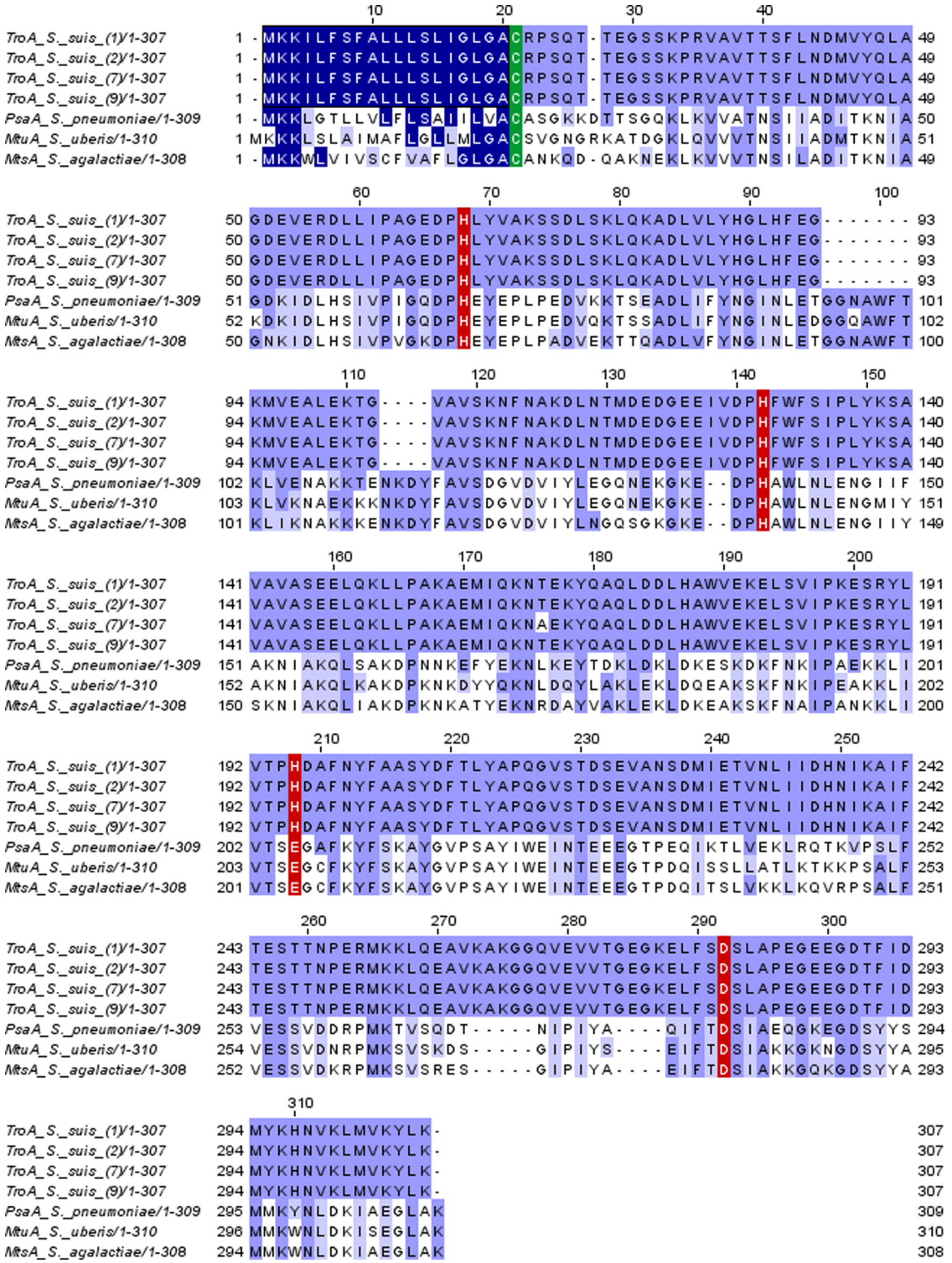

FIG. 1. Sequence features of *S. suis* TroA. ClustalW alignment of the TroA protein present in a serotype 1 (isolate 6388), a serotype 2 (isolate P1/7), a serotype 7 (isolate 7919), and a serotype 9 (isolate 8067) *S. suis* isolate with orthologous in *S. pneumoniae* (PsaA), *S. uberis* (MtuA), and *S. agalactiae* (MtsA). The residues corresponding to the metal binding residues of PsaA are marked in red, the cysteine (C) of the lipobox is marked in green, and the signal peptide is marked in dark blue.

