Interactions of *Streptococcus suis* with the innate immune system

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Interacties van *Streptococcus suis* met het aangeboren immuunsysteem

(met een samenvatting in het Nederlands)

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Chapter 1

General Introduction

Streptococcus suis - Disease and pathogenesis

Streptococcus suis is considered as one of the most important pig pathogens worldwide. *S. suis* bacteria cause systemic disease that often manifests as meningitis, septicemia, endocarditis, pneumonia, enteritis and/or arthritis. For unknown reasons, piglets in the early stages of weaning are most affected by *S. suis.* Besides causing invasive disease, *S. suis* is able to colonize mucosal surfaces of the upper respiratory tract, mainly tonsils and nasal cavities, resulting in asymptomatic carriage of *S. suis*. Different serotypes of *S. suis*, but also different isolates of the same serotype are able to colonize the host simultaneously [1]. Transmission of *S. suis* between pigs occurs horizontally, usually via the nasal or oral route, [2] and vertically [3]. Carrier rates of over 90% have been described [4,5]. With a disease incidence of up to 10% and a case-fatality rate of about 50% under certain conditions, *S. suis* is considered a serious threat to pig health and welfare. The loss of production and applied control measurements is significant economic burden to the pig industry with costs estimated to be around 200 million euro/year worldwide.

S. suis isolates can be distinguished in 33 serotypes on the basis of antigenic differences in the composition of the polysaccharide capsule that surrounds the bacterium. The majority of *S. suis* bacteria isolated from diseased pigs worldwide belong to serotype 2, but isolates of other serotypes, including serotypes 1, 3, 7, 9, and 14 cause disease as well [1,6,7,8]. In recent years serotype 9 isolates spread very efficiently in the European and Asian pig populations [1,9,10]. The successful dissemination of serotype 9 isolates may be the result of increased colonization abilities.

Besides being a pig pathogen, *S. suis* occasionally infects humans resulting in similar disease manifestations as are seen in pigs. Although in Western countries human infections are rare, human outbreaks emerged in 1998 and in 2005 in China [11,12]. The outbreak in 2005 affected more than two hundred individuals with a case-fatality rate of nearly 20% [12]. During the outbreaks several human patients displayed streptococcal toxic shock-like syndrome (STSS) [11,12]. In Vietnam *S. suis* is currently the leading etiologic agent of adult meningitis [13,14]. The zoonotic nature of *S. suis* likely relates to close contacts between humans and infected pigs or contaminated pork products [14,15,16,17]. Especially the consumption of 'high risk' dishes, such as raw pig

blood and undercooked meat, is an important risk factor [18]. Transmission between humans has not been reported. The majority of isolates from diseased humans belong to serotype 2 [19]. Interestingly, the serotype 2 strains responsible for the human outbreaks are distinct from the porcine serotype 2 strains by the presence of a 89 kb genomic fragment (89 kb pathogenicity island) encoding an extra two-component signal transduction system involved in virulence [20].

To cause systemic disease *S. suis* needs to migrate to the bloodstream. It is assumed that bacteria that colonize the upper respiratory tract of pigs traverse the mucosal barriers by damaging the mucosal epithelia. There is evidence that suilysin, the major toxin of *S. suis*, is involved in the destruction of mucosal barriers [21,22,23]. In humans, *S. suis* is supposed to reach the bloodstream by crossing the epithelial layers of the gut after consumption of contaminated food [18] or through abrasions of the skin [24]. In the bloodstream *S. suis* travels freely or in association with phagocytes [25,26]. At this stage the capsule of *S. suis* likely prevents macrophage- and neutrophil-mediated phagocytosis [27]. To cause meningitis, *S. suis* has to migrate from the bloodstream to the central nervous system (CNS). *S. suis* is able to access the CNS by trespassing the blood brain barrier [28] or the blood-cerebrospinal fluid barrier [29,30]. Although specific bacterial surface adhesions [31] and suilysin [26] are expected to facilitate these processes, the exact migration mechanisms are not known. Once *S. suis* bacteria have entered the bloodstream and tissues and/or the CNS, an inflammatory response may develop. Normally, the inflammatory response leads to enhanced bacterial clearance but occasionally the inflammatory response becomes overactive resulting in the development of STSS. The bacterial components triggering the inflammatory responses are largely unknown.

Control and prevention of *S. suis* infections

The use of antibiotics is the golden standard to treat and control *S. suis* infections in both pigs and humans. The main antibiotics used in the pig industry to treat streptococcal infections are β-lactams and trimethoprim-sulphonamide combinations. In humans, penicillin G, ampicillin and amoxicillin are commonly used to treat *S. suis* infections specifically [14]. Especially in the early phases of disease the use of antibiotics and anti-inflammatory agents is very effective.

Unfortunately, the extended use of antibiotics in the animal production industry results in the selection of antibiotic resistant bacteria, including antibiotic resistant *S. suis* strains [32,33,34,35]. Although the emergence of multi-drug resistant strains of *S. suis* is still limited compared to *Staphylococcus aureus* and *Streptococcus pneumoniae* [36,37], it is expected that, with unaltered use of antibiotics, antibiotic resistance in the *S. suis* species will continue to increase.

Besides treating infected animals and humans with therapeutics, preventive measures may be effective against *S. suis* as well. Although evidence is limited, husbandry systems with an all-in-all-out animal flow may reduce the spread of *S. suis* between pig herds. Furthermore, the use of balanced diets may reduce infection rates within pig herds [38,39]. For human risk groups, education might be the key factor for immediate prevention. An improvement of hygiene during pig handling and a decrease in the consumption of raw pig-products is expected to result in a reduction of the number of *S. suis* infections in humans [18]. The lack of detailed knowledge on the interaction between *S. suis* and its host limits the development of new rational prevention strategies.

In pig herds with *S. suis* problems, formalin-killed bacteria (bacterins) are commonly used for vaccination. Unfortunately, the bacterin-based vaccines only provide protection towards invasive disease of the autologous serotype that is used for bacterin preparation [40,41] and do not induce cross-protection against heterologous serotypes [41,42]. Furthermore, serotype 9 based bacterins are recently shown not to reduce colonization and transmission among pigs [43]. Human vaccines are not available at all. It is generally assumed that protein based sub-unit vaccines have the best potential to provide protection towards multiple serotypes of *S. suis*. Such a strategy has already effectively been applied in horses to provide protection against *Streptococcus equi* [44]. In pigs, protective immune responses against *S. suis* infections are detected for a relatively small number of protein antigens. Immunization of pigs with a combination of the native muramidase released protein (MRP) and extracellular factor protein (EF) provide a protective immune response for serotype 2 challenge [42]. Furthermore, the recombinant produced surface antigen one (SAO) protein [45], the recombinant produced 6-phosphogluconate-dehydrogenase (6PGD) protein [46], and the native suilysin protein [47] may provide some degree of protection [48]. The SAO and suilysin protein however, are not widely spread and/or

conserved among *S. suis* serotypes and strains. Thus, the future challenge in *S. suis* vaccine development is to identify the right (combination of) protein antigens that confer protection against multiple serotypes.

S. suis - The pathogen

S. suis is a facultative anaerobic Gram-positive coccus containing a circular chromosome of about 2 million base pairs. The cell wall of *S. suis* consists of a cytoplasmic membrane and a thick peptidoglycan layer surrounded by a polysaccharide capsule (Figure 1). The *S. suis* genome encodes for approximately 2,000 genes, of which 1,300-1,400 belong to the core genome that is present in all isolates [33,49,50,51,52]. With a pan-genome of about 4,000 genes [52] *S. suis* is considered to be a genetically heterogenic species. The majority of genes encodes for proteins involved in metabolic and structural processes [53,54,55,56] and facilitates bacterial duplication in just 30 minutes under optimal laboratory conditions. Inter-strain variations between *S. suis* genomes are observed at multiple levels and include insertions, deletions, inversions, rearrangements and single nucleotide polymorphisms.

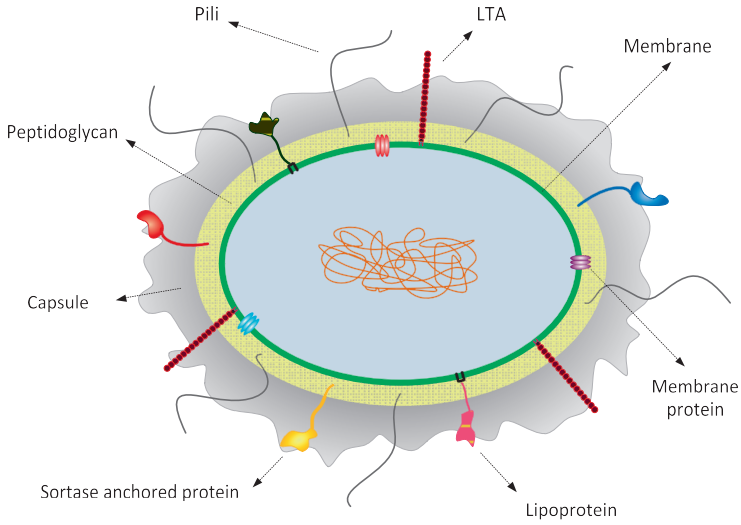

Fig. 1. ***Schematic representation of the S. suis bacterium.*** *The cytoplasm containing the bacterial DNA is surrounded by the cell wall consisting of a membrane and a thick peptidoglycan layer. Attached to the cell wall are various surface structures like LTA, sortase anchored proteins (like pili), lipoproteins and membrane proteins. The outermost structure is the polysaccharide capsule.*

The polysaccharide capsule of *S. suis* serotype 2 consists of five different sugars, namely galactose, glucose, *N*-acetylglucosamine, rhamnose, and *N*-acetylneuraminic acid (sialic acid) [57]. Capsule structures of other serotypes have not been characterized. The genes involved in the production of the serotype 2 capsule are located on the *cps*2 locus [27,58] and capsule thickness may be affected by the host environment [59]. Genes involved in sialic acid synthesis are present in serotypes 1, 2, 14, 16, 27 and 1/2 and absent in other serotypes [58,60]. The capsule is considered to be the most important virulence factor of *S. suis* [27,61], although recent observations show that non-encapsulated strains can be isolated from pigs suffering from endocarditis [62].

The peptidoglycan layer consists of alternating residues of β-(1,4) linked *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM). Peptidoglycan strands are cross-linked by peptide chains to form a rigid 3-dimensional structure giving bacterial strength and stability. Furthermore, peptidoglycan is involved in binary fission during bacterial cell reproduction. The β-lactam antibiotics, such as penicillin, interfere with the formation of peptidoglycan by inhibiting the crosslinking of peptidoglycan monomers [63], eventually resulting in bacterial lysis. Intertwined with the peptidoglycan structure and anchored in the cytoplasmic membrane are the lipo-teichoic acids (LTAs). LTA consists of teichoic acids and long chains of ribitol phosphate and is considered the equivalent of lipopolysaccharide (LPS) found in Gram-negative bacteria [64]. LTA regulates the bacterial surface charge and the activity of autolytic cell wall enzymes. Autolysins cleave the β(1,4) bond between the NAM and NAG residues of peptidoglycan allowing daughter cell separation [65]. In several species including *S. suis*, LTA is modified by D-alanylation to modulate bacterial charge, assisting in resistance to antimicrobial peptides [66].

The cytoplasmic membrane forms the lipid barrier between the cytosol and the extracellular environment and comprises many proteins involved in solute transport and other vital bacterial functions. The cytoplasmic membrane also holds the transport machinery to secrete proteins to the bacterial exterior. In recent years, protein secretion has received increasing attention as 'surface proteins' may be targets for recognition by the host and thus have potential as sub-unit vaccine antigens [67]. Three important classes of surface proteins are

the sortase anchored proteins, the lipoproteins, and the membrane bound proteins.

Sortase anchored proteins are transported across the cytoplasmic membrane and then covalently anchored to the peptidoglycan by sortase enzymes that recognize the conserved C-terminal amino acid anchoring motif LPXTG. The most well-known sortase anchored protein of *S. suis* is the MRP protein [68]. MRP is present in variable molecular weights variants [1], is highly immunogenic [41,42,69], and is able to provide a protective immune response in pigs [42]. Furthermore, MRP is associated to virulent isolates but does not contribute to virulence [70]. Despite the significant amount of data on MRP distribution and immunogenicity, no specific function for the protein has been described. Recently, also pili, assembled and anchored by sortases, have been identified and characterized in *S. suis* [71,72,73,74]. In general, these hair-like structures protruding from the surface of Gram-positive bacteria contribute to host-cell adhesion and pathogenesis [75]. The thus far characterized *S. suis* pili are not widely distributed among the *S. suis* serotypes and do not seem to contribute to virulence, at least in mice models [74]. Some other interesting sortase anchored proteins of *S. suis* include the SAO protein [45,48], the opacity-factor (OFS protein) [76], the alpha-glucan-degrading enzyme ApuA [77] and a hyaluronidase enzyme [78,79].

Bacterial lipoproteins are anchored in the cell wall with a *N*-terminal lipid moiety. The anchoring of lipoproteins in Gram-positive bacteria is mediated by two different enzymes, the prolipoprotein diacylglyceryl transferase (Lgt) enzyme and the lipoprotein signal peptidase (Lsp) enzyme. Lgt mediates the addition of a diacylglyceryl moiety to the cysteine residue of the lipobox (LXXC) of immature lipoproteins [80,81]. Subsequently, the Lsp enzyme cleaves the secretion signal peptide resulting in mature cell membrane anchored lipoproteins [82,83]. Several Gram-positive lipoproteins are described to fulfill various functions including nutrient uptake and transport, signal transduction, conjugation, antibiotic resistance and protein folding [84,85]. So far, knowledge about *S. suis* lipoproteins is limited. Interestingly, a recent study showed up-regulation of three putative divalent-cation transporter lipoproteins upon divalent cation-deprivation. One of these lipoproteins also provided a protective immune response in mice [86].

S. suis and the innate immune system

The ability to colonize and invade host tissues and cause disease not only relies on the *S. suis* gene content but also involves the reactivity of the host immune system. The immune system in vertebrates can be divided into the adaptive immune system and the evolutionary more ancient innate immune system. The adaptive immune system is very specific, highly adaptable, comprises memory and generates long lasting immunity, but requires days to weeks to respond to a primary challenge. In contrast, the innate immune system is relatively non-specific and responds directly to invading microbes but does not generate immunological memory. It seems that the constituents and functionality of the porcine and human innate immune system are very similar. The innate and adaptive immune systems are closely linked and work in synergy to defend the host against infections. Since acute *S. suis* infections are accompanied by a strong inflammatory response [87], the innate immune system likely plays an important role in early pathology. Although the various components of the innate immune system are tightly connected, the system roughly consists of the complement system, antimicrobial peptides/proteins, modulating cytokines and chemokines, and phagocytic cells [88].

After breaching physical and biochemical barriers, microbes are sensed by the innate immune system by pattern recognition receptors (PRRs). These sensors of the immune system recognize conserved microbial structures, also known as pathogen associated molecular patterns (PAMPs) [89]. A variety of host cells express PRRs including antigen presenting cells (APCs), endothelial cells, epithelial cells, and B- and T-cells. PRR expression is not a static event as their distribution and regulation of expression can differ per cell type, location, and time. The main bacterial PAMPs include LPS, peptidoglycan monomers, flagellin, DNA, lipoproteins and glucans. So far, four major PRRs families have been identified: the Toll-like receptors (TLR), the nucleotide oligomerization domain (NOD)-like receptors (NLR), the retinoic acid-inducible gene I (RIG-I)-like receptors (RLR), and the C-type lectins.

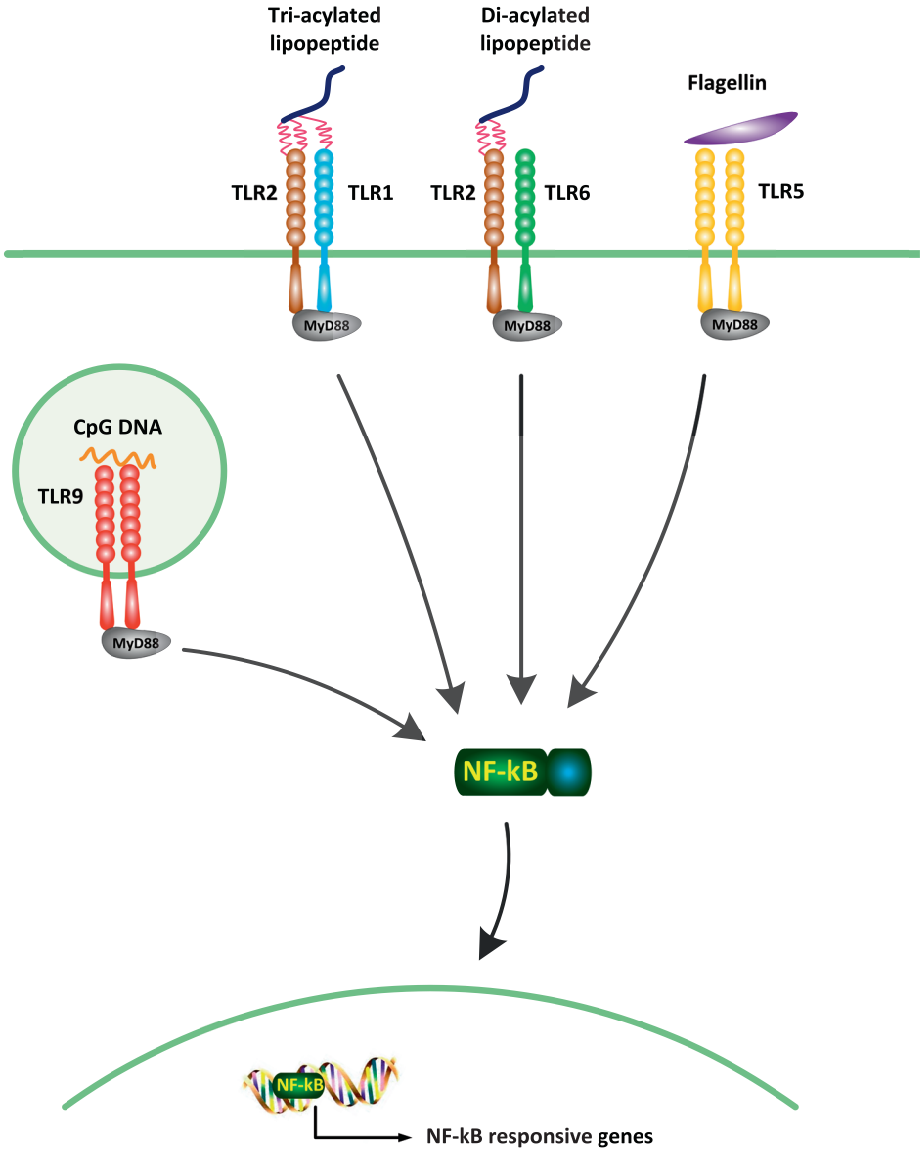

Fig. 2. ***Schematic representation of TLR-mediated sensing of Gram-positive ligands.*** *TLR2 in combination with TLR1 mainly recognizes tri-acylated lipopeptides, TLR2 in combination with TLR6 mainly recognizes di-acylated lipopeptides, TLR5 recognizes flagellin and TLR9 (endosome) recognizes CpG DNA. After TLR activation the MyD88-dependent signaling cascade is initiated resulting in nuclear translocation of NF-κB. NF-κB promotes transcription of inflammatory cytokines and chemokines.*

The TLR class of PRRs is most extensively studied and is involved in bacterial, viral, fungal, and parasite recognition [90,91,92]. Pigs and humans express ten different TLRs [93,94,95,96] with amino acid sequence identities levels between homologous porcine and human TLRs of about 70%. TLRs are membrane-spanning receptors expressed on the surface of a cell or within endosomes. The proteins contain a horseshoe shaped ligand binding domain and an intracellular signaling domain. The TLRs specialized in recognizing bacterial PAMPs include TLR1, TLR2, TLR4, TLR5, TLR6, and TLR9. Most TLRs function as a homodimer, but TLR2 can form heterodimers with other TLRs. The dominant ligand of TLR4 is LPS, whereas TLR5 recognizes flagellum monomers and TLR9 recognizes CpG DNA (most often found in bacteria). TLR2 in combination with TLR1 or TLR6 recognize tri-acylated and di-acylated lipoproteins, respectively [97,98]. In Figure 2 the main ligands and TLRs involved in the sensing of Gram-positive bacteria are depicted. At the start of the research described in this thesis the TLRs and ligands involved in sensing *S. suis* were largely unknown.