TroA expression is regulated by manganese availability. Bacteria often tightly regulate the expression of their high-affinity metal binding lipoproteins at the transcriptional level (3, 53). To evaluate whether *troA* expression is regulated by the environmental manganese concentration, we isolated RNA of wild-type *S. suis* serotype 9 strain 8067 grown in porcine serum with or without additional manganese and determined the mRNA levels of *troA* and two unrelated lipoproteins (homol-

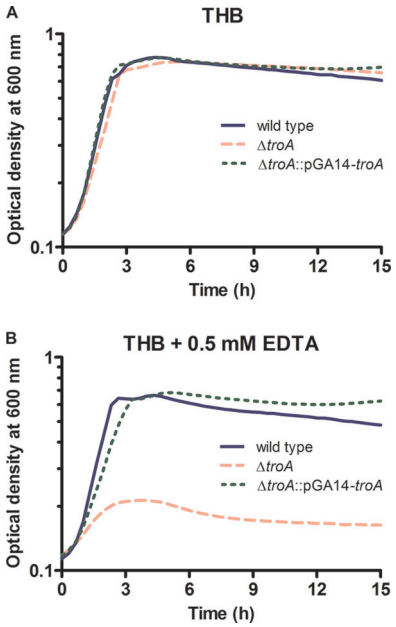

FIG. 2. Growth characteristics of the Δ*troA* mutant. Growth of wild-type, Δ*troA*, and Δ*troA*::pGA14-*troA* mutant bacteria in THB (A) and in THB supplemented with 0.5 mM EDTA (B) was followed by measuring optical densities at the indicated time points. Values represent the mean of three independent experiments. At almost all time points (three per hour) standard deviations were a maximum of 30% of the indicated values.

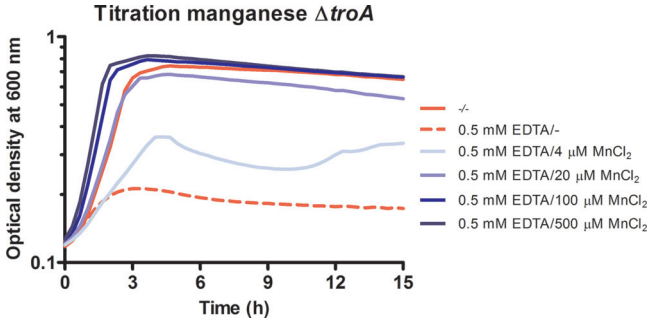

FIG. 4. Minimal manganese concentration to maintain growth of Δ*troA* mutant. Growth of Δ*troA* mutant bacteria in THB supplemented with 0.5 mM EDTA and various concentrations of manganese chloride was followed by measuring optical densities at the indicated time points. Values represent the mean of three independent experiments. At almost all time points (three per hour) standard deviations were a maximum of 30% of the indicated values.

ogous to SSU0934 and SSU1078 in *S. suis* strain P1/7) (18). *troA* transcript levels were significantly reduced in bacteria grown in porcine serum supplemented with manganese compared to bacteria grown in normal porcine serum. Transcript levels of the SSU0934 and SSU1078 genes were unaffected by the addition of manganese (Fig. 6). The negative correlation between manganese concentration and *troA* expression strongly suggests that *troA* transcription is regulated by manganese.

Loss of TroA results in increased susceptibility to oxidative stress. Previously, in *S. pneumoniae* a PsaA mutant was shown to be hypersensitive to oxidative stress (45). In addition, a manganese-dependent superoxide dismutase was identified and characterized in *S. suis* (29). To evaluate whether TroA affects the sensitivity of *S. suis* to oxidative stress, we analyzed the survival of wild-type and mutant bacteria in the presence of 25 mM H_2O_2 in THB. The Δ*troA* mutant bacteria were significantly more susceptible to H_2O_2 than the wild-type and the Δ*troA*::pGA14-*troA* mutant bacteria (Fig. 7). These results indicate that TroA supports *S. suis* survival during increased exposure to oxygen radicals.

TroA is an important virulence factor. To test the virulence of the Δ*troA* mutant bacteria in systemic infection, we used a murine infection model broadly used to assess virulence of *S. suis* mutants (4, 9, 50). Ten CD1 mice were inoculated intraperitoneally with 10^9 CFU of wild-type or Δ*troA* mutant bacteria. Five control mice were injected with D-PBS. The complemented Δ*troA* mutant was not included as it showed wild-type behavior in all *in vitro* assays. The wild-type strain induced severe disease (including apathy, lethargy, and/or nerve disorders) in 60% of the mice within 3 days postinfection (Fig. 8A). The Δ*troA* mutant bacteria did not induce specific clinical signs, except for a few observations of dull coats. All severely diseased mice died naturally or were euthanized because of irreversible disease progression. All control mice remained healthy. Bacterial counts in the liver, spleen, and brain of severely diseased mice in the wild-type-infected group reached ~10^8 CFU/gram of tissue (Fig. 8B). Mice containing high CFU levels in one organ also showed high levels of CFU counts in the other organs tested, indicating the presence of a systemic infection in these mice. In the organs of the Δ*troA* mutant-infected mice and the control mice, no bacteria could be detected. These results indicate that the TroA lipoprotein is an important virulence factor of *S. suis* in mice.

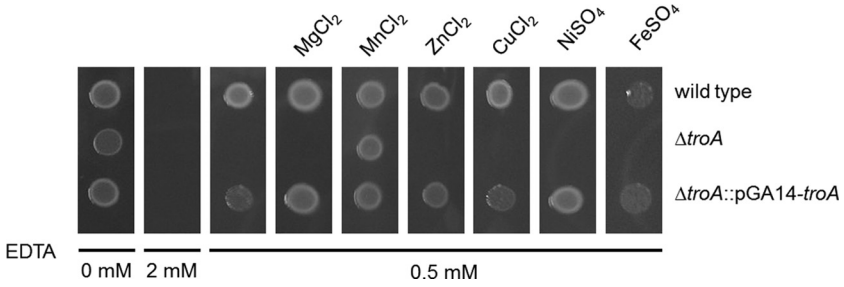

FIG. 3. Involvement of TroA in manganese acquisition. Growth of wild-type, Δ*troA*, and Δ*troA*::pGA14-*troA* mutant bacteria spotted (3 μl of 10^5 CFU/ml) on Colombia agar plates supplemented with EDTA (2 or 0.5 mM) and different trace metals (0.5 mM).

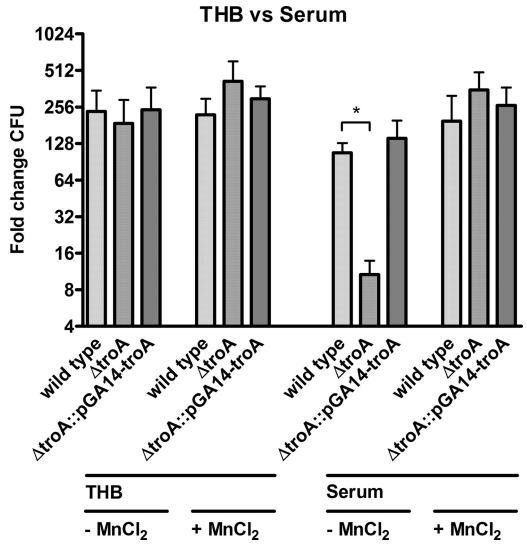

FIG. 5. Growth of Δ*troA* mutant in porcine serum. Growth of wild-type, Δ*troA*, and Δ*troA*::pGA14-*troA* mutant bacteria in THB and in 100% porcine serum (10^4 CFU/ml at start) with or without addition of 0.5 mM $MnCl_2$ (+ $MnCl_2$). Relative bacterial growth was determined by dividing the number of CFU after 3 h of incubation by the number of CFU at the start. Values represent the means ± standard deviations of three experiments performed in duplicate. *, $P < 0.05$ compared to wild-type levels.

DISCUSSION

In this study we investigated the functional properties of a lipoprotein of *S. suis* which was previously found to be dominantly present in a bacterial fraction that activates innate immunity (47). Based on sequence homology, the protein is annotated as being part of the TroA superfamily of metal binding lipoproteins. Here, we provide evidence that TroA of *S. suis* is (i) involved in manganese acquisition, (ii) subject to environmental regulation by manganese at the transcript level, (iii) required for efficient growth in environments with low manganese availability, (iv) involved in efficient oxidative stress response, and (v) required for virulence in mice.