Once PRRs are activated by specific PAMPs, a signaling cascade is initiated which involves the recruitment of adaptor molecules and a chain of phosphorylation reactions. The signaling cascade eventually results in the translocation of transcription factors to the nucleus that initiate the transcription of genes encoding effector molecules such as cytokines, chemokines, and antimicrobial peptides/proteins [99]. In general, TLR activation mediated by Gram-positive PAMPs activate the MyD88 dependent signaling cascade resulting in the nuclear translocation of the Nuclear Factor κappa B (NF-κB) transcription factor (Figure 2). NF-κB promotes the expression of a broad panel of pro-inflammatory cytokines and chemokines including interleukin-1β, tumor necrosis factor-α, interleukin-6 and interleukin-8. These signaling proteins fulfill various functions such as initiating systemic inflammation, increasing mucus production, and recruitment of phagocytic cells. At the beginning of the research presented in this thesis, it has been reported that cell wall fractions of *S. suis* serotype 2 isolates trigger the production of pro-inflammatory cytokines and chemokines in various cell types including murine macrophages, human endothelial brain cells, human monocytes and porcine white blood cells [100,101,102,103]. However, the nature of the bacterial factors that activated this innate immune response was still obscure. Once located at the site of

infection, immune cells such as neutrophils, monocytes, macrophages, dendritic cells and mast cells, are able to produce antimicrobial peptides/proteins, specific proteases and reactive oxygen radicals to facilitate microbial killing. Antimicrobial peptides are 12-50 amino acids in length and are able to disrupt bacterial membranes or interfere with bacterial metabolism [104]. Although the highest concentration of antimicrobial peptides is present in neutrophil granules, antimicrobial peptides are also secreted in the extracellular space. Besides antimicrobial peptides, the host produces antimicrobial proteins. One important antimicrobial protein that is present in high concentrations (>500 μg/ml) in several bodily secretions such as tears, mucus, milk and saliva [105,106] and also in neutrophil granules [107,108,109], is the peptidoglycan degrading enzyme lysozyme. Lysozyme weakens the bacterial cell wall through hydrolysis of the (1,4)-β-linkages between the NAM and NAG residues of the peptidoglycan layer resulting in bacterial lysis [110]. Deficiencies in lysozyme or lysozyme production have been shown to increase the susceptibility of hosts to streptococcal disease [111]. Several bacterial species have evolved mechanisms to resist the hydrolytic activity of lysozyme. An effective strategy to reduce the bactericidal effects of lysozyme in Staphylococci [112] and certain Streptococci [113] is the modification of peptidoglycan. At the start of the research described in this thesis it was not known whether *S. suis* also employs this or other mechanisms to become resistant to lysozyme.

Besides these direct actions to eliminate invading microbes the innate immune system also orchestrates the antigen specific B- and T- cell (adaptive) response. Antigen presenting dendritic cells (DCs) play a central role in connecting the innate and adaptive immune responses. PRR activation on DCs initiates the maturation of DCs which enables these cells to activate naïve T- and B-cells. Produced antibodies can facilitate opsonophagocytosis and complement-mediated bacterial killing. Several bacterial species including Streptococci and Staphylococci have evolved mechanisms to evade opsonophagocytosis and/or the action of the complement system [114,115]. Whether *S. suis* also contains factors that interfere with opsonophagocytosis and/or complement activation remains to be established, although capsule-deficient *S. suis* strains display higher levels of innate activation compared to wild type strains in human monocytes and macrophages [100,116].

Aim of the study

The overall aim of the study described in this thesis is to better understand the interactions of *S. suis* with the host innate immune system. To this end, the ability of *S. suis* to activate human TLR complexes and porcine PBMCs is investigated and the bacterial components responsible for activation are identified and characterized. Particularly the role of lipoproteins and Lgt-mediated lipoprotein processing in innate immune system activation is investigated. Furthermore, the levels and the molecular basis of resistance to the antimicrobial protein lysozyme are examined. It is expected that knowledge of i) the components of *S. suis* able to activate the innate immune system, and ii) the strategies that *S. suis* uses to escape innate immunity, increases understanding of the molecular basis of the pathogenesis of *S. suis* infections. This information may enable the development of novel prevention strategies, including the development of vaccines providing protection towards multiple serotypes.

Outline of this thesis

The general introduction (**this Chapter**) provides an overview of the *S. suis* associated societal problems, the characteristics of the pathogen, and the current knowledge about innate host defense mechanisms against *S. suis*. In **Chapter 2** the interaction of *S. suis* with several innate immune receptor complexes is reported and the human TLR2/6 complex is identified as a primary receptor to sense *S. suis*. In **Chapter 3** the contribution of *S. suis* lipoproteins and lipoprotein processing in the activation of porcine peripheral blood mononucleated cells is studied by determining the production of pro-inflammatory cytokines and chemokines after stimulation with wild type and Lgt-deficient *S. suis* strains. In **Chapter 4,** a lipoprotein abundantly present in an innate activating fraction, designated TroA, is further characterized and shown to be involved in manganese acquisition and virulence in mice. **Chapter 5** describes a study in which the molecular mechanisms of lysozyme resistance in *S. suis* are explored using both a targeted approach, based on known factors involved in lysozyme resistance in other bacteria, and a unbiased approach based on comparative whole genome analysis. In **Chapter 6,** the results of the various studies are discussed and placed in a broader context. New insights into the molecular basis of the pathogenesis of

S. suis infections are discussed, including the implications for future prevention strategies.

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Chapter 2

Differential activation of the Toll-like receptor 2/6 complex by lipoproteins of *Streptococcus suis* serotypes 2 and 9

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Abstract

Streptococcus suis causes invasive infections in pigs and occasionally in humans. Worldwide, *S. suis* serotype 2 is most frequently isolated from diseased piglets, but the less virulent serotype 9 is emerging, at least in Europe. We compared the activation of human Toll-like receptors (hTLRs) by *S. suis* serotype 2 and 9 strains to better understand the role of the innate immune response in fighting *S. suis* infections. Neither live nor heat-killed log phase grown *S. suis* activated the hTLR1/2, hTLR2/6 and hTLR4/MD-2 complexes. However, the hTLR2/6 complex was specifically activated by both serotypes after disruption of the cell wall synthesis using penicillin. Activation levels of the hTLR2/6 complex were higher for serotype 9 strains compared to serotype 2 strains suggesting intrinsic differences in cell wall composition between both serotypes. The hTLR2/6 activating fractions decreased in molecular size after digestion with proteinase K and were sensitive for lipoprotein lipase digestion and NaOH hydrolysis, indicating lipoprotein(s) as active component(s). Overall, our results indicate that *S. suis* lipoproteins activate TLR2/6 but not TLR1/2 and that the clinically different serotypes 2 and 9 display differential release of TLR ligand when cell wall integrity is compromised.

Introduction

Streptococcus suis is primarily a pathogen of pigs and may cause meningitis, endocarditis, septicemia, pneumonia and arthritis. Occasionally *S. suis* causes disease in humans with clinical manifestations similar to those seen in piglets [1,2,3]. Although the human *S. suis* infections were a rare event in the past [4], recently an outbreak with over 200 cases was reported in Sichuan, China [5,6,7]. Most of the human *S. suis* cases follow direct exposure to pigs or pork meat [1,7,8]. So far, no transmissions between human individuals have been reported.

As of now, 33 capsular serotypes of *S. suis* have been described with differences in virulence between serotypes as well as within serotypes. Worldwide, the vast majority of porcine and human isolates belong to serotype 2. In Europe, serotype 9 isolates are emerging and contribute substantially to *S. suis* associated disease in pigs [9,10], despite their reduced virulence [11].

S. suis infections are accompanied by a strong inflammatory response, suggesting an important role of the innate immune system in *S. suis* infection pathology. *S. suis* serotype 2 and especially its cell wall components trigger the production of pro-inflammatory cytokines by murine macrophages, human brain microvascular endothelial cells and porcine blood cells [12,13,14]. At which stages of infection these highly stimulating *S. suis* derived cell wall components become available for recognition and thereby initiate the production of pro-inflammatory cytokines and chemokines is still largely unknown. Furthermore, the specific ligands able to activate pattern recognition receptors (PRRs) such as the Toll-like receptors (TLRs) are not yet identified for *S. suis*.

In the present study we examined the involvement of several TLR complexes in recognizing components of *S. suis* serotypes 2 and 9 in a HeLa transfection system. Our results indicate that both serotypes express lipoproteins that activate the hTLR2/6 complex.

Materials and methods

Bacterial strains

Three virulent wild type *S. suis* serotype 2 strains and three virulent wild type *S. suis* serotype 9 strains (Table 1) were grown on Colombia agar plates (Oxoid Ltd., London, United Kingdom) containing 6% horse blood at 37°C and 5% CO_2. Liquid cultures were grown in Todd-Hewitt broth (TH broth) (Oxoid Ltd., London, United Kingdom) for 18 h at 37°C without agitation. Prior to the experiments, bacteria were pelleted by centrifugation at 4500 × *g* for 10 min and resuspended to 10^9 CFU/ml in Dulbecco's phosphate buffered saline (D-PBS).

Cells

The HeLa 57A cell line stably transfected with a nuclear factor-kappa B (NF-κB) luciferase reporter construct [15] was generously provided by Dr. R.T. Hay (Institute of Biomolecular Sciences, University of St. Andrews, St. Andrews, Scotland, UK). Cells were routinely propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C and 10% CO_2.

Expression vectors and reagents

The expression vectors pTracer-hTLR1, pTracer-hTLR2, pTracer-hTLR6, pTracer-hCD14 containing hTLR1, hTLR2, hTLR6 and hCD14, respectively [16] and lipopolysaccharide (LPS) from *Salmonella enterica* serovar Enteritidis (here referred as *S.* Enteritidis) [17] were kindly provided by Dr. A.M. Keestra (Utrecht University, The Netherlands). The expression vectors pUNO-hTLR4, pUNO-hMD-2 containing hTLR4 and hMD-2, respectively, and the synthetic lipopeptides FSL-1 and PAM_3CSK_4 were purchased from InvivoGen (Toulouse, France).

Transfection and stimulation of cells

HeLa 57A cells were grown in 48 well tissue culture plates in DMEM containing 5% FBS until 50% confluence was reached. Cells were transfected with FuGENE 6 (Roche Diagnostics, Almere, The Netherlands) at a lipid to DNA ratio of 3:1. Expression plasmids carrying the TLR genes were added to a concentration of

67.5 ng/well/plasmid. Empty vector was added in variable amounts to equalize the total transfected plasmid DNA to 250 ng/well. The pTKLacZ vector was used for normalization of the transfection efficiency. After 48 h of incubation at 37°C, medium was replaced with fresh medium. Subsequently, cells were stimulated for 5 h with 10^6 CFU/ml live, 10^7 CFU/ml heat-killed (60°C, 45 min), 10^7 CFU/ml penicillin-treated whole bacteria or bacterial derived supernatant. Stimulation with penicillin-treated whole bacteria was performed by adding live untreated bacteria to transfected HeLa cells in the presence of 30 µg/ml penicillin (Sigma-Aldrich, Zwijndrecht, The Netherlands). The tri-acylated lipopeptide PAM_3CSK_4 (100 ng/ml), the di-acylated lipopeptide FSL-1 (100 ng/ml) and *S.* Enteritidis derived LPS (100 ng/ml) served as TLR specific controls for, respectively, the hTLR1/2, hTLR2/6 and hTLR4/hMD-2 complexes. Cells transfected with empty vector were used to determine TLR2 and TLR4 independent NF-κB activation. The HeLa cells only responded to the TLR ligands Pam_3CSK_4, FSL-1 and LPS after transfection with the specific TLRs, indicating that the tested TLRs are not constitutively expressed by the used HeLa cells.

Luciferase assay

After stimulation, cells were washed twice with D-PBS and lysed in 0.1 ml Reporter Lysis Buffer (E397A, Promega, Leiden, The Netherlands) according the manufactures description. Luciferase activity was determined with a Victor 1420 multilabel counter (PerkinElmer, Groningen, The Netherlands) by incubating 20 µl of lysed cells with 50 µl of luciferase assay substrate (Promega). Due to variation in transfection efficiency within and between experiments, luciferase activity was normalized by determination of β-galactosidase activity with the β-galactosidase assay (E2000, Promega). In addition, relative fold activation was calculated as the normalized reporter activity of the test samples divided by the normalized activity of un-stimulated samples. Results represent the mean ± SEM of three strains per serotype of at least two independent experiments. Statistical significance of a difference between two means was evaluated using a Student's unpaired t-test when data were normally distributed. When data was not normally distributed a Mann–Whitney test was performed. A two-tailed P value of <0.05 was taken as significant.

Table 1. *S. suis* strains used in this work.

Strain	Serotype	Reference
S 3881 (10)	2	[18]
S 3973 (P1/7	2	[19]
S 4005 (3)	2	[18]
S 7997	9	Smith *et al*. (unpublished)
S 8017	9	Smith *et al*. (unpublished)
S 8067	9	Smith *et al*. (unpublished)

Release of TLR2/6 activating components

Serotype 2 and serotype 9 strains were grown for 18 h at 37°C in TH broth, resuspended to 10^9 CFU/ml in D-PBS and diluted 1:10 in TH broth or in a chemical-defined medium (CDM) consisting of a 1:1 mixture of HAM-F12 nutrient mixture (21765, Invitrogen, Breda, The Netherlands) and NCTC-109 medium (N1140, Sigma–Aldrich). The combination of these two cell culture media results in the formation of a CDM that contains all amino acids, a broad range of inorganic salts, several vitamins and glucose as the main carbon source. At an optical density of 0.4 (600 nm), penicillin was added to the culture to a final concentration of 30 µg/ml. After 2 h incubation at 37°C, cells and medium were separated by centrifugation (4500 × *g*, 10 min). The supernatants were 0.2 mm filter sterilized and directly used for stimulation of transfected cells. CDM supernatants of serotype 2 and serotype 9 strains after penicillin treatment were also concentrated using Amicon Ultra-15 centrifugal filter devices with a 100 NMWL cut-off (Millipore, Amsterdam, The Netherlands). Concentrated fractions (referred as Amicon concentrated fractions) were frozen for later treatment or used directly for stimulation.

Proteinase K, lipoprotein lipase and NaOH treatments

Proteins present in the activating fractions (40 times, v/v, Amicon concentrated) of one serotype 2 (3881) and one serotype 9 strain (8067) were digested (1.5 h at 56°C) with 200 mg/ml of proteinase K (Sigma-Aldrich). Inactivation of proteinase K, before HeLa cell stimulation, was performed by addition of 10 mM phenylmethanesulphonylfluoride (PMSF, Sigma-Aldrich). Lipoproteins present in

the 40 times (v/v) Amicon concentrated fractions were digested with lipoprotein lipase or treated with NaOH. Lipoprotein lipase (Sigma-Aldrich) was added to a final concentration of 100 mg/ml and incubated for 18 h at 37°C. NaOH treatment was performed by incubation of the fractions with 0.2N NaOH for 2 h at room temperature followed by neutralization with 37% (v/v) HCl. Positive control fractions were obtained by similar treatments, but without addition of enzyme or NaOH.

SDS-PAGE, silver staining and protein concentration

Amicon concentrated supernatants of penicillin-treated *S. suis* were separated by 4-12% Bis-Tris NuPAGE gels (Invitrogen) under reducing (DTT and 5 min 95°C) or non-reducing (without DTT and heating) conditions. Proteins were visualized with the Silver staining kit Plus One from GE Healthcare (Uppsala, Sweden). Protein concentration of samples was determined with the Bradford protein assay (Biorad, Veenendaal, The Netherlands).

Elution from acrylamide gel

Proteinase K-digested or undigested Amicon concentrated supernatants, corresponding to 150 µg of total protein, of a penicillin-treated *S. suis* serotype 9 strain (8067), were separated with SDS-PAGE under non-reducing conditions. As a positive control 10 µg of FSL-1 was separated as well. Subsequently, gels were cut into 1 cm strips of 7 different molecular size ranges. Each strip was homogenized in a 1% (w/v) SDS solution to elute components. Fractions were centrifuged (12,000 × *g*, 10 min) to remove the homogenized gel. Five volumes of cold acetone were added and the mixture was incubated for 18 h at -20°C. Precipitate was collected by centrifugation at 12,000 × *g* and 4 °C for 10 min. Pellets were dissolved in 200 ml of 10 mM Tris-HCl, pH 8.

Results

S. suis activates human TLR2/6

HeLa cells expressing the hTLR1/2, hTLR2/6 or hTLR4/hMD-2 complexes, all in combination with hCD14 to increase ligand capturing [20,21,22] were stimulated with live, heat-killed or penicillin-treated serotype 2 and serotype 9 strains (three strains per serotype, Table 1). No activation of the tested complexes was observed after stimulation with live or heat-killed *S. suis*. However, after disruption of the cell wall by penicillin, *S. suis* was able to activate the hTLR2/6 complex within 5 h of stimulation (Fig. 1). TLR2/6 dependent NF-κB activation was significantly higher for serotype 9 strains compared to serotype 2 strains. Penicillin by itself (i.e., without *S. suis*) caused no activation of HeLa cells (data not shown). Similarly, HeLa cells transfected with empty vector, hTLR1/2 or hTLR4/MD-2, all showed only basal levels of NF-κB activation with penicillin-treated *S. suis*, indicating that *S. suis*-derived components specifically activated the TLR2/6 complex. Penicillin minimum inhibitory concentration (MIC) values did not differ significantly between serotypes 2 and 9 (data not shown).

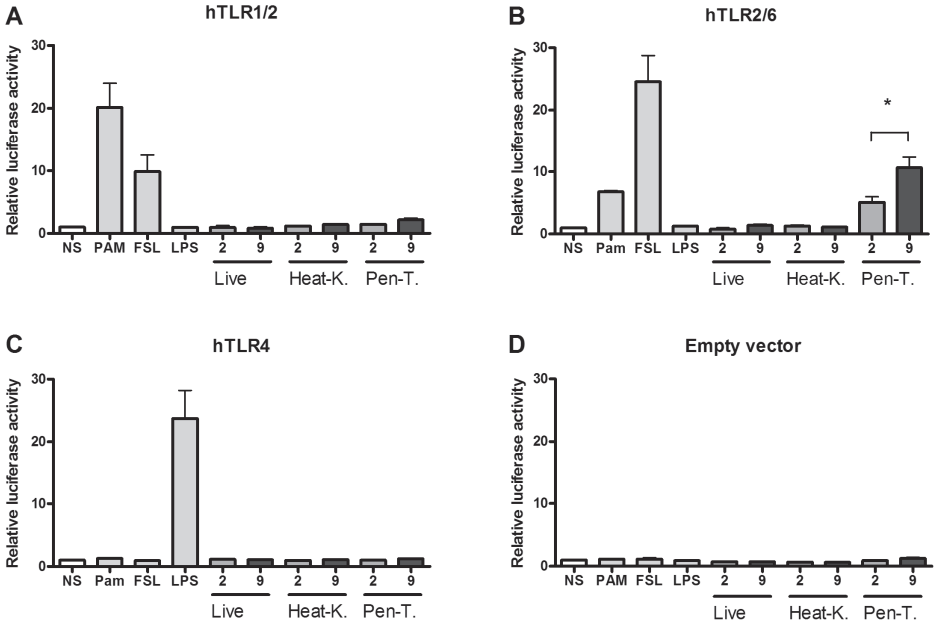

Fig. 1. ***Specific activation of hTLR2/6 by penicillin-treated S. suis.*** *HeLa 57A cells expressing hTLR1/2 (A), hTLR2/6 (B), hTLR4/MD-2 (C) and control cells transfected with empty vector (D) were stimulated (5 h) with live, heat-killed (Heat-K.) or penicillin-treated (Pen-T.) S. suis serotype 2 and 9 strains. PAM_3CSK_4 (PAM), FSL-1 (FSL) and S. Enteritidis LPS were used as TLR specific controls. Data represent relative fold activation calculated by dividing the normalized reporter activity of the test samples by the normalized activity of non-stimulated (NS) samples. Values represent the mean ± SEM of three strains per serotype of two independent experiments. *P < 0.05.*

S. suis releases hTLR2/6 activating components into the medium upon penicillin treatment

To evaluate whether *S. suis* released hTLR2/6 activating components into the medium after penicillin treatment, we added penicillin to log phase grown bacteria in TH broth or CDM and collected the supernatants. Similar as was observed with penicillin-treated whole bacteria (Fig. 1B) supernatants of penicillin-treated *S. suis* induced NF-κB activation in hTLR2/6 transfected HeLa cells (Fig. 2A). Supernatants of serotype 9 strains activated the hTLR2/6 transfected cells more than serotype 2 strains, resembling the stimulation profile of the penicillin-treated whole bacteria (Fig. 1B). Mean NF-κB activation levels were lower for *S. suis* grown in CDM compared to TH broth (Fig. 2A), despite that

growth rates of *S. suis* in CDM were only slightly decreased compared to TH broth (data not shown). In all cases, stimulation of cells transfected with empty vector yielded only background levels of NF-κB activity (Fig. 2B). Although penicillin treatment resulted in the most potent hTLR2/6 activation, activation could also be obtained by concentrated supernatants of untreated stationary phase bacteria (data not shown), suggesting that TLR2/6 activating components are released during natural infection as well.