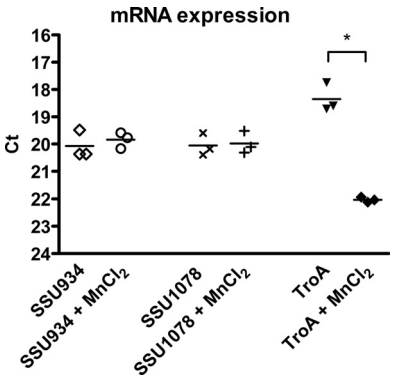

FIG. 6. TroA RNA expression. Transcript levels of TroA (SSU1869 in P1/7) as measured by quantitative real-time PCR in *S. suis* strain 8067 after growth in porcine serum with or without additional 0.5 mM manganese chloride ($MnCl_2$). Homologues of two other P1/7 lipoproteins, SSU0934 and SSU1078, served as negative controls. Data are depicted as C_T values and correspond to means ± standard deviations of three independently grown cultures. A horizontal line indicates the median for each group.

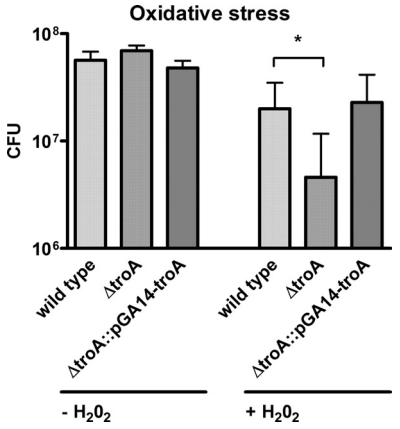

FIG. 7. Role of TroA in oxidative stress. Oxidative stress tolerance of wild-type, Δ*troA*, and Δ*troA*::pGA14-*troA* mutant bacteria in the presence of 25 mM H_2O_2 (+ H_2O_2) in THB. Values represent the means ± standard deviations of three experiments performed in duplicate. *, $P < 0.05$ compared to wild-type levels.

To investigate the involvement of TroA in metal acquisition, we created a Δ*troA* mutant strain and evaluated growth of this

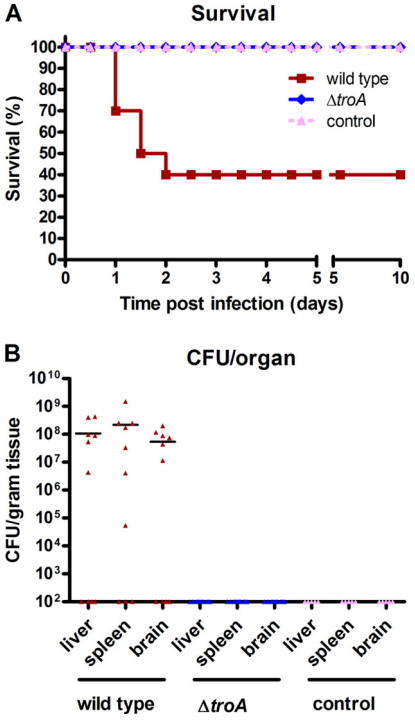

FIG. 8. Role of TroA in *S. suis* virulence. (A) Survival of wild-type- and Δ*troA* mutant-infected mice. CD1 mice (10 mice/group) were intraperitoneally inoculated with wild-type or Δ*troA* mutant bacteria or D-PBS. Irreversibly diseased mice were euthanized during the course of the experiment. A Gehan-Breslow-Wilcoxon test revealed a significant difference between wild-type and Δ*troA* survival rates of infected mice ($P < 0.05$). (B) Bacterial counts within liver, spleen, and brain of all mice determined after natural death or euthanization. The horizontal line indicates the median for each group. Mice showing high CFU counts in one organ also showed high CFU counts in the other organs. Detection limit, 1.0×10^2 CFU/g of tissue.

strain in EDTA-chelated medium. EDTA binds metal ions with various affinities; however, at the 0.5 mM concentration used here, EDTA is expected to reduce the entire pool of free available divalent metal ions. Since growth of wild-type bacteria was maintained in the EDTA-chelated medium and since growth of Δ*troA* mutant bacteria was strongly inhibited, we were able to investigate, by supplementing the medium with specific cations, which cation(s) is dominantly scavenged by the TroA protein. We showed that growth of Δ*troA* mutant bacteria is restored by adding ≥20 μM manganese chloride to the EDTA-chelated medium. The reduced growth of the Δ*troA* mutant bacteria in porcine serum confirmed that manganese availability within the host is <20 μM (27) and requires TroA expression. The Δ*troA* mutant still showed some growth in chelated medium and in porcine serum. This may indicate that *S. suis* carries a limited internal pool of manganese, perhaps related to the presence of the Dps-like peroxide resistance protein (Dpr) (16).

Recognition of *S. suis* by the host innate immune system may initiate or strengthen the production of host factors involved in creating an environment low in bacterial nutrients including essential trace metals (21). This so-called nutritional immunity has been shown to be a potent defense mechanism to fight *Staphylococcus aureus* infections. Within *S. aureus* abscesses, manganese and zinc levels are very low due to increased production of the metal binding host protein calprotectin (14). Interestingly, a recent transcriptome study of swine spleen showed a strong upregulation of calprotectin (also named S100A8/S100A9) after *S. suis* challenge (25). Our observations of reduced disease and mortality and of reduced bacterial recovery from organs of Δ*troA* mutant-infected mice strongly suggest that TroA is an important virulence factor and contributes to overcome nutritional immunity of the host.

We used a well-established intraperitoneal mouse infection model (4, 9, 50) to assess virulence of the Δ*troA* mutant. This demonstrated that TroA is required to cause systemic disease in mice. The reduced virulence of the Δ*troA* mutant is in agreement with experimental data obtained with orthologous mutants in other streptococci. A manganese binding lipoprotein-deficient *S. uberis* strain (MtuA mutant) was unable to cause mastitis in a bovine infection model (36), and PsaA mutant bacteria of *S. pneumoniae* were nonvirulent in various infection models (8, 26, 31). This study underscores that manganese acquisition mediated by high-affinity manganese binding lipoproteins contributes to growth and virulence of streptococci within the host.

TroA is predicted to be the scavenger protein of an ABC transport system. The expression of scavenger proteins, especially those involved in metal acquisition, is often tightly regulated (10, 20, 28). The reduced transcription of TroA in porcine serum supplemented with manganese and the upregulation of TroA transcripts *in vivo* (53) strongly suggest the presence of a manganese-dependent TroA transcriptional regulatory mechanism. Probably, the putative metal-dependent transcription regulator (SSU1870 in *S. suis* strain P1/7), located directly downstream of TroA, is involved in repressing transcript levels of TroA in the presence of high manganese. SSU1870 shares >50% amino acid sequence identity with several metallo-regulatory proteins in other streptococci (http://www.ncbi.nlm.nih.gov/), and the putative structure of SSU1870 (data not shown) closely resembles the manganese-responsive transcriptional regulator ScaR of *Streptococcus gordonii* (38). It is likely that the TroA transcriptional repressor protein, which contains putative metal binding sites, becomes activated after binding of intracellular manganese ions and thereby helps to ensure an optimal availability of intracellular manganese.

In the absence of TroA we observed an increased sensitivity of *S. suis* to oxidative stress. Similar to PsaA in S. *pneumoniae*, TroA of *S. suis* might directly increase oxidative stress tolerance as part of a signal transduction pathway that regulates redox homeostasis (43). Alternatively, inactivation of TroA may reduce intracellular manganese levels and the activity of the manganese-dependent superoxide dismutase (29). Thus, inactivation of TroA not only affects the capabilities of *S. suis* to grow in manganese-limiting environments but may also reduce its oxidative stress tolerance. Besides regulation of stress responses, the availability of manganese has been shown to influence competence, physiology, and general metabolism in *S. pneumoniae* as well (30). Whether manganese availability influences such processes in *S. suis* awaits further study.

In the course of this study the crystal structure of *S. suis* TroA was elucidated (56). The results show that TroA is able to bind manganese and zinc with nanomolar affinity. Both trace metals were able to stabilize the protein against thermal unfolding and induced distinct conformational changes upon binding of the metal (56). The protein contains nine α-helices and eight β-sheets and looks very similar to the structures of MtsA of *Streptococcus pyogenes* (39) and PsaA of *S. pneumoniae* (24) though sequence identity levels are relatively low. The observed binding of manganese to TroA is in agreement with our study; however, we did not observe a role in zinc binding or transport. Possibly, other zinc-specific transport systems, including high-affinity zinc binding lipoproteins present in *S. suis* (3), are able to sustain sufficient intracellular zinc levels in the absence of TroA.