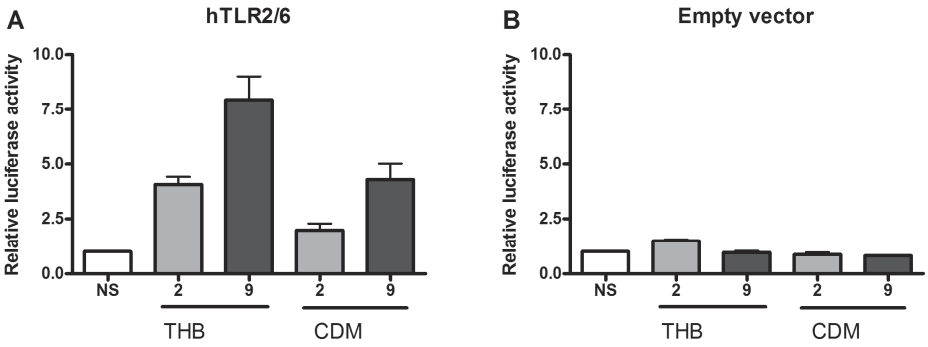

Fig. 2. ***hTLR2/6 induced activation by supernatant of penicillin-treated S. suis.*** *HeLa 57A cells expressing hTLR2/6 were stimulated with supernatant of penicillin-treated S. suis serotype 2 and 9 strains grown in TH broth or CDM (A). Control cells were transfected with empty vector and stimulated with the same fractions (B). Data represent relative fold activation calculated by dividing the normalized reporter activity of the test samples by the normalized activity of non-stimulated (NS) samples. Values represent the mean ± SEM of three strains per serotype of two independent experiments.*

Characterization of the *S. suis* TLR2/6 ligands

To investigate the nature of the hTLR2/6 activating components of *S. suis*, concentrated CDM culture supernatants of penicillin-treated *S. suis* were digested with proteinase K. Proteinase K treatment did not significantly reduce the hTLR2/6 activating capacity of both serotypes (Fig. 3A), while cells transfected with empty vector remained unresponsive (data not shown). To confirm proteinase K digestion, samples were separated by SDS-PAGE and proteins were visualized by silver staining. The majority of proteins were digested by proteinase K in both serotypes resulting in disappearance of proteins in the higher and medium molecular size range (Fig. 3B). The silver staining also showed more intense staining for the supernatant of the penicillin-treated

serotype 9 strain compared to the serotype 2 strain, suggesting a difference in protein release upon penicillin treatment of the serotypes. Despite being resistant to proteinase K digestion (Fig. 3A), the hTLR2/6 activating component(s) might still be of proteinaceous nature as several lipoproteins have been described to maintain TLR2 activating capacity after proteinase K digestion [23,24].

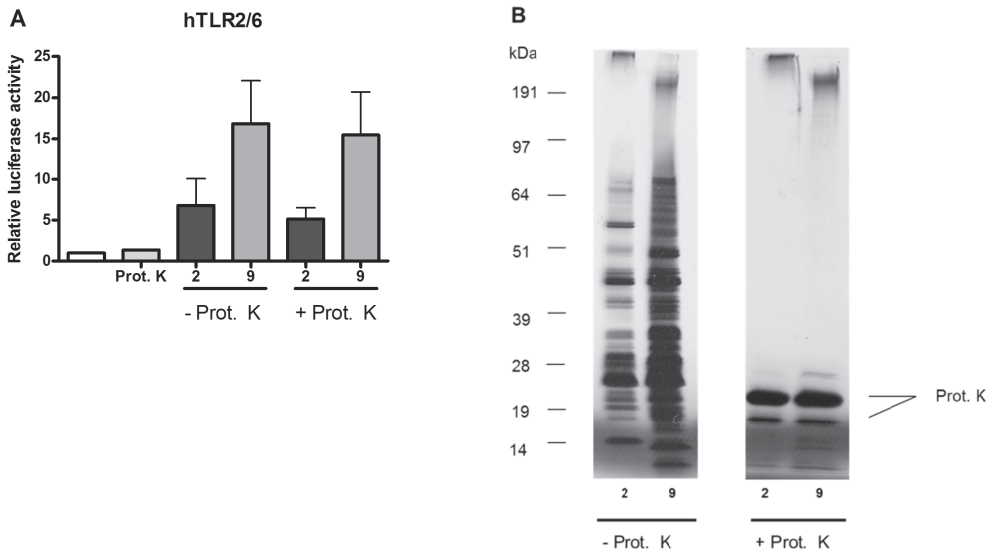

Fig. 3. ***hTLR2/6 activation by proteinase K-digested supernatant of penicillin-treated S. suis.*** *(A) HeLa 57A cells expressing hTLR2/6 were stimulated with proteinase K-digested supernatant of penicillin-treated serotype 2 and 9 strains. Undigested samples were used as a positive control and proteinase K itself as a negative control. Data represent relative fold activation as calculated by dividing the normalized reporter activity of the test samples by the normalized activity of non-stimulated (NS) samples. Values represent the mean ± SD of four values obtained from two independent experiments performed in duplicate. (B) Protein silver staining of proteinase K-digested and undigested supernatants of serotype 2 and 9 strains.*

The hTLR2/6 ligand of *S. suis* has a protein backbone

To further examine whether the proteinase K-resistant TLR2/6 stimulatory activity of penicillin-treated *S. suis* has a protein component we separated proteinase K-digested and undigested fractions by SDS-PAGE (non-reduced conditions) and tested the hTLR2/6 stimulating activity of different molecular size ranges. The hTLR2/6 activating capacity of the positive control FSL-1, was

observed in the low molecular size fraction of approximately 5-15 kDa (Fig. 4C). The hTLR2/6 activating capacity of the undigested sample was observed in a broad molecular size range of 10-200 kDa with peak activity at approximately 30 kDa (Fig. 4A). After proteinase K digestion the hTLR2/6 activating capacity clearly shifted towards a lower molecular size range (Fig. 4B). The shift in molecular size of the active components after proteinase K treatment strongly suggests the TLR2/6 activating component(s) of *S. suis* has a protein backbone.

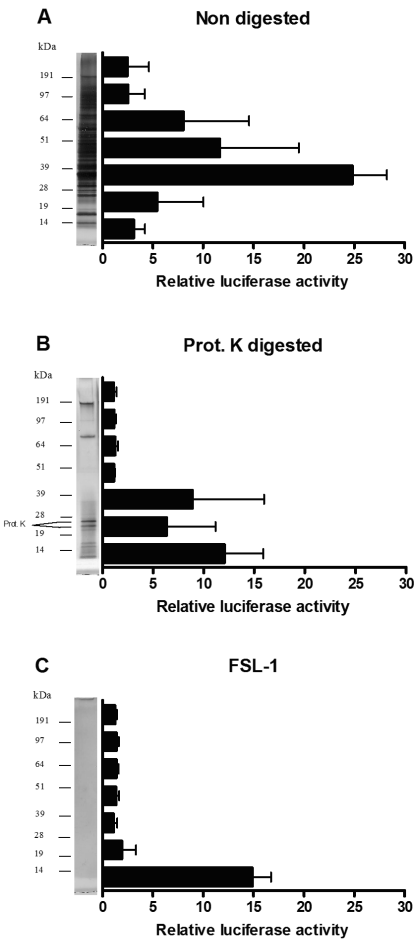

Fig. 4. ***Molecular size range of hTLR2/6 activating components before and after proteinase K digestion.*** *Undigested (A) or proteinase K-digested (B) supernatants of the penicillin-treated serotype 9 S. suis strain 8067 (150 µg of protein) was separated by SDS-PAGE. After cutting of the gel into 7 pieces of different molecular size ranges and purification of the proteins, NF-κB activation by the different fractions was measured in hTLR2/6 transfected cells. FSL-1 (C) was separated in parallel and served as a positive control. Data represent relative fold activation as calculated by dividing the normalized reporter activity of the test samples by the normalized activity of non-stimulated samples. Values represent the mean ± SD of four values obtained from two independent experiments performed in duplicate. Each bar corresponds to one of the 7 molecular size ranges of the separated fractions, indicated by the SDS-PAGE profile.*

Sensitivity of the *S. suis* TLR2/6 ligand(s) to lipase and NaOH treatment

As TLR2/6 ligands are often lipoproteins, we tested whether the hTLR2/6 activating proteins of *S. suis* were lipoproteins. Therefore, we digested the activating fractions with lipoprotein lipase or treated the fractions with NaOH to cleave of the acyl-chains present at the *N*-terminal cysteine of lipoproteins. Lipoprotein lipase treatment reduced the hTLR2/6 stimulating activity of both serotypes 2 and 9 to approximately 50% (Fig. 5). NaOH treatment reduced hTLR2/6 activation of both serotypes to approximately 30%. These results strongly suggest that *S. suis* lipoproteins are the primary ligands recognized by hTLR2/6.

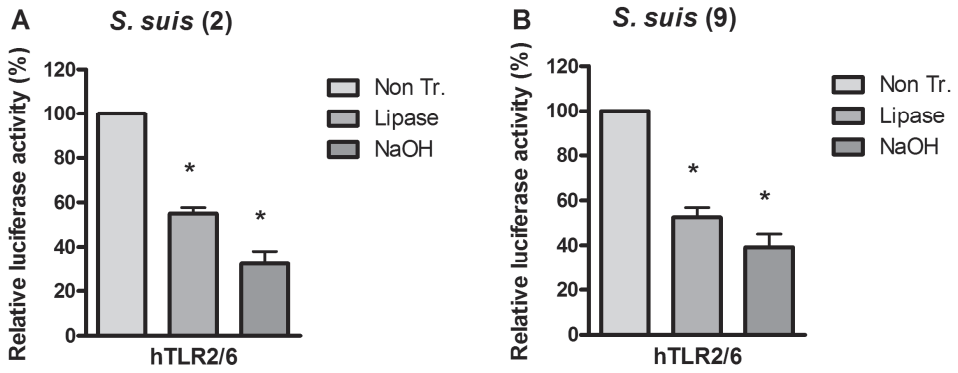

Fig. 5. ***Lipoprotein lipase and NaOH treatment of supernatant of penicillin-treated S. suis.*** *Supernatants of penicillin-treated S. suis serotype 2 (A) and serotype 9 (B) strains were concentrated by Amicon centrifugal devices and treated with lipoprotein lipase or NaOH. Subsequently, hTLR2/6 stimulating activity of the fractions was determined. Activity levels of untreated samples were set to 100%. Values represent the mean ± SEM of 4 independent experiments. *P < 0.05.*

Comparable hTLR2/6 activation of *S. suis* serotypes 2 and 9 with equivalent amounts of released protein

Serotype 9 strains activated the hTLR2/6 complex more than serotype 2 strains (Fig. 1B). In addition, we showed that upon penicillin-treatment serotype 2 strains release a lower total amount of protein compared to serotype 9 strains (Fig. 3B). To evaluate whether the difference in TLR2/6 activating capacity between serotypes 2 and 9 was maintained after correction for the amounts of

protein released, we stimulated hTLR2/6 transfected cells with supernatant of penicillin-treated bacteria adjusted for protein concentration. After protein normalization no significant difference was observed between the two serotypes (Fig. 6), suggesting that the difference in hTLR2/6 stimulation between serotypes 2 and 9 may be related to differential release rather than to intrinsic differences of the hTLR2/6 ligand(s).

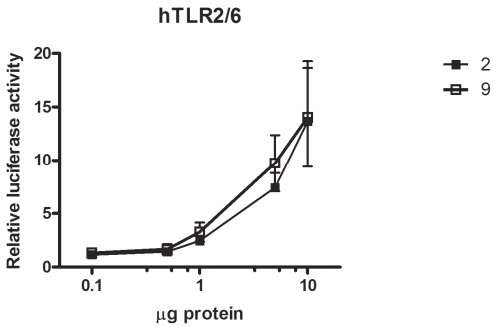

Fig. 6. ***Titration curve of hTLR2/6 activating proteins of S. suis serotype 2 and 9.*** *Supernatant of penicillin-treated S. suis serotype 2 and 9 strains were concentrated by Amicon centrifugal devices and equalized for protein concentration. A protein concentration–response relationship for stimulation of hTLR2/6 was determined for both serotypes. Data represent relative fold activation as calculated by dividing the normalized reporter activity of the test samples by the normalized activity of non-stimulated samples. Values represent the mean ± SD of four values obtained from two independent experiments performed in duplicate.*

Discussion

In this study, we provide evidence that two pathogenic serotypes (2 and 9) of *S. suis* stimulate the hTLR2/6 complex, resulting in activation of NF-κB. The ligand was at least one lipoprotein as evidenced by its susceptibility to proteinase, lipase and NaOH. The differential release of TLR2/6 ligand after penicillin treatment of serotype 2 and 9 strains suggests the existence of intrinsic differences in cell wall composition between these serotypes.

The novel finding that *S. suis* activates the TLR2/6 but not the TLR1/2 complex and that this effect is conferred by a bacterial lipoprotein is consistent with the general concept that the TLR2 complex has specificity for di- or tri-acylated peptides. TLR2 must form heterodimers with TLR1 or TLR6 to signal [25]. The TLR1/2 complex is activated by tri-acylated lipopeptides [26,27] while the TLR2/6 complex is activated by di-acylated lipopeptides [28]. Considering

this, the specific activation of TLR2/6 by *S. suis* is likely caused by at least one di-acylated lipoprotein. Lipidation of proteins is well established for Gram-positive species and is achieved by the lipoprotein diacylglyceryl transferase (Lgt) [29,30]. Lgt couples a diacylglycerol moiety via a thioester bond to the thiol group of the cysteine present in the lipobox in the C-terminal part of the *N*-terminal signal sequence of a protein [29,30]. After lipidation, a lipoprotein signal peptidase (Lsp) is able to cleave of the signal peptide [31], followed by anchoring of the lipoprotein in the cell wall. The synthesis of tri-acylated lipoproteins requires the *N*-acyltransferase (Lnt) that acylates the amine group of the cysteine residue in the lipobox. Interestingly, no Lnt homologues in streptococcal species, including *S. suis*, have thus far been identified, which may explain the lack of activation of TLR1/2 by *S. suis*. In published genomes of *S. suis* [19,32] we found more than 40 putative lipoproteins. All of these are putative TLR2/6 ligands.

We investigated the interaction of *S. suis* with human TLR complexes in a human cell based transfection system. Because our primary interest was to study TLR activation by different *S. suis* serotypes we started this work using the human TLR system. Furthermore, the human TLR read out systems have been validated in contrast to the porcine TLR read out systems. In a later stage we would like to concentrate on the interaction of *S. suis* with porcine TLR complexes.

We tested live, heat-killed and penicillin-treated bacteria for their capability to activate the human TLR1/2, TLR2/6 and TLR4/MD-2 complexes. In general, lipopeptides are believed to be the main TLR2 ligands. There might be a role for peptidoglycan and or lipo-teichoic acid, however TLR2 recognition of these components is, at least partially, suspected to be due to contaminating lipopeptides [33]. In addition to TLR2, interaction with TLR4 was evaluated because *S. suis* produces suilysin, a homologue to pneumolysin of *Streptococcus pneumoniae*. This toxin has been reported to interact with TLR4 and confers resistance to *S. pneumoniae* infection [34].

In our hands, NF-κB activation was only observed after stimulating cells expressing the hTLR2/6 complex with penicillin-treated *S. suis*. Presumably the amount of hTLR2/6 activating components released from log phase bacteria was beneath the detection level of the system, nevertheless TLR2/6 activating components could be detected in the absence of antibiotic in concentrated

stationary phase cultures. The molecular basis of increased release of TLR2 ligands after penicillin treatment is unknown, although the effect has also been reported for *S. pneumoniae* [35]. Presumably, upon penicillin treatment, the bacterial cell wall becomes less rigid resulting in the release of cell wall associated molecules that are able to activate TLR2/6. Our data imply that *in vivo* activation of TLR2/6 by *S. suis* may particularly occur after changes to the peptidoglycan layer.

The present study is the first that shows that *S. suis* lipoprotein(s), presumably di-acylated lipoproteins, are the ligands that activate the TLR2/6 complex. Our data underpin previous suggestions that TLR2 plays a role in *S. suis* associated disease [36]. Monocytes treated with a monoclonal antibody against TLR2 but not TLR4 showed a strong inhibition of cytokine and chemokine production after stimulation with *S. suis* [36]. Furthermore, TLR2-deficient mouse macrophages showed a decrease IL-6 and MCP-1 production after stimulation with encapsulated *S. suis* [36]. Why *S. suis* did not stimulate TLR4 by means of suilysin remains unclear.

In our assays, one consistent difference between serotype 2 and serotype 9 strains was the enhanced activation of the TLR2/6 complex by serotype 9 strains. This could be due to enhanced protein release by serotype 9 strains after penicillin treatment. As growth rates and penicillin MIC values for both serotypes were quite similar, other factors such as the composition of the cell wall or metabolic differences may also have contributed to this enhanced activation of TLR2/6. A correlation between increased TLR2/6 activation and reduced virulence might be present. It can be speculated that the less virulent serotype 9 strains are better cleared by the innate immune system due to better recognition by TLRs resulting in less severe clinical problems. Nevertheless serotype 9 strains seem to have evolved mechanisms to spread more easily among the pig population compared to serotype 2 strains. To reveal the complete picture of the correlation between virulence, activation of the innate immune system and spreading awaits further study.

Acknowledgements

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Chapter 3

Lgt processing is an essential step in *Streptococcus suis* lipoprotein mediated innate immune activation

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Abstract

Streptococcus suis causes invasive infections in pigs and occasionally in humans. The host innate immune system plays a major role in counteracting *S. suis* infections. The main components of *S. suis* able to activate the innate immune system likely include cell wall constituents that may be released during growth or after cell wall integrity loss, however characterization of these components is still limited. A concentrated very potent innate immunity activating supernatant of penicillin-treated *S. suis* was SDS-PAGE fractionated and tested for porcine peripheral blood mononucleated cell (PBMC) stimulating activity using cytokine gene transcript analysis. More than half of the 24 tested fractions increased IL-1β and IL-8 cytokine gene transcript levels in porcine PBMCs. Mass spectrometry of the active fractions indicated 24 proteins including 9 lipoproteins. Genetic inactivation of a putative prolipoprotein diacylglyceryl transferase (Lgt) gene resulted in deficient lipoprotein synthesis as evidenced by palmitate labeling. The Lgt mutant showed strongly reduced activation of porcine PBMCs, indicating that lipoproteins are dominant porcine PBMC activating molecules of *S. suis*. This study for the first time identifies and characterizes lipoproteins of *S. suis* as major activators of the innate immune system of the pig. In addition, we provide evidence that Lgt processing of lipoproteins is required for lipoprotein mediated innate immune activation.

Introduction

Streptococcus suis causes severe infections in pigs, including meningitis, septicemia, endocarditis, pneumonia and arthritis. Occasionally, *S. suis* infects humans as well, resulting in comparable disease manifestations as are seen in pigs [1,2,3]. To date, 33 serotypes of *S. suis* have been described based on differences in polysaccharide capsule. Isolates even of the same serotype may vary in virulence. The majority of isolates that causes disease belong to serotype 2, although in Europe serotype 9 isolates are emerging [4,5,6].

Based on the existence of a strong inflammatory response during an acute *S. suis* infection, a significant activation of innate immunity is expected early after infection. The innate immune system uses pattern recognition receptors (PRRs) to recognize pathogen associated molecular patterns (PAMPs) of microbes. One group of PRRs able to sense a diverse set of bacterial PAMPs is the Toll-like receptor (TLR) family. Activation of these TLRs results in nuclear translocation of transcription factors (e.g. nuclear factor kappa B, NF-κB) which ultimately causes enhanced production of pro-inflammatory cytokines, chemokines and antimicrobial peptides. Besides these direct mechanisms to eliminate invading microbes, the innate immune system plays a decisive role in initiating and strengthening humoral and cell-mediated protection.