High-affinity metal binding lipoproteins, including *S. suis* TroA, have been shown to be immunogenic (53, 54). Some of these immunogenic lipoproteins possess potential as vaccine candidates. The relatively conserved nature, the large extracellular domains, and the involvement in virulence potentially make these high-affinity metal binding lipoproteins ideal antigens to be contained within a vaccine. Aranda et al., investigated the immunogenicity and protective response of three putative high-affinity metal binding lipoproteins in *S. suis* isolate 89/1591 (3). One of these proteins (SSU0308 in P1/7) induced a significant protective response in mice (3). Interestingly, the TroA orthologue PsaA is one of the most promising vaccine candidates for protection against *S. pneumoniae* infections (40, 44, 51, 55). Therefore, TroA of *S. suis* may have potential as a vaccine candidate as well.

ACKNOWLEDGMENTS

We thank D. Takamatsu for providing the pSET5 plasmid.

This project was financially supported by the Dutch Ministry of Agriculture, Nature and Food Quality (KB-08).

REFERENCES

1. **Altschul, S. F., et al.** 2005. Protein database searches using compositionally adjusted substitution matrices. FEBS J. **272:**5101–5109.
2. **Andrews, S. C., A. K. Robinson, and F. Rodriguez-Quinones.** 2003. Bacterial iron homeostasis. FEMS Microbiol. Rev. **27:**215–237.