The capsule of *S. suis* may be one of the first structures to be recognized by the innate immune system. However, capsule by itself is a poor activator of the innate immune system [7]. Capsule-deficient *S. suis* strains display even higher levels of innate activation compared to wild type strains in human monocytes and macrophages [7,8]. The main components of *S. suis* involved in activating the innate immune system therefore likely include cell wall or cell membrane constituents. Indeed, cell wall extracts of *S. suis* have been shown to be potent cytokine inducers in murine macrophages, human endothelial brain cells, human monocytes and in a porcine whole blood model [8,9,10,11]. Furthermore, we recently provided evidence that components of *S. suis* released after cell wall integrity loss specifically activate the human TLR2/6 complex that mostly recognizes bacterial lipoproteins [12].

Lipoproteins of Gram-positive bacteria are processed by two key enzymes; the prolipoprotein diacylglyceryl transferase (Lgt) enzyme and the lipoprotein signal peptidase (Lsp) enzyme. The Lgt enzyme recognizes a so-called lipobox motif (LXXC) in the C-terminal region of the signal peptide of a premature lipoprotein and transfers a diacylglyceryl moiety to the cysteine residue of the lipobox [13,14]. Subsequently, the Lsp enzyme cleaves the signal peptide resulting in a mature lipoprotein [15,16]. Lipid modification of Gram-positive bacterial lipoproteins via Lgt has been described to be essential for innate immune activation [17,18].

The objective of this study was to identify components of *S. suis* that activate porcine peripheral blood mononucleated cells (PBMCs). We used mass spectrometry and genetically defined lipoprotein-processing defective strains as research instruments.

Material and methods

Ethics Statement

Fresh porcine blood was obtained in accordance with a protocol (2008120.a) approved by the Animal Experiments Committee of the Central Veterinary Institute (Lelystad, The Netherlands), in agreement with the Dutch Experiments on Animals Act (Project code: 2008149).

Bacterial strains and growth conditions

In this study we used a serotype 9 strain (strain 8067, virulent pig isolate, Smith *et al*., unpublished results), which is previously shown to activate the innate immune system via human TLR2/6 more efficiently compared to serotype 2 strains [12]. Wild type bacteria, isogenic mutants as well as complemented mutant strains were grown on Colombia agar plates (Oxoid Ltd, London, United Kingdom) containing 6% horse blood at 5% CO_2 and 37°C. Liquid cultures were grown in Todd-Hewitt broth (THB) (Oxoid Ltd.) for 18 h at 37°C without agitation. *Escherichia coli* were grown on Luria-Bertani (LB) agar plates or in LB broth. When necessary, antibiotics were added to culture media at the following concentrations: for *E. coli*, ampicillin 100 µg/ml; chloramphenicol 8 µg/ml and

spectinomycin 100 µg/ml; for *S. suis*, chloramphenicol 5 µg/ml and spectinomycin 100 µg/ml. For use in stimulation experiments, bacteria were pelleted by centrifugation at 4,500 × *g* for 10 min and resuspended to 10^9 CFU/ml in Dulbecco's phosphate buffered saline (D-PBS).

General DNA techniques

Genomic DNA from *S. suis* was isolated as described previously [19]. PCRs were conducted with Phusion High-Fidelity DNA polymerase (BIOKE, Leiden, The Netherlands). Plasmid DNA was isolated with the Plasmid DNA Purification System (Promega, Leiden, The Netherlands). DNA purifications were performed with the Zymogen clean up 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 [20].

Generation of Δ*lgt* mutant

Primers used in this study are listed in Table S2. Primers 1 and 4 were used to amplify a fragment of the chromosomal DNA of strain 8067 containing the intact *lgt* 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) and ligation mixtures were transformed to *E. coli*. Plasmid DNA (designated pJET-*lgt*) obtained from transformants was then used to replace an internal fragment (about 300 bp) of *lgt* by a Spc resistance cassette. To do this, we used an inverse PCR strategy on pJET-*lgt* 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 digested with *Xma*I and *Sal*I and ligated together. Ligation mixtures were introduced into *E. coli* to generate pJET-*lgt*-*spc*. The entire insert fragment of pJET-*lgt*-*spc* was subsequently amplified using primers 1 and 4 and ligated to the thermo sensitive shuttle vector pSET5 [21], which was linerialized with the *Sma*I restriction enzyme, generating pSET5-*lgt*-*spc*. The pSET5-*lgt*-*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 and 7,8 as well as by Southern blotting.

Complementation of the Δ*lgt* mutant

To complement the Δ*lgt* mutant with an intact *lgt* gene, we constructed an expression plasmid containing the wild type *lgt* gene including its putative promoter. Primers 13 and 14 were used to amplify the *lgt* fragment, which was cloned into pJET1.2 generating pJET1.2-*lgt*-expr. Subsequently, pJET1.2-*lgt*-expr was digested with *Sma*I and *Sal*I and the *lgt* fragment was purified and cloned into pGA14 [22] digested with *Sma*I and *Sal*I, generating pGA14-*lgt*-expr. Finally, the chloramphenicol resistance gene (*cm*) of pSET5, amplified with primers 15 and 16 and digested with *Sal*I, was introduced at the *Sal*I site of pGA14-*lgt*-expr to yield pGA14-*lgt*-expr-*cm*. As a negative control, *cm* was introduced in pGA14 digested with *Sal*I, generating pGA14-*cm*. Both plasmids were subsequently introduced into the Δ*lgt* mutant generating Δ*lgt*::pGA14-*lgt* and Δ*lgt*::pGA14-*cm* respectively. RNA expression of the *lgt* gene in the Δ*lgt*::pGA14-*lgt* mutant was confirmed by quantitative real time PCR.

Growth analysis

Overnight cultures of wild type and mutant bacteria were 1:100 diluted in fresh THB and optical density at 600 nm (OD_{600}) of 400 µl samples was followed in time using the Bioscreen C (Thermo Scientific, Breda, The Netherlands) at 37°C. Overnight cultures of wild type, Δ*lgt* mutant and complemented mutants had similar OD_{600} values and contained the same amounts of CFU.

[^{3}H]palmitate labeling

Bacteria were grown for 18 h at 37°C in THB, pelleted, resuspended to 1.0×10^9 CFU/ml in D-PBS and then diluted 1:20 in chemical defined medium (CDM) consisting of a 1:1 mixture of HAM-F12 nutrient mixture (Invitrogen, Breda, The Netherlands) and NCTC-109 medium (Sigma-Aldrich, Zwijndrecht, The

Netherlands) containing 10 µCi/ml [9,10-^{3H}]palmitic acid (Perkin Elmer, Groningen, The Netherlands). At an optical density of 0.4 (600 nm), penicillin G (Sigma-Aldrich) was added to the culture to a final concentration of 30 µg/ml. After 2 h of incubation at 37°C, the bacteria and medium were separated by centrifugation (4,500 × *g*, 10 min) and the supernatant was 40 times volume concentrated by Amicon Ultra-15 centrifugal filter devices with a 3 kD cut-off (Millipore, Amsterdam, The Netherlands). Subsequently, LDS Sample Buffer (Invitrogen) was added to the concentrated fraction and 30 µl samples were separated using SDS-PAGE. Finally, the gel was fixed, dried, and exposed to an autoradiography film for 24 h.

Generation of (concentrated) bacterial supernatant

Wild type 8067, Δ*lgt* mutant and complemented Δ*lgt* mutant strains were grown for 18 h at 37°C in THB, pelleted, resuspended to 1.0×10^9 CFU/ml in D-PBS and diluted 1:20 in CDM. Penicillin treated supernatant was obtained by adding penicillin G (final concentration of 30 µg/ml) to cultures when OD_{600} values reached 0.4. After 2 h of incubation at 37°C, the bacteria and medium were separated by centrifugation (4,500 × *g*, 10 min) and the supernatants were 0.2 µm filter sterilized. Stationary phase-derived supernatant was obtained by incubating 1:20 diluted CDM cultures for 24 h at 37°C followed by centrifugation (4,500 × *g*, 10 min) and filtration (0.2 µm). Supernatants were directly used for PBMC stimulation or used for further concentration. For this, 10% TCA w/v was added to the supernatants followed by overnight incubation at 4°C. After centrifugation at 30,000 × *g* for 30 min, the pellets were washed with 100% acetone and air dried. Finally, protein pellets were dissolved in LDS Sample Buffer.

Identification of proteins within crude immune stimulatory fraction

Proteins (1 mg) present in a concentrated bacterial supernatant of *S. suis* strain 8067 were separated on a 10% SDS-polyacrylamide gel (15 cm in length, 12 cm wide) under non-reducing conditions (no boiling). One cm of the gel was stained with the Silver staining kit Plus One from GE Healthcare (Uppsala, Sweden) and the remaining gel was cut into 0.5 cm strips of 24 different molecular size ranges. Each strip was homogenized with a mortar in a 1% w/v SDS solution to solubilize proteins. Gel residue was removed by centrifugation (12,000 × *g*, 10 min) and five volumes of cold acetone were added to the supernatant. After overnight incubation at -20°C, precipitate was collected by centrifugation (12,000 × *g* 4°C for 10 min). Pellets were dissolved in 200 µl of 10 mM Tris-HCl pH 8. Fractions (50 µl) were tested for activity using porcine PBMCs and human TLR2/6 expressing HeLa 57A cells. Fractions that showed > 5 fold porcine PBMC activation were once more separated on a 4-12% polyacrylamide gel (Invitrogen), stained with SimpleBlue Safe Stain (Invitrogen), excised from the gel, and identified with mass spectrometry. Briefly, proteins were reduced with dithiothreitol, alkylated with iodoacetamide, and digested with trypsin (Roche) as described [23]. Samples were subjected to nanoflow LC (Eksigent) using C18 reverse phase trap columns (Phenomenex; column dimensions 2 cm x 100 µm, packed in-house) and subsequently separated on C18 analytical columns (Reprosil; column dimensions, 20 cm x 50 µm; packed in-house) using a linear gradient from 0 to 40% B (A = 0.1 M acetic acid; B = 95% (v/v) acetonitrile, 0.1 M acetic acid) in 60 min and at a constant flow rate of 150 nl/min. Column eluate was directly coupled to a LTQ-Orbitrap-XL mass spectrometer (Thermo Fisher Scientific) operating in positive mode, using Lock spray internal calibration. Data were processed and subjected to database searches using MASCOT software (Matrixscience) against Swiss Prot and non-redundant NCBI database with a 10 ppm mass tolerance of precursor and 0.8 Da for the fragment ion.

PBMC isolation and stimulation

Blood of three to four week old piglets from a specific pathogen free (SPF) herd was aseptically collected and mixed with heparin (LEO Pharma, Breda, Netherlands) to a final concentration of 5 IE/ml. Subsequently, PBMCs were isolated with lymphoprep tubes (Lucron Bioproducts, Gennep, Netherlands), according the manufactures instructions. The PBMCs were resuspended to 5.0×10^6 cells/ml in RPMI 1640 supplemented with 2% v/v of homologous serum (from the same animal as the PBMCs) and 30 µg/ml of penicillin. Cells (1 ml) were seeded into 24 well tissue plates. After overnight incubation, cells were stimulated with 50 µl of SDS-PAGE derived fractions, 5.0×10^6 *S. suis* bacteria (in presence or absence penicillin), or 50 µl of *S. suis* derived bacterial supernatant. After stimulation (2 and 4 h) cells were lysed and frozen (-80°C) and stored until RNA isolation and cytokine detection. We used quantitative real time PCR analysis, because the available porcine cytokine ELISAs are much less sensitive, particularly for stimulation experiments that last only 2-4 h. FSL-1 (100 ng/ml) and medium-stimulated cells served as positive and negative controls, respectively.

RNA isolation, cDNA synthesis, and quantitative real time PCR

Total RNA was isolated with the High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany), according the manufactures instructions. RNA quantity and quality was checked with the NANOdrop (Thermo Fisher Scientific, Pitsburgh, USA). To make cDNA, 200 ng RNA was reverse transcribed using OligoDt and Superscript III (Promega), according the manufactures instructions. For quantitative real time PCR analysis of IL-1β and IL-8 cytokines, 5 µl of 20 times diluted cDNA was added to 1 × power cyber green mixture (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands) containing 0.625 µM of forward and reverse primer (Table S2) in a total of 20 µl. Serial dilutions of pGemTeasy plasmids containing the PCR fragment of interest were used as internal standards. 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 sec, annealing at 59°C for 30 sec, and elongation at 72°C for 36 sec. C_t values for the tested cytokines in each sample were expressed as cDNA quantity (ng) using the

internal standards. Subsequently, the IL-1β and IL-8 ng levels were normalized with the ng levels of the house keeping gene *gapdh*. To calculate fold inductions, normalized IL-1β and IL-8 levels of stimulated cells were divided by normalized IL-1β and IL-8 levels of medium-stimulated control cells.

Stimulation of human TLR2/6 transfected HeLa cells

The HeLa 57A cell line, stably transfected with a NF-κB luciferase reporter construct [24], was generously provided by Dr. R.T. Hay (Institute of Biomolecular Sciences, University of St. Andrews, St. Andrews, Scotland, UK). Cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C and 10% CO_2. For transfection experiments, cells were seeded in 48 well tissue culture plates. When 50% confluence was reached, cells were transfected with 250 ng DNA/well using FuGENE 6 (Roche Diagnostics, Almere, The Netherlands) at a lipid to DNA ratio of 3 to 1. For TLR2/6 transfection, expression plasmids carrying the human TLR2, human TLR6 and human CD14 gene [25] were used, kindly provided by Dr. A.M. Keestra (Utrecht University, The Netherlands). Cells transfected with empty vector were used as negative controls and the pTK-LacZ vector was used for normalization of the transfection efficiency. After 48 h of incubation at 37°C, medium was replaced with fresh medium containing 30 μg/ml penicillin (Sigma-Aldrich). Subsequently, cells were stimulated for 5 h with 2.0×10^7/ml bacteria or with 50 μl of SDS-PAGE derived cell wall fractions. The di-acylated lipopeptide FSL-1 (InvivoGen, Toulouse, France) (100 ng/ml) served as a TLR2/6 specific control. After stimulation, cells were washed twice with D-PBS and lysed in 0.1 ml of passive Reporter Lysis Buffer (Promega), according the manufactures description. Subsequently, luciferase activity was determined with a Victor 1420 multilabel counter (PerkinElmer, Groningen, The Netherlands) by incubating 20 μl of lysed cells with 50 μl of luciferase assay substrate (Promega). Luciferase activity was normalized for transfection efficiency by determination of β-galactosidase activity with the β-galactosidase assay (Promega). Relative fold activation was calculated as the normalized reporter activity of the test samples divided by the normalized activity of medium-stimulated control cells.

Statistical analysis

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

Results

***S. suis* activates porcine PBMCs efficiently**

Porcine PBMCs were isolated from pig blood and incubated with *S. suis* and collected bacterial culture supernatant. Penicillin was used to enhance the possible release of PBMC activating components. PBMC activation was determined by measuring changes in IL-1β and IL-8 mRNA transcripts using qRT-PCR. Stimulation of PBMCs with penicillin treated *S. suis* increased IL-1β and IL-8 cytokine transcripts to similar levels as obtained after stimulation with FSL-1, a synthetic lipopeptide (Fig. 1). Activating components were not exclusively cell bound since bacterial culture supernatant stimulated the PBMCs as well (Fig. 1). These results indicate that penicillin-treated *S. suis* is sensed efficiently by the porcine innate immune system and that activating component(s) are released into the supernatant.

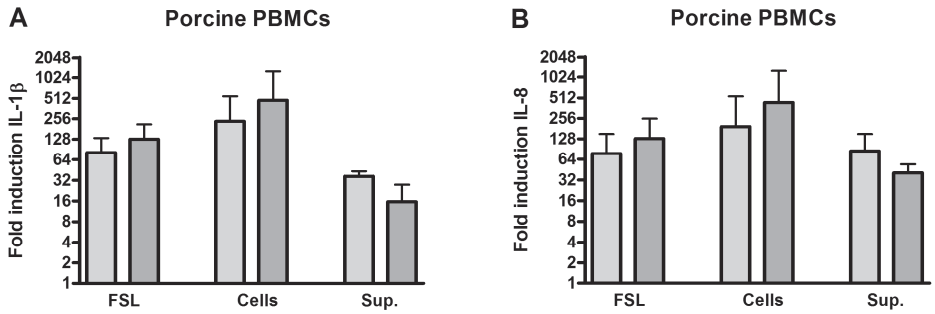

FIG. 1. ***Porcine PBMC stimulation with wild type S. suis.*** *Porcine PBMCs were stimulated with whole S. suis strain 8067 bacteria (cells) in the presence of penicillin or with supernatant (sup.) derived from penicillin treated bacteria. At 2 (light grey bar) and 4 h (dark grey bar) post stimulation IL-1β (A) and IL-8 (B) mRNA expression levels were determined by quantitative real time PCR. The di-acylated lipopeptide FSL was used as a positive control. Data represent fold inductions calculated by dividing the normalized cytokine levels of stimulated cells by the normalized cytokine levels of medium-stimulated negative control cells. Values represent the mean ± SD of two experiments performed in duplicate.*

Identification of innate immunity activating proteins

To gain more insights into the nature of the porcine PBMC activating component(s), we concentrated the supernatant of penicillin-treated *S. suis* and size fractionated it into 24 fractions by SDS-PAGE. The obtained fractions were analyzed for their ability to stimulate porcine PBMCs. More than half of the fractions increased IL-1β and IL-8 cytokine transcript levels as measured by qRT-PCR (Fig. 2A, B). The kinetics of the changes in IL-1β and IL-8 mRNA were very similar. The fractions that caused a more than 5-fold increase in IL-1β and IL-8 mRNA were individually analyzed by mass spectrometry. Mascot scores were determined using the identified peptides in all the fractions simultaneously to increase the sensitivity and specificity of the analysis. A total of 24 *S. suis* proteins with MASCOT scores > 50 (Table 1) were identified. Among these 24 proteins, nine (37.5%) putative lipoproteins were present, including two lipoproteins previously shown to be recognized by porcine convalescent sera [26,27]. In the genome of *S. suis* strain P1/7, 45 putative lipoprotein coding genes are present (Table S1, [28]) which corresponds to 2.5% of the proteome [28]. This large

enrichment of lipoproteins in the porcine PBMC activating fractions suggests that *S. suis* lipoproteins contribute to the observed PBMC activation.

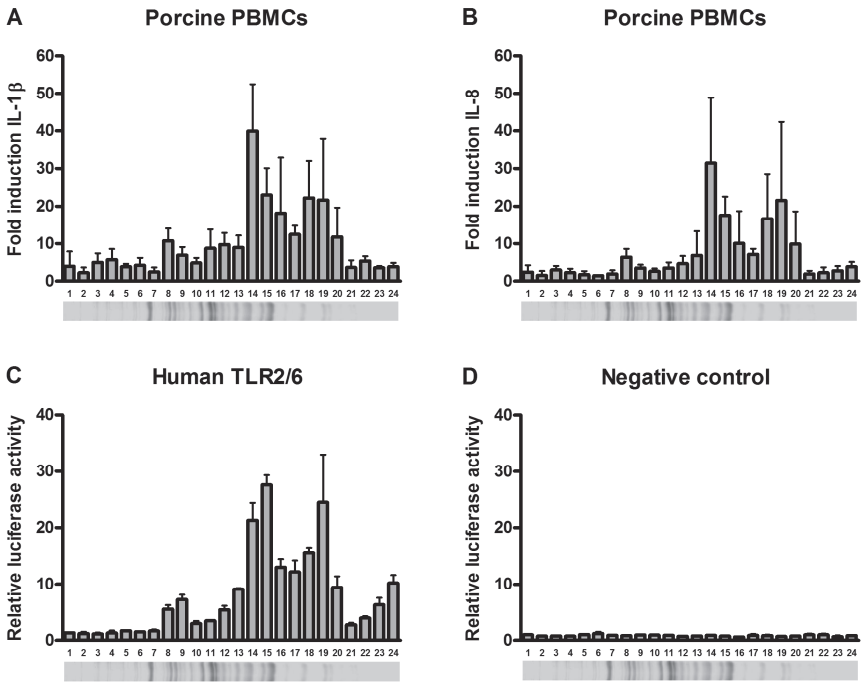

FIG. 2. ***Porcine PBMC and human TLR2/6 stimulation of innate immunity activating fractions.*** *Porcine PBMCs were stimulated with an innate immunity activating fraction of S. suis strain 8067 (concentrated supernatant of penicillin treated bacteria) subdivided into 24 fractions of different molecular sizes. At 4 h post stimulation IL-1β (A) and IL-8 (B) mRNA expression levels were determined by quantitative real time PCR. HeLa 57A cells expressing human TLR2/6 (C), and control cells transfected with vector without insert (D) were stimulated (5 h) with the same 24 fractions. Data represent relative fold activation calculated by dividing the normalized test samples by the normalized activity of medium-stimulated negative control samples. Values represent the mean ± SD of two experiments performed in duplicate. Fractions that induced >5 fold porcine PBMC activation were analyzed by mass spectrometry (Table 1).*

Table 1. Identified proteins within porcine PBMC activating fractions using mass spectrometry

No.	Identified protein	NCBI accession no.	SSU in P1/7	Coverage (%)	peptides matched	# AAs	MW [kDa]	calc. pI	Mascot Score
1	Glyceraldehyde-3-phosphate dehydrogenase	123967422	153	19	30	335	35.6	5.58	1106.37
2*	Basic membrane lipoprotein	81096738	934	12	11	355	36.3	4.93	498.48
3*	High-affinity metal binding protein precursor	146319740	1869	8	6	317	35.5	5.38	298.76
4*	Amino acid ABC transporter, amino acid-binding protein	146318206	503	22	7	280	31.3	4.67	293.96
5	Enoyl-CoA hydratase	146319463	1609	13	5	263	28.6	5.41	271.41
6	Mannose-specific PTS IID	146321637	1585	23	7	303	33.2	8.07	262.69
7*	Amino acid ABC transporter periplasmic protein	146318671	875	22	5	266	28	4.65	262
8	3-oxoacyl-(acyl-carrier-protein) reductase	81097246	1603	26	4	244	25.6	5.47	243.33
9*	Hypothetical protein SSU98_1558	146321405	1364	7	8	380	40.1	4.96	218.37
10	L-lactate dehydrogenase	81096123	927	8	6	327	35.4	5.24	214.96
11	Triosephosphate isomerase	146318185	483	12	3	250	26.6	4.79	204.04
12	Fructose-bisphosphate aldolase	146320177	312	18	4	293	31.1	4.98	200.87
13	Ribosomal protein L1, bacterial and chloroplast form	81097390	1164	25	5	186	19.8	9.35	172.66
14	Phosphoglycerate kinase	146317815	154	8	2	399	42	4.96	161.88
15*	Parvulin-like peptidyl-prolyl isomerase	146321102	1078	9	2	255	27.9	5.16	146.14
16	Elongation factor Ts	81177336	1770	9	2	346	37.2	4.79	134.24
17	Phosphoglycerate mutase 1	81176996	1451	14	3	230	26	5.3	128.5
18*	ABC-type metal ion transport system, periplasmic component/surface antigen	146321629	1577	12	2	283	31	4.67	117.48
19	D-alanine-D-alanine ligase	81096718	1184	6	2	348	38.7	4.79	110.48
20*	Extracellular solute-binding protein, family 3	81096925	1853	11	2	267	28.6	4.58	96.38
21	Hypothetical protein SSU98_0389	146320236	361	19	2	132	15.1	5.94	95.79
22*	ABC transporter substrate-binding protein - maltose/maltodextrin	81097038	1915g	11	2	249	26.6	4.97	95.45
23	Glucokinase ROK	81096801	775	8	2	319	33.4	4.97	84.67
24	Hypothetical protein SSU98_0901	146320748	839	11	2	201	23.4	5.05	67.3

Mass spectrometry identified proteins (of strain 8067) within molecular size fractionated innate immunity stimulating fractions containing > 5 fold porcine PBMC activating capacity. The peptides were searched against the Swiss Prot and non-redundant NCBI database to identify the proteins. In total 24 proteins were identified and ranked by MASCOT score, which indicates the reliability of the identification which is partly correlated with the protein quantity. The corresponding annotated proteins in strain P1/7 [28] are shown in lane 4.

* Annotated as lipoprotein.

Porcine PBMC activating fractions also activate human TLR2/6 expressing HeLa cells

To investigate the specificity of the (lipo)proteins for porcine PBMC activation, we analyzed the same fractions as used in the PBMC experiment to stimulate HeLa cells expressing human TLR2/6 and a NF-κB luciferase reporter [25]. Human TLR2/6 recognizes bacterial lipoproteins including those of *S. suis* [12]. As shown in Fig. 2C, all fractions able to initiate a porcine IL-1β and IL-8 response (Fig. 2A, B) also activated the TLR2/6-expressing HeLa cells (Fig. 2C), while fractions with low activity yielded a poor response in both porcine PBMCs and human TLR2/6-expressing cells. None of the tested fractions was able to activate transfected HeLa cells lacking TLR2/6 expression (Fig. 2D). The comparable

activation of the porcine PBMCs and the human TLR2/6 cell system strongly suggests that lipoproteins have a major role in activating porcine PBMCs, although these results do not exclude that also non-lipoproteins activate porcine PBMCs.

Generation and characterization of a *S. suis* Δ*lgt* mutant

To distinguish between lipoprotein and non-lipoprotein mediated innate immune activation of porcine PBMCs, we constructed a mutant *S. suis* serotype 9 isolate deficient in the expression of the lipoprotein processing enzyme Lgt. Lgt in Gram-positive bacteria is required for lipid modification of the cysteine residue present within the lipobox of prelipoproteins. In the genome of *S. suis* serotype 2 strain P1/7 gene SSU_1418 had been annotated to encode the Lgt protein. This putative *Lgt* protein showed 67% amino acid sequence identity to the Lgt protein of *S. pneumoniae* strain D39 [29]. The *lgt* gene is the second gene transcribed of an operon expressing 4 genes also encoding two putative exported proteins and a phosphorylase enzyme. We inactivated the corresponding *lgt* gene in *S. suis* serotype 9 strain 8067 by homologous recombination generating Δ*lgt* mutant bacteria. A positive control was made by re-introducing an intact *lgt* gene in the Δ*lgt* mutant strain by plasmid complementation generating Δ*lgt*::pGA14-*lgt*. As a negative control we complemented the Δ*lgt* mutant with vector lacking the *lgt* insert, generating Δ*lgt*::pGA14-*cm*. Inactivation of *lgt* resulted in viable *S. suis* bacteria able to grow efficiently in THB after a slightly increased lag phase (Fig. 3).

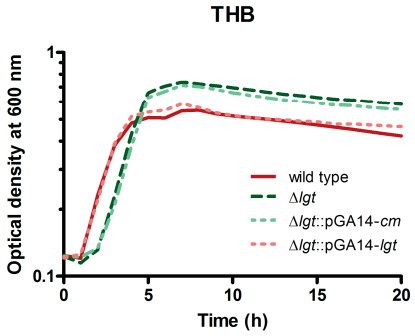

FIG. 3. ***Growth of wild type and Δlgt mutant bacteria.*** *Growth of wild type, Δlgt mutant, and the complemented Δlgt mutant (Δlgt::pGA14-cm; Δlgt::pGA14-lgt) bacteria was assessed in THB by following optical densities in time.*

To verify that lipoprotein processing had been abolished in the Δ*lgt* mutant, lipidation of lipoproteins in the wild type and mutant was analyzed. Bacteria were grown in the presence of [^{3}H]palmitic acid and subsequently treated with penicillin. Similar amounts of protein were released from wild type and (complemented) Δ*lgt* mutant bacteria (Fig. 4). Several radiolabeled proteins were detected in the supernatant of the wild type and the Δ*lgt*::pGA14-*lgt* mutant (Fig. 4), whereas no radiolabeled (lipo)proteins were detected in the supernatant of the Δ*lgt* mutant and the Δ*lgt*::pGA14-*cm* mutant. These data confirm that Lgt is responsible for lipid modification of prelipoproteins in *S. suis*.

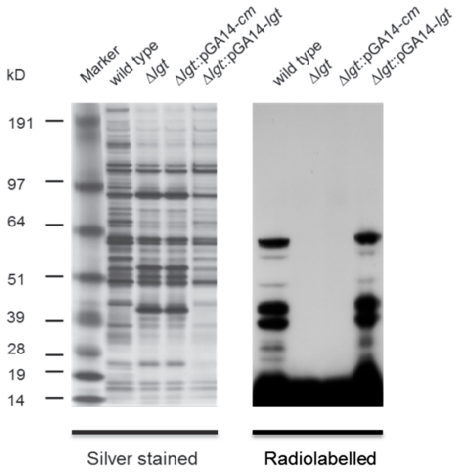

FIG. 4. ***Lipidation of wild type and Δlgt mutant bacteria.*** *Lipidation was assessed by incubating the wild type and mutant bacteria with [^{3}H]palmitic acid, followed by penicillin treatment and SDS-PAGE. Lipidation was visualized using autoradiography. As a control, total protein release of wild type and mutant bacteria was visualized with Silver staining.*

Disruption of *lgt* abolishes activation of human TLR2/6

To investigate whether lipid modification of *S. suis* prelipoproteins is a prerequisite for human TLR2/6 activation, we compared the abilities of the (penicillin-treated) wild type, Δ*lgt* mutant and the complemented Δ*lgt* mutant strains to activate HeLa cells expressing human TLR2/6. Both *S. suis* wild type and the Δ*lgt*::pGA14-*lgt* mutant induced significant TLR2/6 activation (Fig. 5A), in contrast to the Δ*lgt* mutant and the Δ*lgt*::pGA14-*cm* strain. In all cases, stimulation of HeLa cells transfected with the vectors lacking the TLR gene yielded only background levels of NF-κB activity (Fig. 5B). These data indicate that the presence of a protein bound lipid moiety is a prerequisite for activation of human TLR2/6 and that the *S. suis* lipoproteins are the primary ligands that activate the human TLR2/6 complex.

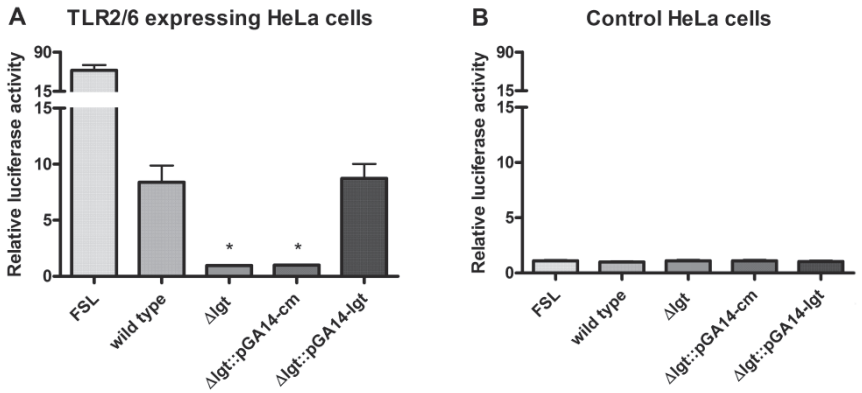

FIG. 5. ***TLR2/6 activating capacity of wild type and Δlgt mutant bacteria.*** *HeLa 57A cells expressing human TLR2/6 (A) and control cells transfected with vector without insert (B) were stimulated with wild type, Δlgt, Δlgt::pGA14-cm, and Δlgt::pGA14-lgt mutant bacteria in the presence of penicillin (30 μg/ml). At 5 h post stimulation, NF-κB luciferase activity was determined. The di-acylated lipopeptide FSL was used as a positive control. Data represent relative luciferase activity calculated by dividing the normalized activity of the test samples by the normalized activity of medium-stimulated negative control samples. Values represent the mean ± SD of three independent experiments performed in duplicate. * P <0.05 compared to wild type level.*

Inactivation of *lgt* reduces PBMC activation

In contrast to the transfected HeLa cells expressing human TLR2/6, porcine PBMC express multiple innate immune receptors that may respond to various *S. suis* components. To assess the contribution of lipoproteins to PBMC activation, we stimulated porcine PBMCs with (penicillin-treated) supernatants and cells of wild type, Δ*lgt* mutant, Δ*lgt*::pGA14-*cm* mutant and Δ*lgt*::pGA14-*lgt* mutant bacteria. Stimulation with the wild type and the Δ*lgt*::pGA14-*lgt* mutant bacterial supernatants resulted in efficient induction of IL-1β and IL-8 mRNA at 2 h and 4 h post stimulation (Figs. 6A and B). As expected, only minimal induction of IL-1β and IL-8 mRNA was observed after stimulation with the Δ*lgt* mutant and Δ*lgt*::pGA14-*cm* mutant derived supernatant. In line with the activation kinetics of the supernatants, PBMCs stimulation with wild type and Δ*lgt*::pGA14-*lgt* mutant bacteria also resulted in efficient induction of IL-1β and IL-8 mRNA at 2 h and 4 h post stimulation (Fig. 6C and D). The IL-1β and IL-8 mRNA levels induced by the Δ*lgt* mutant and the Δ*lgt*::pGA14-*cm* mutant were once more strongly reduced compared to the wild type strain especially at 2 h post stimulation. These results suggest *S. suis* lipoproteins as the principal activators of the porcine PBMC innate immune response.

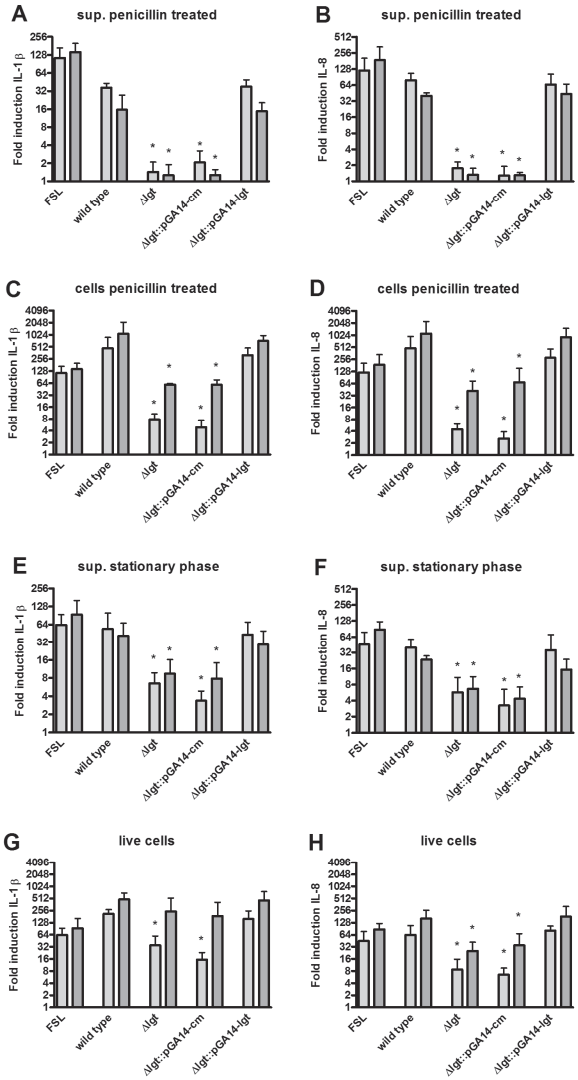

FIG. 6. ***Porcine PBMC activating capacity of wild type and Δlgt mutant bacteria.*** *Porcine PBMCs were stimulated with wild type, Δlgt, Δlgt::pGA14-cm, and Δlgt::pGA14-lgt mutant bacteria. PBMCs were stimulated with supernatants derived from penicillin treated bacteria (A, B), cells in the presence of penicillin (C, D), supernatants of stationary phase grown bacteria in the absence of penicillin (E, F) and cells in the absence of penicillin (G, H). At 2 h (light grey bar) and 4 h (dark grey bar) after stimulation, IL-1β (A, C, E, G) and IL-8 (B, D, F, H) mRNA levels were determined. The di-acylated lipopeptide FSL was used as a positive control. Data represent relative fold activation calculated by dividing the normalized activity of the test samples by the normalized activity of medium-stimulated negative control samples. Values represent the mean ± SD of three independent experiments performed in duplicate. * P <0.05 compared to wild type level.*

Contribution lipoproteins in activating porcine PBMCs in the absence of penicillin

The above results were obtained with penicillin-treated *S. suis* to enhance the release, and enable the identification, of immune activating bacterial factors. To assess the contribution of lipoproteins as activators of the porcine PBMC response in the absence of antibiotics, we stimulated porcine PBMCs with live *S. suis* and supernatants of *S. suis* grown to stationary phase without penicillin. Stimulation of PBMCs with both cells and supernatant of wild type and Δ*lgt*::pGA14-*lgt* mutant bacteria resulted in efficient induction of IL-1β and IL-8 mRNA (Fig. 6E-H). Much less induction of IL-1β and IL-8 mRNA was observed after stimulation with the Δ*lgt* mutant and the Δ*lgt*::pGA14-*cm* mutant cells and supernatants, consistent with the results obtained in the presence of penicillin. Together, these results indicate that *S. suis* lipoproteins are major activators of the innate immune system of the pig.

Discussion

In the present study we identified nine *S. suis* lipoproteins within a fraction able to activate porcine PBMCs efficiently. Disruption of the *lgt* gene required for lipoprotein synthesis strongly reduced activation of porcine PBMCs. This effect was restored after complementation of the gene defect. Altogether, these results provide conclusive evidence that lipoproteins are potent and dominant innate immunity activating molecules of *S. suis*.

The identification of *S. suis* lipoproteins as major activators of porcine PBMCs resulted from detailed analysis of active fractions of bacterial culture supernatant. Mass spectrometry results and the finding that similar fractions activated porcine PBMCs and the human TLR2/6 complex pointed towards possible lipoproteins as activating molecules. We possibly only identified the most abundantly expressed or released lipoproteins of *S. suis* by mass spectrometry. As shown for several bacterial species including *S. suis*, expression levels may vary between different lipoproteins and are influenced by the bacterial environment. In a recent study, three divalent-cation-binding lipoproteins of *S. suis* were shown to be up-regulated after divalent-cation

deprivation *in vitro* [30] and a fourth divalent-cation-binding lipoprotein was shown to be up regulated in mice [27]. Of the nine lipoproteins we identified here, the basic membrane lipoprotein (SSU0934 in *S. suis* P1/7) and a putative high affinity metal binding lipoprotein (SSU1869 in *S. suis* P1/7) have been demonstrated to be recognized by convalescent pig sera [26,27], indicating their expression and immunogenicity *in vivo*.

Porcine PBMCs are expected to express a wide range of PRRs including TLRs. Activation of TLRs by bacterial PAMPs generally results in nuclear translocation of NF-κB followed by transcription of pro-inflammatory cytokines and chemokines such as IL-1β and IL-8. Efficient activation and differences in transcript levels of IL-1β and IL-8 mRNA were already observed at a *S. suis* to PBMC ratio of 1:1 at the start of infection and as early as 2 and 4 h post infection. As expected, other NF-κB dependent cytokines, such as IL-6, TNF-α and IL-10 showed similar kinetics when compared to IL-1β and IL-8 expression profiles (supplementary data, Fig. S1). Cell damage at prolonged infection prevented measurements of accurate cytokine release into the medium. The increase in IL-1β and IL-8 in the porcine PBMCs is likely mediated via porcine TLR2/6, although this could not be measured as the porcine TLR read out systems have not been validated in a porcine cell background. However, we successfully demonstrated that the identified *S. suis* lipoproteins activate human TLR2/6, which has a high level of sequence identity with porcine TLR2/6.

During this study, we initially used penicillin to enhance the release of possible innate immunity activating components of *S. suis*. Penicillin inactivates the penicillin binding proteins essential for the crosslinking of bacterial peptidoglycan, skewing the release of components normally tightly attached to the cell wall or cell membrane. This procedure resulted in increased release of (lipo)proteins in the culture supernatant and facilitated the identification of the innate immunity activating (lipo)proteins. The effect of increased innate sensing after penicillin treatment has also been reported for *S. pneumoniae* [31]. Our finding that PBMC activation also occurred in the absence of penicillin (Fig. 6) excludes adverse effects of penicillin (e.g. cell lysis) on the immune activation. In the absence of penicillin, the effects of immune activation were most pronounced during stationary growth phase. This likely explains the lack of activation of human TLR2/6 by logarithmic phase-derived bacteria [12].