3. **Aranda, J., M. E. Garrido, P. Cortes, M. Llagostera, and J. Barbe.** 2008. Analysis of the protective capacity of three *Streptococcus suis* proteins induced under divalent-cation-limited conditions. Infect. Immun. **76:**1590–1598.
4. **Aranda, J., et al.** 2010. The cation-uptake regulators AdcR and Fur are necessary for full virulence of *Streptococcus suis*. Vet. Microbiol. **144:**246–249.
5. **Arends, J. P., and H. C. Zanen.** 1988. Meningitis caused by *Streptococcus suis* in humans. Rev. Infect. Dis. **10:**131–137.
6. **Baums, C. G., et al.** 2009. *Streptococcus suis* bacterin and subunit vaccine immunogenicities and protective efficacies against serotypes 2 and 9. Clin. Vaccine Immunol. **16:**200–208.
7. **Beineke, A., et al.** 2008. Comparative evaluation of virulence and pathology of *Streptococcus suis* serotypes 2 and 9 in experimentally infected growers. Vet. Microbiol. **128:**423–430.
8. **Berry, A. M., and J. C. Paton.** 1996. Sequence heterogeneity of PsaA, a 37-kilodalton putative adhesin essential for virulence of *Streptococcus pneumoniae*. Infect. Immun. **64:**5255–5262.
9. **Bonifait, L., et al.** 2010. The cell envelope subtilisin-like proteinase is a virulence determinant for *Streptococcus suis*. BMC Microbiol. **10:**42.
10. **Bray, B. A., I. C. Sutcliffe, and D. J. Harrington.** 2009. Expression of the MtsA lipoprotein of *Streptococcus agalactiae* A909 is regulated by manganese and iron. Antonie Van Leeuwenhoek **95:**101–109.
11. **Bungener, W., and R. Bialek.** 1989. Fatal *Streptococcus suis* septicemia in an abattoir worker. Eur. J. Clin. Microbiol. Infect. Dis. **8:**306–308.
12. **Chen, C., et al.** 2007. A glimpse of streptococcal toxic shock syndrome from comparative genomics of *S. suis* 2 Chinese isolates. PLoS One **2:**e315.
13. **Clamp, M., J. Cuff, S. M. Searle, and G. J. Barton.** 2004. The Jalview Java alignment editor. Bioinformatics **20:**426–427.
14. **Corbin, B. D., et al.** 2008. Metal chelation and inhibition of bacterial growth in tissue abscesses. Science **319:**962–965.
15. **Gebhardt, C., J. Nemeth, P. Angel, and J. Hess.** 2006. S100A8 and S100A9 in inflammation and cancer. Biochem. Pharmacol. **72:**1622–1631.
16. **Haikarainen, T., et al.** 2011. Magnetic properties and structural characterization of iron oxide nanoparticles formed by *Streptococcus suis* Dpr and four mutants. J. Biol. Inorg. Chem. **16:**799–807.
17. **Hoa, N. T., et al.** 2011. The antimicrobial resistance patterns and associated determinants in *Streptococcus suis* isolated from humans in southern Vietnam, 1997–2008. BMC Infect. Dis. **11:**6.
18. **Holden, M. T., et al.** 2009. Rapid evolution of virulence and drug resistance in the emerging zoonotic pathogen *Streptococcus suis*. PLoS One **4:**e6072.
19. **Johnson, S. R., B. M. Steiner, D. D. Cruce, G. H. Perkins, and R. J. Arko.** 1993. Characterization of a catalase-deficient strain of *Neisseria gonorrhoeae*: evidence for the significance of catalase in the biology of *N. gonorrhoeae*. Infect. Immun. **61:**1232–1238.
20. **Johnston, J. W., D. E. Briles, L. E. Myers, and S. K. Hollingshead.** 2006. Mn^{2+}-dependent regulation of multiple genes in *Streptococcus pneumoniae* through PsaR and the resultant impact on virulence. Infect. Immun. **74:**1171–1180.
21. **Kehl-Fie, T. E., and E. P. Skaar.** 2010. Nutritional immunity beyond iron: a role for manganese and zinc. Curr. Opin. Chem. Biol. **14:**218–224.
22. **Kovacs-Simon, A., R. W. Titball, and S. L. Michell.** 2011. Lipoproteins of bacterial pathogens. Infect. Immun. **79:**548–561.
23. **Lanie, J. A., et al.** 2007. Genome sequence of Avery's virulent serotype 2 strain D39 of *Streptococcus pneumoniae* and comparison with that of unencapsulated laboratory strain R6. J. Bacteriol. **189:**38–51.
24. **Lawrence, M. C., et al.** 1998. The crystal structure of pneumococcal surface antigen PsaA reveals a metal-binding site and a novel structure for a putative ABC-type binding protein. Structure **6:**1553–1561.
25. **Li, R., et al.** 2010. Response of swine spleen to *Streptococcus suis* infection revealed by transcription analysis. BMC Genomics **11:**556.
26. **Marra, A., S. Lawson, J. S. Asundi, D. Brigham, and A. E. Hromockyj.** 2002. *In vivo* characterization of the *psa* genes from *Streptococcus pneumoniae* in multiple models of infection. Microbiology **148:**1483–1491.
27. **Miller, K. B., S. M. Newman, Jr., J. S. Caton, and J. W. Finley.** 2004. Manganese alters mitochondrial integrity in the hearts of swine marginally deficient in magnesium. Biofactors **20:**85–96.
28. **Nepomuceno, M. F., M. Tabak, and A. E. Vercesi.** 2002. Opposite effects of Mn(III) and Fe(III) forms of meso-tetrakis(4-*N*-methyl pyridiniumyl) porphyrins on isolated rat liver mitochondria. J. Bioenerg. Biomembr. **34:**41–47.
29. **Niven, D. F., A. Ekins, and A. A. al-Samaurai.** 1999. Effects of iron and manganese availability on growth and production of superoxide dismutase by *Streptococcus suis*. Can. J. Microbiol. **45:**1027–1032.