In *S. suis* a significant group of lipoproteins is predicted to have substrate binding and transport functions (Table S1) which suggests lipoproteins to be involved in nutrient acquisition. Interestingly, the *in vitro* growth data of the Δ*lgt* mutant bacteria suggest that nutrient acquisition mediated by lipoproteins is not critical for growth in rich media such as THB or that lipid modification of lipoproteins is not essential for lipoprotein function. The *in vitro* growth ability of the *S. suis* Δ*lgt* mutant bacteria resembles observation of several other *lgt* mutants in streptococcal species such as *S. pneumoniae*, *S. equi*, *S. agalactiae*, *S. sanguinis* and *S. uberis* [17,32,33,34,35]. Probably lipoproteins without lipid moiety are still anchored in the bacterial membrane and able to fulfill (partly) their roles in nutrient acquisition. The observations of reduced innate immune activation, observed for the *S. suis* Δ*lgt* mutant strain, is in agreement with observations in other Gram-positive bacterial species, including *Staphyloccoccus aureus*, *Listeria monocytogenes* and *S. agalactiae* [17,18,36].

On the basis of our results, the absence of lipoprotein lipidation may benefit *S. suis* as it may aid to evade sensing by the innate immune system. On the other hand, the absence of lipoprotein lipidation might affect lipoprotein functionality, which may affect *in vivo* growth and virulence characteristics, interactions with components in the host, and interactions with other surrounding *S. suis* bacteria. These growth effects complicate the interpretation of *in vivo* studies on the effect of *S. suis* on the innate immune response. In *S. sanguinis* and *S. pneumoniae* inactivation of Lgt processing of lipoproteins have been shown to moderately reduce virulence [32,33]. In *S. agalactiae* and *S. aureus* Δ*lgt* mutant bacteria became hypervirulent at a low dose [17,37]. Whether virulence of the *S. suis* Δ*lgt* mutant is affected compared to wild type bacteria and whether this is caused by an altered innate immune response or growth characteristics awaits further study.

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Supporting information

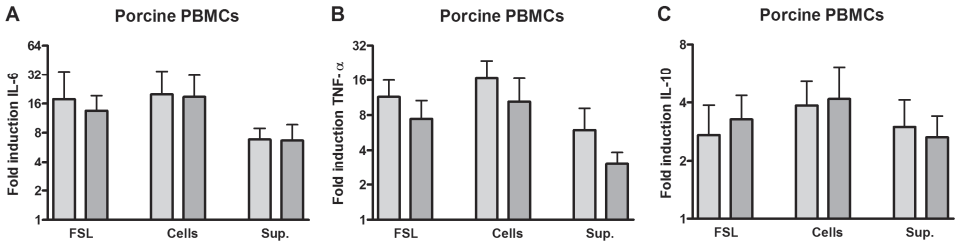

Fig. S1. ***IL-6, TNF-α and IL-10 cytokine responses of porcine PBMCs stimulated with wild type S. suis.*** *Porcine PBMCs were stimulated with whole S. suis strain 8067 bacteria (cells) in the presence of penicillin or with supernatant (sup.) derived from penicillin treated bacteria. At 2 (light grey bar) and 4 h (dark grey bar) post stimulation IL-6 (A), TNF-α (B) and IL-10 (C) mRNA expression levels were determined by quantitative real time PCR. The diacylated lipopeptide FSL was used as a positive control. Data represent fold inductions calculated by dividing the normalized cytokine levels of stimulated cells by the normalized cytokine levels of medium-stimulated negative control cells. Values represent the mean ± SD of two experiments performed in duplicate.*

Table S1. Putative lipoproteins of *S. suis* strain P1/7

nr	SSU	Signal peptide	# AA	kD	Putative function
1	115	MRKVGLLFLSVSALLLGAC	503	55	zinc-binding protein AdcA precursor
2	152	MRKKLKFSLVAVAC	663	73	metalloendopeptidase
3	164	MKKIGRYVTLLAAAGLLAAC	424	47	extracellular solute-binding lipoprotein
4	205	MAKYMKYKNFIWFIC	343	38	sulfonate/nitrate/taurine transport system substrate-binding
5	232	MHKQPLFWTTIAGAVLSFILGVTC	176	19	unknown
6	284	MASKGDCFMLKKVLASALLVSTLTLAAC	291	32	extracellular solute-binding lipoprotein
7	308	MLKKVIRGCFVALFGFVLAAC	306	34	zinc transport system substrate-binding protein
8	370	MVNLLKTTTIKKALLITANILLAGAIIAC	728	80	penicillin-binding protein 1A
9	477	MKKISIMLMVVFSLFLAAC	121	13	unknown
10	**503**	**MNIKKMMLGALALVTSLTLAAC**	**278**	**31**	**extracellular amino acid-binding protein**
11	606	MKKFLAIFSLFVGLVFLTAC	309	34	ferrichrome-binding protein precursor
12	656	MKWNKKLALSAVLVASLFTLSAC	205	23	unknown
13	698	MIMKRTILLCLPTILLLAAC	251	28	putative D-alanyl-D-alanine carboxypeptidase
14	798	MKRKYLALALGLLTIMSLSAC	249	27	unknown
15	801	MAVLKYSKVLLLVLLIATGLSC	262	29	unknown
16	812	MNKKKILASLTLLASTALLFAC	279	31	unknown
17	836	MKKKVISMLALGSIILTAC	125	14	unknown
18	**875**	**MKKILALAATVLAGLTLAAC**	**266**	**29**	**extracellular amino acid-binding protein**
19	876	MKKYIMMGLVLLATMTLSAC	289	32	extracellular amino acid-binding protein
20	**934**	**MNKKLVGLGLAAAAAVSLAAC**	**355**	**39**	**unknown**
21	943	MRKVSRGVLTILLSATVLSAC	409	45	unknown
22	953	MKKKQKLGIAALFLLSSMALSGC	289	32	extracellular phosphate-binding lipoprotein
23	**1078**	**MKQTKKILAGAVTLFAAVTLAAC**	**333**	**37**	**foldase protein PrsA precursor**
24	1092	MYYKLVKKLATISVASMGLLTLAAC	324	36	unknown
25	1093	MGFKKIILSSVALLSAVTLAAC	319	35	unknown
26	1170	MKHTFGKTVVTLLASTVLLAAC	539	59	extracellular solute-binding protein
27	1177	MKKILVLALASSILLTAC	267	29	cyclophilin type peptidyl-prolyl cis-trans isomerase
28	1207	MKQSKKGKSMKKLILCIGLC	251	28	unknown
29	1300	MKLIGNILRWIWIRTFWGLIWLSMVLLAC	219	24	zinc-dependent metalloprotease
30	1303	MRKCLAICSLLCCLFLVGC	134	15	unknown
31	**1364**	**MKTRKFAVALATFASAALLAAC**	**389**	**43**	**extracellular amino acid-binding protein**
32	1372	MKMKTFLKCASVCAFASFLVAC	411	45	multiple sugar-binding protein precursor
33	1390	MGKKKTTLFALTGIILSCHLFLAGC	858	94	Streptococcal histidine triad-family protein
34	1459	MRPRKAGNLNIWKWLFLAQLSLLIGC	198	22	unknown
35	1559	MKKIASLLFVGLLMVLLVAC	349	38	thiamine biosynthesis lipoprotein
36	1560	MKTTKVVLKSLVVLGAVFALVAC	159	17	unknown
37	**1577**	**MKLKKLFSLATAALSVGVLAAC**	**283**	**31**	**D-methionine transport system substrate-binding protein**
38	1639	MKTNLKKLLYSGVTLMSLGVLAAC	384	42	unknown
39	1640	MKIFISKLMLVSTVVLLAAC	223	25	unknown
40	1664	MKKTTKLFALAGVTLLSASVLAAC	596	66	peptide/nickel transport system substrate-binding protein
41	1707	MKTWKKFAYSCVTLASAAALAAC	493	54	extracellular solute-binding lipoprotein
42	**1853**	**MKKIAMFTSLLFAGIVLAAC**	**267**	**29**	**amino-acid ABC transporter extracellular-binding protein**
43	**1869**	**MKKILFSFALLLSLIGLGAC**	**307**	**34**	**extracellular metal cation-binding protein**
44	**1915**	**MKHNLLKSVALLAASTAVLAAC**	**419**	**46**	**putative maltose/maltodextrin-binding protein precursor**
45	1933	MKKKAWLGLLMSLLAVLFLVAC	505	56	fumarate reductase flavoprotein subunit

Bold: Identified in porcine PBMC stimulating fraction.

Table S2. Primer sequences

Primer nr.	Application	Primer name	Sequence (5'-3')
1	Δ*lgt* mutant	LgtF-partB-2_ApaI	GG*gggccc* CCCCCAACCCCTAGCTTATGGTAT
2	Δ*lgt* mutant	LgtR-partB-2_SalI	TA*gtcgac* TAAAGAAATTTCCCCAACGACC
3	Δ*lgt* mutant	LgtF-partA_XmaI	TC*cccggg* GATAGAAGGCTTGCGGACGGACAG
4	Δ*lgt* mutant	LgtR-partA_SacII	CC*ccgcgg* GGTGAGTTGAGACAGCGCCTTATTTT
5	Δ*lgt* mutant	Over LgT heen F	GAGGTGGGCAGGTGTTGAGTTAGC
6	Δ*lgt* mutant	Over LgT heen R	GTTGTCTTTCCGCTACCACTCG
7	Δ*lgt* mutant	Falling out LgT F	TGCCAGCCTTTATCCGAGACC
8	Δ*lgt* mutant	Falling out LgT R	AAAGGCGACCACAACCATACCA
9	Spectinomysin	SpecF_SalI	GC*gtcgac* GCAGGTCGATTTTCGTTCGT
10	Spectinomysin	SpecR_XmaI	AT*cccggg* ATGCAAGGGTTTATTGTTTTCTAA
11	pSET5 vector	RepA-F	GGGCGTATCTATGGCTGTCA
12	pSET5 vector	RepA-R	CTCCCCTAAGGCGAATAAAAG
13	Expression *lgt*	Expr-Lgt-F-SmaI	GG*cccggg* CCTCTTACTTGGATAGTCGCCTG
14	Expression *lgt*	Expr-Lgt-R1-SalI	CG*gtcgac* CGATGGACAAGGCAATAATCAAGAC
15	Expression *lgt*	Chloramp-F-SalI	AG*gtcgac* CTTGGTCTGACAGTTACCAATGC
16	Expression *lgt*	Chloramp-R-SalI	GG*gtcgac* CCGAGGCTCAACGTCAATAAAGC
17	Real time PCR	pIL-1βFabi	GGCCGCCAAGATATAACTGA
18	Real time PCR	pIL-1βRabi	GGACCTCTGGGTATGGCTTTC
19	Real time PCR	pIL-8Fabi	TTCGATGCCAGTGCATAAATA
20	Real time PCR	pIL-8Rabi	CTGTACAACCTTCTGCACCCA
21	Real time PCR	pGAPDH-F2abi	TGCCAACGTGTCGGTTGT
22	Real time PCR	pGAPDH-R2abi	TGTCATCATATTTGGCAGGTTTCT

Sequences in *Italic* correspond to restriction sites

Chapter 4

TroA of *Streptococcus suis* is required for manganese acquisition and full virulence

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Abstract

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.

Introduction

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 [1,2,3]. Although human infections are exceptional, a large outbreak in humans was reported in 2005 in China, with 215 cases and 39 deaths [4]. 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 [5,6,7]. 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* [8,9], and bacterin-based vaccines do not provide protection against multiple serotypes [10].

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 [11]. 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 [12,13]. This recruitment of trace metals by host proteins has recently been regarded as a mechanism of "nutritional immunity" [14]. 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 [15].

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 [16,17]. 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 [18]. 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 [19].

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 Colombia 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 [20].

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 [21]. 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 [22].

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 *Xma*I and *Sal*I, 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 [23], which was linearized with the *Sma*I 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.

Table 1. Primer sequences

Primer nr.	Application	Primer name	Sequence (5'-3')
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	Spectinomysin	SpecF_SalI	GC*gtcgac* GCAGGTCGATTTTCGTTCGT
10	Spectinomysin	SpecR_XmaI	AT*cccggg* ATGCAAGGGTTTATTGTTTTCTAA
11	pSET5 vector	RepA-F	GGGCGTATCTATGGCTGTCA
12	pSET5 vector	RepA-R	CTCCCCTAAGGCGAATAAAAG
13	Expression *troA*	Expr-TroA-F-BamHI	AG*ggatcc* CTTCTCTGCGATGAGTTTATTGGTAA
14	Expression *troA*	Expr-TroA-R-SalI	AT*gtcgac* ATTTTCGCAGAGCACCAATGC
15	Expression *troA*	Chloramp-F-SalI	AG*gtcgac* CTTGGTCTGACAGTTACCAATGC
16	Expression *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

Sequences in *italic* correspond to restriction sites

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 *Sal*I and *Bam*HI, and the *troA* fragment was purified and cloned into pGA14 [24] digested with *Sal*I and *Bam*HI, generating pGA14-*troA*-expr. Finally, the chloramphenicol resistance gene (*cat*) of pSET5, amplified with primers 15 and 16 and digested with *Sal*I, was introduced at the *Sal*I 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, Nieuwerkerk aan den 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 SSU0934 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*. [25] 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 post infection. 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 [17]. 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 [26] (Fig. 1). In *Streptococcus pneumoniae* the closest TroA orthologue is known as the pneumococcal surface adhesin A protein (PsaA) [27], and in *Streptococcus uberis* and *Streptococcus agalactiae*, the closest TroA orthologues are known as MtuA [28] and MtsA [29], 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 [30].

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.

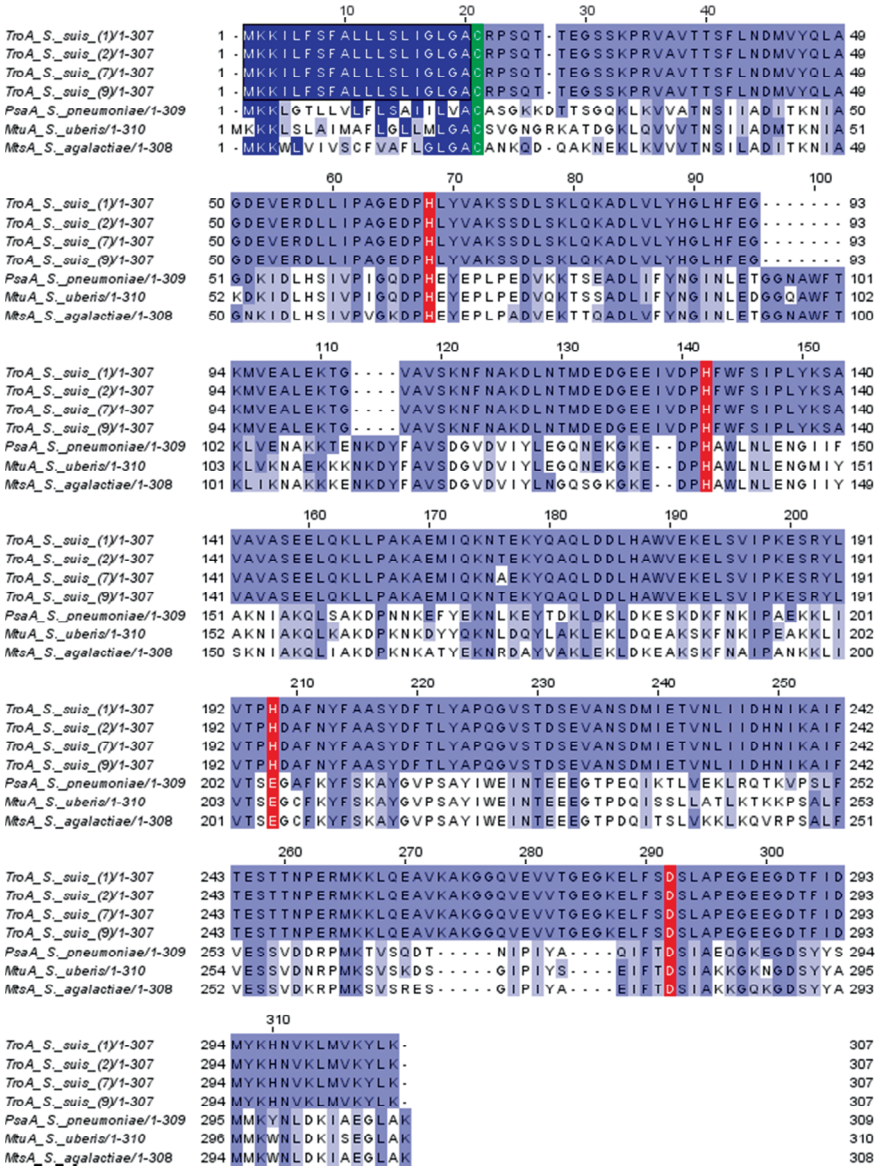

Fig. 1. ***Sequence features of S. suis TroA.*** *ClustalW alignment of the TroA protein present in a serotype 1 (isolate 6388), a serotype 2 (isolate P1/7), a serotype 7 (isolate 7919), and a serotype 9 (isolate 8067) S. suis isolate with orthologous in S. pneumoniae (PsaA), S. uberis (MtuA), and S. 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.*

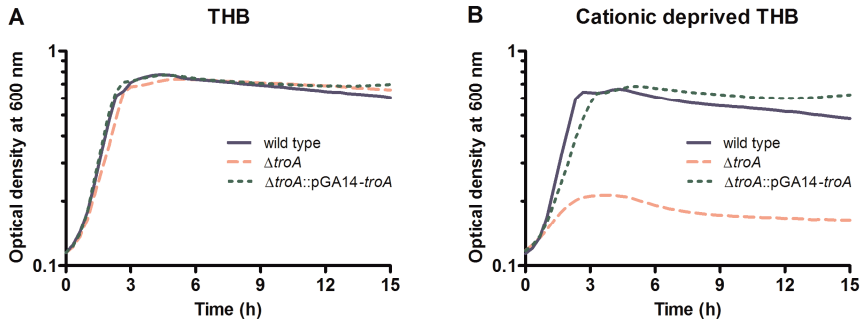

Fig. 2. ***Growth characteristics of the ΔtroA mutant.*** *Growth of wild type, ΔtroA, and ΔtroA::pGA14-troA mutant bacteria in THB (A) and in THB supplemented with 0.5 mM EDTA (B) was followed by measuring optical densities at the indicated time points. Values represent the mean of three independent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

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.

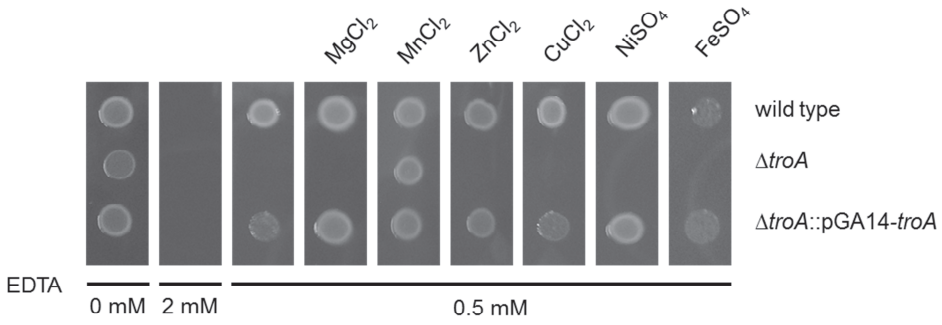

Fig. 3. ***Involvement of TroA in manganese acquisition.*** *Growth of wild type, ΔtroA, and ΔtroA::pGA14-troA mutant bacteria spotted (3 µl of 10^5 CFU/ml) on Colombia agar plates supplemented with EDTA (2 or 0.5 mM) and different trace metals (0.5 mM).*

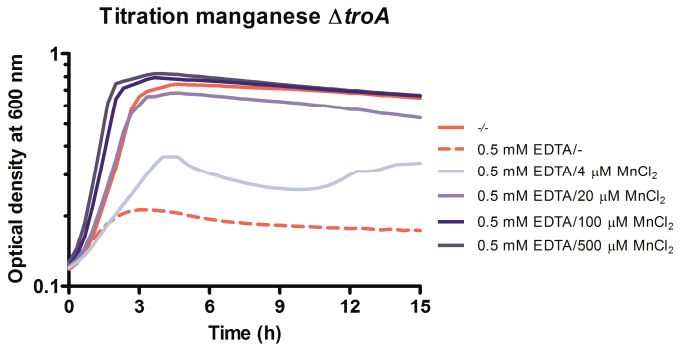

Fig. 4. ***Minimal manganese concentration to maintain growth of ΔtroA mutant.*** *Growth of ΔtroA mutant bacteria in THB supplemented with 0.5 mM EDTA and various concentrations of manganese chloride was followed by measuring optical densities at the indicated time points. Values represent the mean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

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 [31].

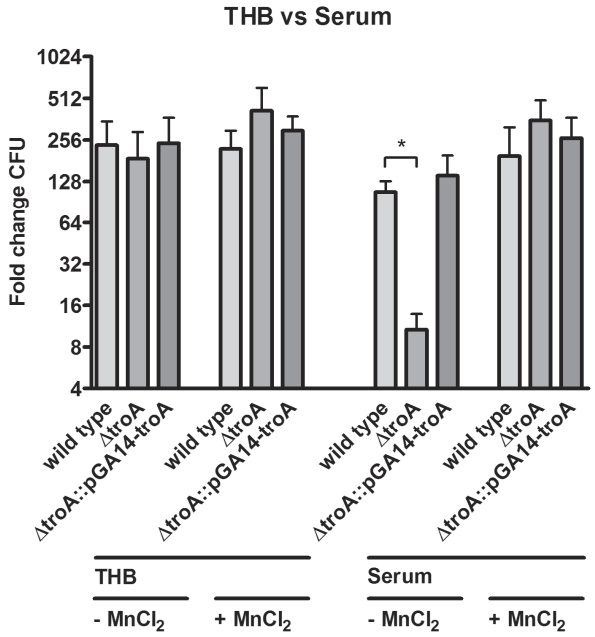

Fig. 5. ***Growth of ΔtroA mutant in porcine serum.*** *Growth of wild type, ΔtroA, and ΔtroA::pGA14-troA mutant bacteria in THB and in 100% porcine serum (10^4 CFU/ml at start) with or without addition of 0.5 mM $MnCl_2$ (+ $MnCl_2$). Relative bacterial growth was determined by dividing the number of 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.*

TroA expression is regulated by manganese availability

Bacteria often tightly regulate the expression of their high-affinity metal binding lipoproteins at the transcriptional level [18,32]. 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 (homologous to SSU0934 and SSU1078 in *S. suis* strain P1/7) [17]. *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.

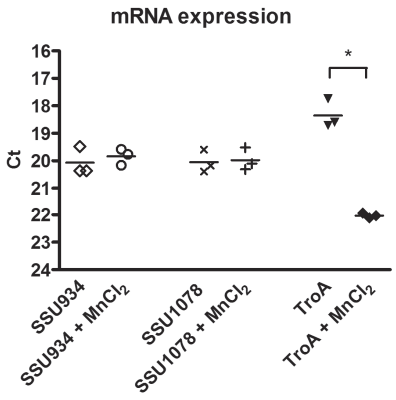

Fig. 6. ***TroA RNA expression.*** *Transcript levels of TroA (SSU1869 in P1/7) as measured by quantitative real-time PCR in S. suis strain 8067 after growth in porcine serum with or without additional 0.5 mM manganese chloride ($MnCl_2$). Homologues of two other P1/7 lipoproteins, SSU0934 and SSU1078, 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.*

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* [33]. 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.

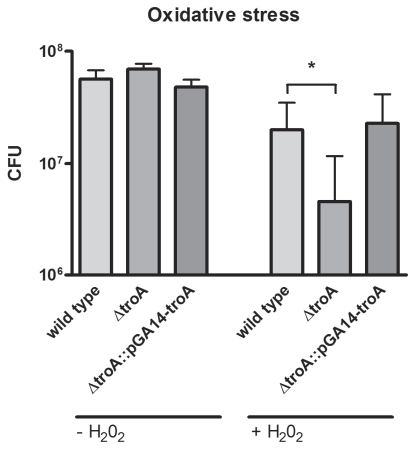

Fig. 7. ***Role of TroA in oxidative stress.*** *Oxidative stress tolerance of wild type, ΔtroA, and ΔtroA::pGA14-troA mutant bacteria in the presence of 25 mM H_2O_2 (+ H_2O_2) in THB. Values represent the means ± standard deviations of three experiments performed in duplicate. *, P < 0.05 compared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

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 [34,35,36]. 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 post infection (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.

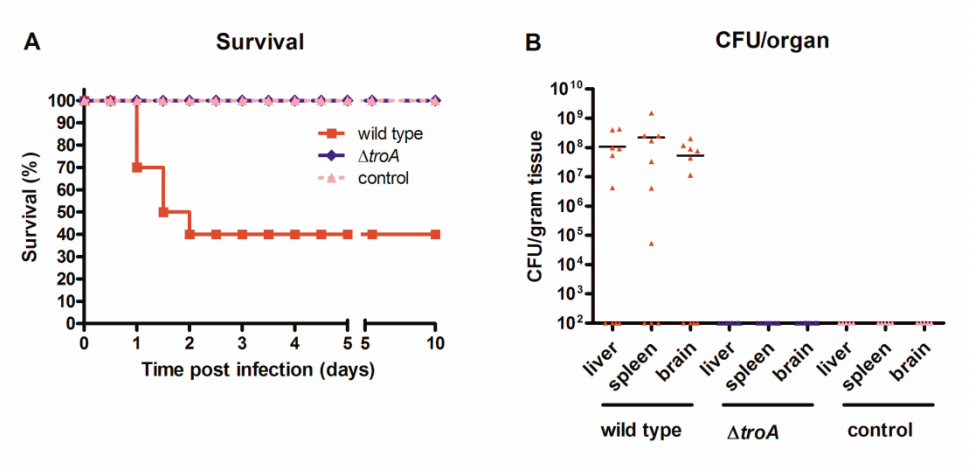

Fig. 8. ***Role of TroA in S. suis virulence.*** *(A) Survival of wild type- and ΔtroA mutant-infected mice. CD1 mice (10 mice/group) were intraperitoneally inoculated with wild type or ΔtroA mutant bacteria or D-PBS. Irreversibly diseased mice were euthanized during the course of the experiment. A Gehan-Breslow-Wilcoxon test revealed a significant difference between wild type and ΔtroA 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.*

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 [19]. 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.

To investigate the involvement of TroA in metal acquisition, we created a Δ*troA* mutant strain and evaluated growth of this 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 [31] 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) [37].

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 [38]. 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 [12]. Interestingly, a recent transcriptome study of swine spleen showed a strong upregulation of calprotectin (also named S100A8/S100A9) after *S. suis* challenge [39]. 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 [34,35,36] 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 [40], and PsaA mutant bacteria of *S. pneumoniae* were nonvirulent in various infection models [41,42,43]. 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 [44,45,46]. The reduced transcription of TroA in porcine serum supplemented with manganese and the upregulation of TroA transcripts [32] 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 [47]. 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 [48]. Alternatively, inactivation of TroA may reduce intracellular manganese levels and the activity of the manganese-dependent superoxide dismutase [33]. 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 [49]. 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 [50]. 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 [50]. The protein contains nine α-helices and eight β-sheets and looks very similar to the structures of MtsA of *Streptococcus pyogenes* [51] and PsaA of *S. pneumoniae* [52] 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* [18], 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 [32,53]. 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 [18]. One of these proteins (SSU0308 in P1/7) induced a significant protective response in mice [18]. Interestingly, the TroA orthologue PsaA is one of the most promising vaccine candidates for protection against *S. pneumoniae* infections [30,54,55,56]. Therefore, TroA of *S. suis* may have potential as a vaccine candidate as well.

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Chapter 5

Lysozyme resistance in *Streptococcus suis* is highly variable and multifactorial

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Abstract

Streptococcus suis is an important infectious agent for pigs and occasionally for humans. The host innate immune system plays a key role in preventing and eliminating *S. suis* infections. One important constituent of the innate immune system is the protein lysozyme, which is present in a variety of body fluids and immune cells. Lysozyme acts as a peptidoglycan degrading enzyme causing bacterial lysis. Several pathogens have developed mechanisms to evade lysozyme-mediated killing. In the present study we compared the lysozyme sensitivity of various *S. suis* isolates and investigated the molecular basis of lysozyme resistance for this pathogen. The lysozyme minimal inhibitory concentrations of a wide panel of *S. suis* isolates varied between 0.3 to 10 mg/ml. By inactivating the *oatA* gene in a serotype 2 and a serotype 9 strain, we showed that OatA-mediated peptidoglycan modification partly contributes to lysozyme resistance. Furthermore, inactivation of the *murMN* operon provided evidence that additional peptidoglycan cross-linking is not involved in lysozyme resistance in *S. suis*. Besides a targeted approach, we also used an unbiased approach for identifying factors involved in lysozyme resistance. Based on whole genome comparisons of a lysozyme sensitive strain and selected lysozyme resistant derivatives, we detected several single nucleotide polymorphisms (SNPs) that were correlated with the lysozyme resistance trait. Two SNPs caused defects in protein expression of an autolysin and a capsule sugar transferase. Analysis of specific isogenic mutants, confirmed the involvement of autolysin activity and capsule structures in lysozyme resistance of *S. suis*. This study shows that lysozyme resistance levels are highly variable among *S. suis* isolates and serotypes. Furthermore, the results show that lysozyme resistance in *S. suis* can involve different mechanisms including OatA-mediated peptidolycan modification, autolysin activity and capsule production.

Introduction

Streptococcus suis is an important pig pathogen causing severe infections including meningitis, septicemia, endocarditis, pneumonia and arthritis. *S. suis* is also a zoonotic agent displaying comparable disease manifestations in humans as are seen in pigs [1,2,3]. The host innate immune system is an important factor in the prevention and elimination of *S. suis* infections, the involvement of pattern recognition receptors (PRRs) in sensing *S. suis* has recently been described [4,5]. However, little is known about the role of effector molecules of the innate immune system in counteracting *S. suis* infections. One important effector molecule, with anti-bacterial activity, is the protein lysozyme.

Lysozyme is found in high concentrations (>500 μg/ml) in several bodily secretions including tears, mucus, milk and saliva [6,7]. In addition neutrophil granules contain significant amounts of the protein [8,9,10]. Lysozyme weakens bacterial peptidoglycan layers by hydrolysis of the [1,4]-β-linkages between N-acetylmuramic acid (NAM) and N-acetyl-D-glucosamine (NAG) residues. Extensive hydrolysis results in bacterial lysis. Deficiencies in lysozyme, by gene inactivation, have been shown to increase susceptibility to streptococcal disease [11].

To survive in hostile environments comprising high levels of lysozyme, bacteria have evolved mechanisms to resist lysozyme digestion. In streptococcal species the peptidoglycan modifying N-acetylglucosamine deacetylase PgdA and the peptidoglycan O-acetyltransferase OatA (designated Adr in *S. pneumoniae* [12]), confer lysozyme resistance. Both enzymes directly change the NAM and NAG structures of peptidoglycan and reduce lysozyme affinity to the peptidoglycan layer [12,13,14]. In addition, the tRNA dependent ligases MurM and MurN, encoded by the *murMN* operon, can increase resistance to lysozyme by introducing extra peptide cross-linking in the peptidoglycan layer [15,16]. Occasionally, such molecular changes to the peptidoglycan layer are accompanied with changes in bacterial morphology [17].

For *S. suis* the lysozyme sensitivity between and within serotypes has not been investigated systematically. Furthermore, limited data is available about the involvement of peptidoglycan modifying enzymes of *S. suis* in lysozyme

resistance. So far, only the role of a PgdA homologue in lysozyme resistance of a serotype 2 strain has been reported [18]. In the latter study, an isogenic *pgdA* mutant showed an unaltered lysozyme resistance phenotype compared to wild type bacteria *in vitro* and a strongly reduced virulence *in vivo*. The genome sequences of various *S. suis* serotype 2 strains suggests the presence of an O-acetyltransferase (OatA) gene and the absence of a pneumococcal *murMN* operon homologue in *S. suis* [19,20]. However, recent sequence analysis of a *S. suis* serotype 9 strain suggests the existence of a *S. suis murMN* operon [21].

The objective of the present study was to determine lysozyme resistance levels in a panel of *S. suis* isolates and to investigate the molecular basis of this resistance. To accomplish this, we focussed on homologues of well-known peptidoglycan modifying enzymes. In addition, we also used an unbiased approach based on comparative whole genome analysis.

Materials and methods

Bacterial strains

We used a panel of *S. suis* serotype 1, 2, 7, and 9 isolates as depicted in Table S1. Wild type bacteria, isogenic mutants and complemented mutant strains were grown on Colombia agar plates (Oxoid Ltd, London, United Kingdom) containing 6% horse blood at 5% CO_2 and 37°C. Bacterial suspensions were grown in Todd-Hewitt broth (THB) (Oxoid Ltd.) for 18 h at 37°C without agitation. *Escherichia coli* were grown on Luria-Bertani (LB) agar plates and in LB broth. Antibiotics were added to culture media at the following concentrations: for *E. coli*, ampicillin 100 µg/ml; chloramphenicol 8 µg/ml and spectinomycin 100 µg/ml; for *S. suis*, chloramphenicol 5 µg/ml and spectinomycin 100 µg/ml when necessary.

Lysozyme MIC assay

Lysozyme MICs were determined by spotting 8 µl (containing ≈ 5×10^4 CFU) bacterial suspension in Dulbecco's phosphate buffered saline (D-PBS) on Colombia agar plates containing two-fold increasing concentrations of lysozyme (L6876, Sigma-Aldrich, Zwijndrecht, The Netherlands). Subsequently, growth

was evaluated after incubation at 5% CO_2 and 37°C for 24 h. The minimal concentration in which > 99,9% of bacteria were unable to grow was designated the MIC value.

Detection *oatA*, *pgdA*, *murM* and *murN* genes

Overnight cultures in THB were diluted 1:10 in D-PBS and directly used as template in PCR analysis. Primers (Table S1) used to detect *oatA* (SSU1504 in P1/7), *pgdA* (SSU1448 in P1/7), *murM* (SSUD12_0367 in D12) and *murN* (SSUD12_0368 in D12) genes were designed to bind at relatively conserved sequence regions, based on the available *S. suis* genomes in the NCBI database and based on a preliminary *S. suis* serotype 9 genome sequence (H. E. Smith *et al.*, unpublished results). In a final volume of 20 µl, forward and reverse primers (final concentration; 0.25 µM, Table S1) were mixed with 1 × Phusion High-Fidelity DNA polymerase master mix (BIOKE, Leiden, The Netherlands) and 2 µl template. PCR conditions were as follows: denaturation for 2 min at 98°C, followed by 35 cycles of 15 s of denaturation at 98°C, 15 s of annealing at 55°C, and 30 s elongation. Amplification of the specific genes was verified on ethidium bromide based 1% agarose gels.

THB growth

Overnight THB cultures (containing similar amounts of CFUs) of wild type and mutant strains were diluted 1:100 in 400 µl fresh THB with or without the addition of 500 µg/ml lysozyme. Subsequently, the optical density at 600 nm was followed in time using a Bioscreen C instrument (Thermo Scientific, Breda, The Netherlands) at 37°C.

Generation 10-Lys-R1 and 10-Lys-R2 strains

Serotype 2 strain 10 (lysozyme sensitive) was plated on Colombia agar plates containing two-fold increasing concentrations of lysozyme and allowed to grow for 24 h at 37°C and 5% CO_2 (passage one). Subsequently, colonies growing on plates containing the highest lysozyme concentration were collected, re-suspended in 100 µl D-PBS and used to inoculate a new set of plates (passage two). Yet again, colonies growing on plates containing the highest lysozyme

concentration were collected and used to inoculate a new set of plates (passage three). After ≤ four passages highly lysozyme resistant clones were obtained.

Construction mutants and complemented mutants

General DNA techniques

Chromosomal *S. suis* DNA was isolated as previously described [22]. Phusion High-Fidelity DNA polymerase master mix was used to amplify specific fragments. Plasmid DNA was isolated with the Plasmid DNA Purification System (Promega, Leiden, The Netherlands). DNA purifications were performed with the Zymogen clean up 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 [23]. Primers used in this study are listed in Table S1.

Generation of oatA and murMN mutants

To inactivate the *oatA* gene in serotype 2 strain 10 and serotype 9 strain 8067 and to inactivate the *murMN* operon in strain 8067 we used an inverse PCR strategy. Briefly, primer pairs 1/4 respectively 9/12 were used to amplify chromosomal fragments of the *oatA* and the *murMN* operon with flanking regions of about 0.7-1.5 kb. The fragments were subsequently cloned into pJET1.2 (Fermentas, St. Leon-Rot, Germany) according the manufactures instructions. Subsequently, the generated pJET1.2 plasmids were used as template for an inverse PCR using primer pairs 2/3 or 10/11 to replace an internal fragment by a fragment encoding a spectinomycin resistance mechanism (*spc*, amplified with primers 17/18) as described previously [5,24]. The *spc* gene was oriented in the same direction as the gene of interest. The resistance cassette containing the flanking regions of *oatA* and/or *murMN* were subsequently amplified using primer pairs 1/4 and 9/12. Finally, these fragments were ligated to the thermo sensitive shuttle vector pSET5 [25], which was linerialized with the *Sma*I restriction enzyme. The pSET5 plasmids were than used to inactivate the *oatA* gene of strain 10 and strain 8067 and the *murMN* operon of strain 8067 as previously described [5,24] generating 10-Δ*oatA*, 8067-Δ*oatA* and 8067-Δ*murMN*. Mutants were confirmed to have the expected genotype by PCR using primer pairs 5/6, 7/8, 13/14, 15/16.

MurMN complementation

To complement strain 10 with the *murMN* operon of strain 8067 we constructed an expression plasmid containing the 8067 *murMN* operon including its putative promoter region. Primers 33 and 34 were used to amplify the *murMN* fragment which was cloned into pJET1.2 generating pJET1.2-*murMN*. Subsequently, the pJET1.2-*murMN* plasmid was digested with *Sal*I and a chloramphenicol resistance gene (*cat*) of pSET5, amplified with primers 15 and 16 and also digested with *Sal*I was cloned upstream. The entire fragment (*murMN-cat*) was amplified using primers 35 and 36 and subsequently cloned into pGA14 [26], which is able to replicate in *S. suis*. To do this pGA14 was digested with *Hin*DIII and *Sac*I, made blunt and ligated to the *murMN-cat* PCR fragment, generating pGA14-*murMN-cat*. The plasmid was introduced into *S. suis* strain 10 generating 10::pGA14-*murMN*. RNA expression of the *murMN* operon was confirmed by quantitative real time PCR.

SSU0475 inactivation

To inactivate SSU0475 (autolysin) in *S. suis* strain 10 we used an overlap extension PCR strategy. Briefly, three fragments (flanking left, *spc*, flanking right) were generated using primers pairs 19/20, 21/22 and 23/24. Subsequently, the three fragments were mixed and used as template in a fusion PCR mixture containing primers 19 and 24. The resulting fragment flanking left-*spc*-flanking right was cloned into pJET1.2 according the manufactures instructions. Subsequently, the plasmid was directly used for electroporation into strain 10 followed by spectinomycin selection. Double cross over mutants were identified by PCR using primer pairs 25/26, 27/28 and 31/32.

SSU0519 complementation

To complement strain 10-lysR-2 with an intact copy of SSU0519 (*cps*2E) we constructed a plasmid containing the promoter region and ribosomal binding site of SSU0514 fused with the start codon of SSU0519. In addition the spectinomycin resistance gene (*spc*) was added upstream the fragment for positive selection. Briefly, the three fragments (promoter region SSU0514, SSU0519, *spc*) amplified with primer pairs 37/38, 39/40 and 41/42 were generated and used as template in an overlap extension PCR reaction containing primers 37 and 42. The resulting

fragment was subsequently ligated into the thermo sensitive shuttle vector pSET5 [25], which was linerialized with the *Sma*I restriction enzyme. The pSET5 plasmid was subsequently introduced into strain 10-lysR-2 at 37°C allowing single cross over events generating 10-lysR-2-*cps*2E.

Sequence analysis

Highly purified DNA of parent strain 10, strain 10-lysR-1 and strain 10-lysR-2 was isolated [22] and used for paired-end Illumina sequence analysis (BaseClear, Leiden, The Netherlands). The reads were 50 or 75 bp in length and across all bases Illunina quality scores (1.5 encoding) were above 30 and no over-representative k-mers were observed. Of each individual strain the average insert size was about 250 bp. Using CLCbio software, the reads were subsequently mapped against the serotype 2 reference strain P1/7 [19], resulting in a general coverage > 100, and unmapped reads were assembled *de novo*. Subsequently, single nucleotide polymorphisms (SNPs), insertions and deletions (InDels) of the individual strains were identified and the genetic changes in 10-LysR-1 and 10-LysR-2, obtained due to the lysozyme resistance selection procedure were identified.

Morphological analysis

Crystal violet staining

Exponentially growing wild type or mutant bacteria (in THB) were heat fixed onto glass slides, washed with water, stained with crystal violet and again washed with water. Bacteria were directly visualized using a Zeiss microscope (1000×).