30. **Ogunniyi, A. D., et al.** 2010. Central role of manganese in regulation of stress responses, physiology, and metabolism in *Streptococcus pneumoniae*. J. Bacteriol. **192:**4489–4497.
31. **Paton, J. C.** 1998. Novel pneumococcal surface proteins: role in virulence and vaccine potential. Trends Microbiol. **6:**85–88.
32. **Peetermans, W. E., B. G. Moffie, and J. Thompson.** 1989. Bacterial endocarditis caused by *Streptococcus suis* type 2. J. Infect. Dis. **159:**595–596.
33. **Perez-Martinez, G., et al.** 1992. Protein export elements from *Lactococcus lactis*. Mol. Gen. Genet. **234:**401–411.
34. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
35. **Silva, L. M., et al.** 2006. Virulence-associated gene profiling of *Streptococcus suis* isolates by PCR. Vet. Microbiol. **115:**117–127.
36. **Smith, A. J., et al.** 2003. MtuA, a lipoprotein receptor antigen from *Streptococcus uberis*, is responsible for acquisition of manganese during growth in milk and is essential for infection of the lactating bovine mammary gland. Infect. Immun. **71:**4842–4849.
37. **Smith, H. E., H. J. Wisselink, U. Vecht, A. L. Gielkens, and M. A. Smits.** 1995. High-efficiency transformation and gene inactivation in *Streptococcus suis* type 2. Microbiology **141:**181–188.
38. **Stoll, K. E., et al.** 2009. Characterization and structure of the manganese-responsive transcriptional regulator ScaR. Biochemistry **48:**10308–10320.
39. **Sun, X., et al.** 2009. Crystal structure and metal binding properties of the lipoprotein MtsA, responsible for iron transport in *Streptococcus pyogenes*. Biochemistry **48:**6184–6190.
40. **Tai, S. S.** 2006. *Streptococcus pneumoniae* protein vaccine candidates: properties, activities and animal studies. Crit. Rev. Microbiol. **32:**139–153.
41. **Takamatsu, D., M. Osaki, and T. Sekizaki.** 2001. Thermosensitive suicide vectors for gene replacement in *Streptococcus suis*. Plasmid **46:**140–148.
42. **Tettelin, H., et al.** 2005. Genome analysis of multiple pathogenic isolates of *Streptococcus agalactiae*: implications for the microbial "pan-genome." Proc. Natl. Acad. Sci. U. S. A. **102:**13950–13955.
43. **Tseng, H. J., A. G. McEwan, J. C. Paton, and M. P. Jennings.** 2002. Virulence of *Streptococcus pneumoniae*: PsaA mutants are hypersensitive to oxidative stress. Infect. Immun. **70:**1635–1639.
44. **Wang, S., et al.** 2010. Immune responses to recombinant pneumococcal PsaA antigen delivered by a live attenuated *Salmonella* vaccine. Infect. Immun. **78:**3258–3271.
45. **Ward, P. N., et al.** 2009. Evidence for niche adaptation in the genome of the bovine pathogen *Streptococcus uberis*. BMC Genomics **10:**54.
46. **Weinberg, E. D.** 2009. Iron availability and infection. Biochim. Biophys. Acta **1790:**600–605.
47. **Wichgers Schreur, P. J., J. M. Rebel, M. A. Smits, J. P. van Putten, and H. E. Smith.** 2011. Lgt processing is an essential Step in *Streptococcus suis* lipoprotein mediated innate immune activation. PLoS One **6**:e22299.
48. **Wisselink, H. J., H. E. Smith, N. Stockhofe-Zurwieden, K. Peperkamp, and U. Vecht.** 2000. Distribution of capsular types and production of muramidase-released protein (MRP) and extracellular factor (EF) of *Streptococcus suis* strains isolated from diseased pigs in seven European countries. Vet. Microbiol. **74:**237–248.
49. **Wisselink, H. J., K. T. Veldman, C. Van den Eede, S. A. Salmon, and D. J. Mevius.** 2006. Quantitative susceptibility of *Streptococcus suis* strains isolated from diseased pigs in seven European countries to antimicrobial agents licensed in veterinary medicine. Vet. Microbiol. **113:**73–82.
50. **Wu, T., et al.** 2011. Trigger factor of *Streptococcus suis* is involved in stress tolerance and virulence. Microb. Pathog. **51:**69–76.
51. **Xu, J., et al.** 2011. Intranasal vaccination with chitosan-DNA nanoparticles expressing pneumococcal surface antigen A (PsaA) protects mice against nasopharyngeal colonization by *Streptococcus pneumoniae*. Clin. Vaccine Immunol. **18:**75–81.
52. **Yu, H., et al.** 2006. Human *Streptococcus suis* outbreak, Sichuan, China. Emerg. Infect. Dis. **12:**914–920.
53. **Zhang, A., et al.** 2009. Identification of three novel *in vivo*-induced expressed antigens during infection with *Streptococcus suis* serotype 2. FEMS Microbiol. Lett. **295:**17–22.
54. **Zhang, A., C. Xie, H. Chen, and M. Jin.** 2008. Identification of immunogenic cell wall-associated proteins of *Streptococcus suis* serotype 2. Proteomics **8:**3506–3515.
55. **Zhang, Q., Q. Ma, Q. Li, W. Yao, and C. Wang.** 2011. Enhanced protection against nasopharyngeal carriage of *Streptococcus pneumoniae* elicited by oral multiantigen DNA vaccines delivered in attenuated *Salmonella typhimurium*. Mol. Biol. Rep. **38:**1209–1217.
56. **Zheng, B., et al.** 2011. Insight into the Interaction of Metal Ions with TroA from *Streptococcus suis*. PLoS One **6:**e19510.