TEM analysis

TEM analysis was performed as previously described with some modifications [27]. Briefly, exponentially growing wild type or mutant bacteria (in 10 ml THB) were harvested by centrifugation, washed with D-PBS and re-suspended in 1 ml D-PBS. Subsequently, bacteria were fixed in cacodylate buffer (0.1 M cacodylate, 5% w/v glutaraldehyde, 0.15% ruthenium red) for 2 h at room temperature, immobilized and pelleted in 2% agarose. One mm^3 pieces of the bacterial pellet were washed with cacodylate buffer (0.1 M cacodylate) and post-fixed with 2%

v/v osmium tetraoxide (overnight at room temperature). Finally, the pieces were dehydrated in graded series of ethanol (50, 70, 95, and 100%) and embedded in Spurr low-viscosity resin (Aurion, Wageningen, The Netherlands) according the manufactures instructions. The samples were cut using a diamond knife and post-stained with uranyl acetate and lead citrate and viewed in an electron microscope (Philips CM 10) at 60 kV.

Results

Heterogeneous lysozyme resistance levels in *S. suis*

To investigate lysozyme resistance levels in the *S. suis* species, the lysozyme minimal inhibitory concentration (MIC) was determined for a broad panel of *S. suis* isolates belonging to serotypes 1, 2, 7 or 9. The lysozyme MICs varied between 0.3 and 10 mg/ml, as measured by a plate assay (Fig. 1 and Table S2). In general, isolates belonging to serotypes 7 and 9 resisted higher levels of lysozyme compared to serotype 1 isolates and the majority of the serotype 2 isolates. The differences in lysozyme resistance among the serotype 2 isolates correlated with clusters A and B identified by comparative genome hybridization (CGH) for this serotype [28]. Taken together, these results indicate that lysozyme resistance varies between and within different *S. suis* serotypes and suggest that resistance levels correlate with serotype-related genetic backgrounds.

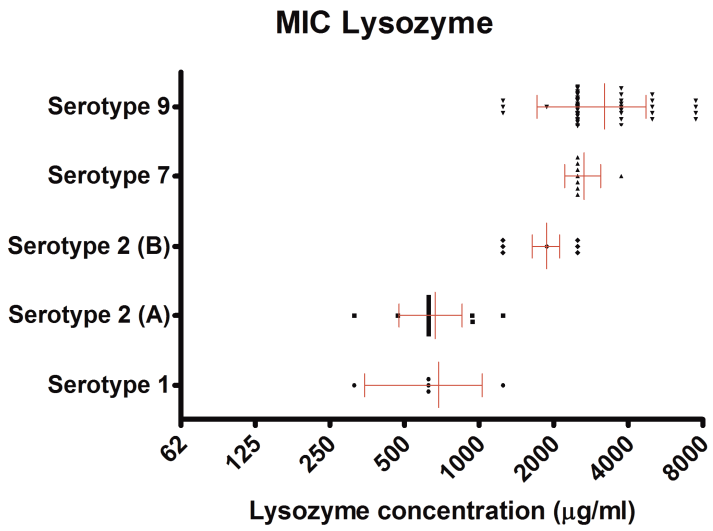

FIG. 1. ***Lysozyme MIC levels of S. suis.*** *Wild type serotype 1, 2, 7 and 9 isolates (Table S2) were spotted onto Colombia agar plates containing two-fold increasing concentrations of lysozyme (start concentration: 62.5 μg/ml). Growth was assessed 24 h later and MICs were determined. Serotype 2 strains were separated into two (A and B) different genetic clusters based on CGH data [28]. Each isolate is represented by a dot and one dot represents the mean of two independent observations. The red lines represents the mean resistance level and SD of the indicated groups.*

Distribution of genes that encode peptidoglycan modifying enzymes

To assess the presence in *S. suis* of genes encoding peptidoglycan modifying enzymes, known to be involved in lysozyme resistance in other streptococcal species, we performed gene specific PCRs (*pgdA*, *oatA*, and *murMN*) on the same panel of *S. suis* isolates used above. PCRs yielded products of the expected size for the *pgdA* and *oatA* genes in all tested isolates (Table 1, Table S1), suggesting that *pgdA* and *oatA* are widely distributed in the *S. suis* species. A putative *murMN* operon (presence product of the expected size) could be detected in about half of the isolates tested. The putative *murMN* operon is predominantly present in serotype 9 isolates with relative high lysozyme resistance levels, however the majority of relatively high lysozyme resistant serotype 7 and 2 isolates lack the operon. Furthermore, the *murMN* operon could not be detected in all lysozyme sensitive serotype 1 and 2 isolates (Table 1, Table S1). Based on these data, no

apparent correlation could be detected between *pgdA*, *oatA* and/or *murMN* presence and the level of lysozyme resistance.

Table 1. Distribution of genes encoding peptidoglycan modifying enzymes in different *S. suis* serotypes

Serotype	*oatA*	*pgdA*	*murMN*
1	(5/5)	(5/5)	(0/5)
2 (CGH A)	(19/19)	(19/19)	(0/19)
2 (CGH B)	(7/7)	(7/7)	(4/7)
7	(8/8)	(8/8)	(1/8)
9	(55/55)	(55/55)	(53/55)

Serotype 2 strains were separated into two (A and B) different genetic clusters based on CGH data [22]. The numbers between brackets represent the number of positive PCR products compared to the total number of analyzed isolates.

OatA but not MurM and MurN contributes to lysozyme resistance

To investigate the role of cell wall modification to *S. suis* lysozyme resistance in more detail, we constructed isogenic *oatA* and *murMN* mutants. *OatA* and *murMN* mutant strains were constructed of serotype 9 strain 8067, a relative high lysozyme resistant strain (MIC 2.5 mg/ml). In addition, an oatA mutant strain and a *murMN* complemented strain were constructed of serotype 2 strain 10, a relative low lysozyme resistant strain (MIC 0.3 mg/ml). PgdA mutants were not constructed in the present study as this gene has recently been reported not to affect *S. suis* lysozyme resistance [18]. Comparisons of the lysozyme MICs of mutant and parent strains indicated that lysozyme MICs of the 8067-Δ*murMN* mutant and the *murMN* complemented strain 10 (10::pGA14-*murMN*) were identical to those of their parent strains (Fig. 2). However, the *oatA* mutants (10-Δ*oatA* and 8067-Δ*oatA*) displayed increased sensitivity to lysozyme compared to their parent strains (Fig. 2). These results strongly suggest that the presence of OatA and not MurM and MurN increases lysozyme resistance in *S. suis*. Since the lysozyme MIC levels of strain 10-Δ*oatA* and strain 8067-Δ*oatA* differ significantly, it is unlikely that OatA is solely responsible for the lysozyme resistant phenotypes in *S. suis*.

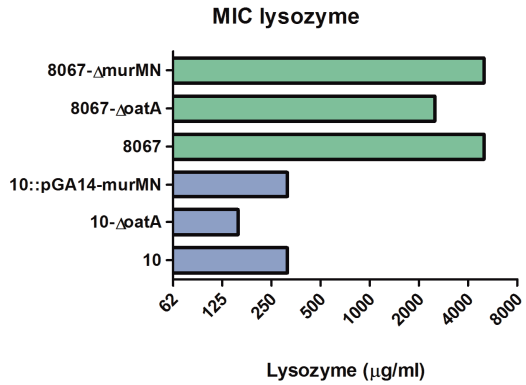

FIG. 2. ***Lysozyme MIC levels of OatA and MurMN mutants.*** *Strains 10-ΔoatA, 8067-ΔoatA, 8067-ΔmurMN, 10::pGA14-murMN and wild type strain 10 and 8067 were spotted onto Colombia agar plates containing two-fold increasing concentrations of lysozyme. Growth was assessed 24 h later and MICs were determined. Green bars represent wild type and mutant derivatives of serotype 9 strain 8067 and blue bars represent wild type and mutant derivatives of serotype 2 strain 10. Values represent the mean of three independent observations.*

Selection for lysozyme resistance

To discover additional genetic factors involved in lysozyme resistance in *S. suis*, we took advantage of the observation that a lysozyme sensitive strain can acquire higher lysozyme resistance levels by passage in the presence of sub-lethal concentrations of lysozyme. As shown in Fig. 3, the lysozyme sensitive strain 10 (serotype 2) acquired step-wise higher lysozyme resistance levels during passage on plates with successive increasing concentrations of lysozyme. After four passages the lysozyme MIC of the isolate was even higher compared to the MIC of the natural lysozyme resistant isolates of serotypes 2, 7 and 9 (Fig 1). In two independent rounds of passaging two lysozyme resistant strains, designated 10-LysR-1, and 10-LysR-2, were obtained. After sub-culturing in the absence of lysozyme, the strains remained equally resistant to lysozyme. For both strains we compared the growth rate in THB with the growth rate in THB supplemented with 500 μg/ml lysozyme. In THB growth rates of strain 10-LysR-1 and 10-LysR-2 roughly resembled those of the wild type strain, although strain 10-LysR-1 displayed an extended lag phase (Fig. 4A). In the presence of lysozyme, only the selected strains 10-LysR-1 and 10-LysR-2 were able to grow efficiently (Fig. 4B), consistent with the selected lysozyme-resistant phenotype.

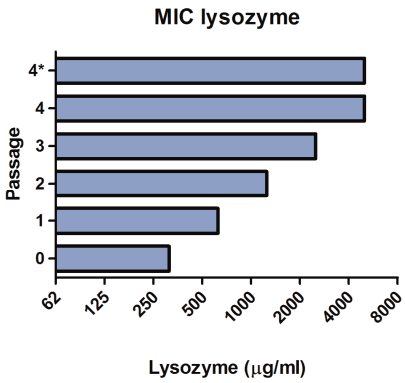

FIG. 3. ***Lysozyme MIC levels of strain 10 after passaging.*** *Lysozyme MIC of strain 10 passaged four times onto Colombia agar plates containing two-fold increasing concentrations of lysozyme (start concentration: 62.5 μg/ml). Within 4 passages the lysozyme MIC increased towards levels observed in natural lysozyme resistant strains (compare with Figure 1). * Lysozyme MIC level after sub-culturing the lysozyme resistant strain in the absence of lysozyme. Values represent the MIC levels of one selection procedure.*

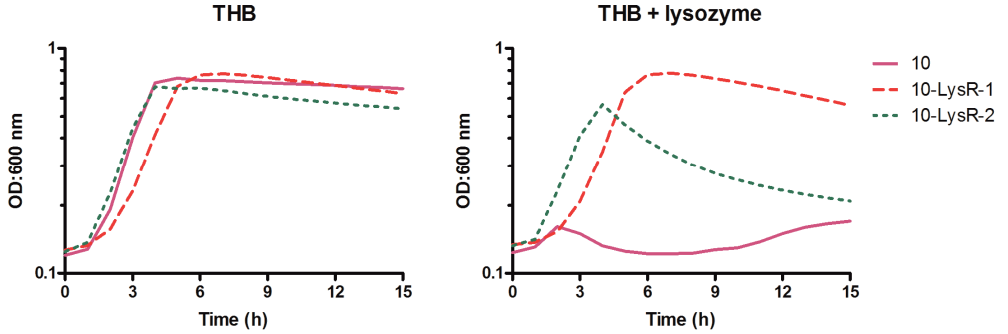

FIG. 4. ***Growth curves of wild type, 10-LysR-1 and 10-LysR-2 S. suis strains.*** *Growth of S. suis strain 10 (wild type), strain 10-LysR-1 and strain 10-LysR-2 in THB (A) and in THB supplemented with 500 μg/ml lysozyme (B). Values represent the mean of three independent experiments. At almost all time points (three per hour) SD values were maximal 30% of the indicated values.*

Identification of gene polymorphisms associated with increased lysozyme resistance

Since the acquired lysozyme resistance phenotype of strains 10-LysR-1 and 10-LysR-2 remained stable during sub-culture in the absence of lysozyme (Fig. 3), we expected to find changes in the genome sequences of these strains. To identify these genomic alterations we performed Illumina whole genome sequence analysis of strain 10-LysR-1, strain 10-LysR-2 and of parent strain 10. The paired end sequence reads were mapped to reference strain P1/7 [19] and the unmapped reads were assembled *de novo*. Subsequently, SNPs and insertions and deletions of each individual strain were identified relative to reference strain P1/7. Finally, by subtraction, differences in the genome sequences of strain 10-LysR-1, strain 10-LysR-2 and the parent strain 10 were identified. As shown in Table 2, strain 10-LysR-1 and strain 10-LysR-2 both had acquired 3 SNPs during the selection procedure. No insertions or deletions resulting from the selection were detected. The SNPs present in strain 10-LysR-1 were at different loci compared to the SNPs in strain 10-LysR-2, suggesting lysozyme resistance can be acquired via different routes and/or mechanisms. Of the 6 identified SNPs in the two lysozyme resistant strains, 5 were present in protein coding regions and resulted in amino-acid substitutions. In strain 10-LysR-1 one SNP resulted in the amino-acid substitution of His-136-Asn in gene SSU0383 (protein phosphatase), one SNP resulted in the substitution of Arg-215-Ser in gene SSU1292 (membrane protein), and one resulted in the substitution of the start codon (Met-1-Ile) of gene SSU0475 (glycosyl hydrolase family protein; putative autolysin). In strain 10-LysR-2 one SNP resulted in the substitution of Thr-138-Ile in SSU1566 (TrkA family transport protein) and one resulted in the introduction of a stop codon (Leu-211-Stop) in the Cps2E protein (SSU0519, sugar transferase involved in capsule synthesis). The third SNP was located in a non-coding region between gene SSU0319 and gene SSU0320.

Table 2. Genomic differences observed between parent strain 10 and its selected lysozyme resistant derivatives 10-LysR-1 and 10-LysR-2

Strain	Reference Position	Variation Type	Reference	Allele Variations	Gene in P1/7	Amino Acid Change
10-LysR-1	409567	SNP	C	A	SSU0383	His136Asn
	507081	SNP	C	T	SSU0475	Met1Ile
	1323604	SNP	G	T	SSU1292	Arg215Ser
10-LysR-2	339081	SNP	T	G		
	557107	SNP	T	G	SSU0519	Leu211Stp
	1571782	SNP	C	T	SSU1566	Thr138Ile

SSU0475 (autolysin) and SSU0519 (capsule) involved in lysozyme resistance

From the 6 identified SNPs, the SNPs in SSU0475 (putative autolysin) and SSU0519 (cps2E) putatively result in defects in protein expression due to an inactivated start codon and the introduction of a premature stop codon, respectively. To demonstrate the power of this unbiased search for genes involved in a particular trait and to verify the role of these genes in lysozyme resistance, we selected the latter two genes and tested isogenic mutants with defects in gene SSU0475 (10-Δ*0475*) and gene SSU0519 (capsule mutant, 10-Δ*cps*2EF) [29] for lysozyme resistance. In addition we tested the lysozyme resistant phenotype of strain 10-LysR-2 complemented with an intact copy of the SSU0519 gene. We were unable to introduce an intact gene copy of SSU0475 into strain 10-LysR-1. As shown in Fig. 5 the lysozyme MICs of strains 10-Δ*0475* and 10-Δ*cps*2EF were increased compared to wild type strain 10 and thus resembled the phenotypes of the passage-selected resistant strains. In contrast introduction of an intact copy of gene SSU0519 in strain 10-LysR-2 caused a decrease in lysozyme resistance compared to the selected resistant parent strain, confirming the involvement of the *cps*2E gene in lysozyme resistance. Altogether these results indicate that both SSU0475 and SSU0519 are involved in lysozyme resistance. Since the level of lysozyme resistance of 10-Δ*0475* and 10-Δ*cps*2EF was increased but not as high as observed for strains 10-LysR-1 and 10-LysR-2, it is tempting to speculate that the other identified SNP containing genes are involved in lysozyme resistance as well.

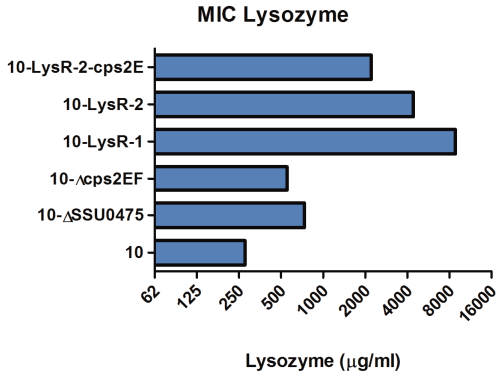

FIG. 5. ***Lysozyme MIC levels of autolysin and capsule mutant strains.*** *Strain 10 (wild type), strain 10-LysR-1, 10-LysR-2, 10-Δ0475, 10-Δcps2EF and 10-LysR-2-cps2E were spotted onto Colombia agar plates containing two-fold increasing concentrations of lysozyme. Growth was assessed 24 h later and MICs were determined. Values represent three independent observations.*

Identified SNPs and genes affect bacterial morphology

Whether the identified SNPs affect more than solely the observed increase in lysozyme resistance, we tested whether the SNPs caused changes in bacterial morphology. Hereto strain 10, strain 10-LysR-1, strain 10-LysR-2, strain 10-Δ*0475*, strain 10-Δ*cps*2EF and strain 10-LysR-2-*cps*2E were stained with crystal violet and examined by light microscopy and processed for viewing by transition electron microscopy (TEM). Both light microscopy (Fig 6A) and TEM (Fig 6B) of strain 10-LysR-1 revealed increased bacterial chain lengths and cluster formation and more bacterial cell shape diversity compared to the wild type strain. Strain 10-Δ*0475* showed also increased chain lengths and heterogeneity in bacterial shape. No major differences in chain lengths were observed between strain 10-LysR-2, strain 10-LysR-2-*cps*2E and the wild type strain. However, TEM analysis clearly indicated reduced amounts of capsule for strain 10-LysR-2 and (as expected) for the isogenic mutant strain 10-Δ*cps*2EF. Thus acquiring lysozyme resistance may be accompanied by alterations (such as loss of classical cell shape or capsule) that may change bacterial behaviour and characteristics besides lysozyme resistance.

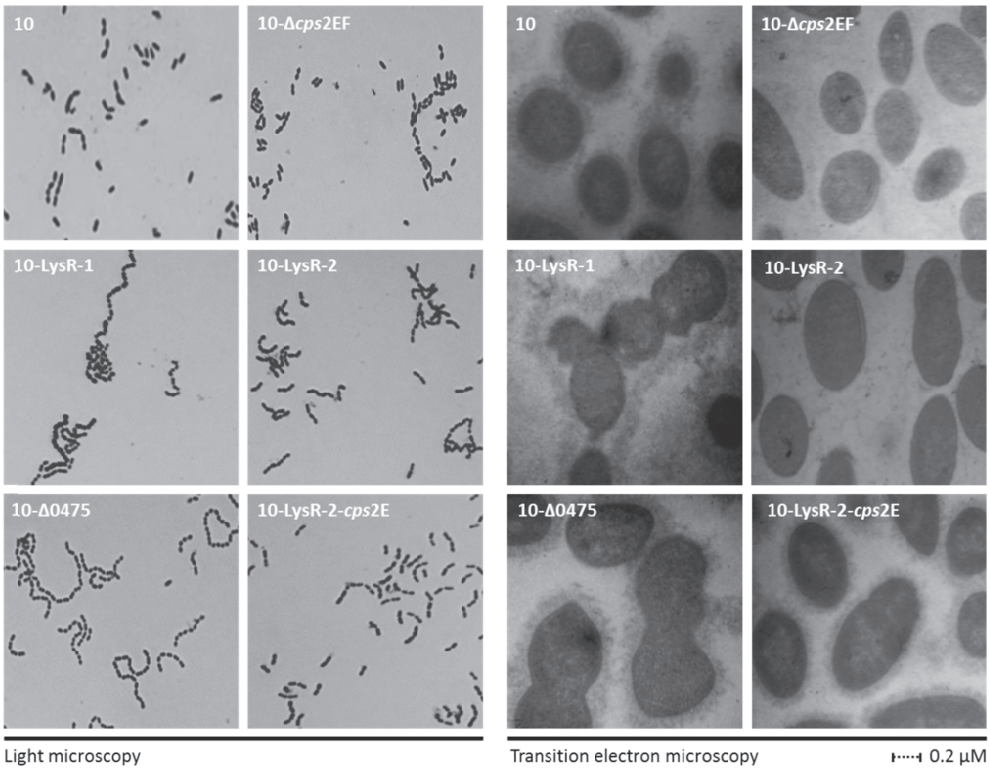

FIG. 6. ***Bacterial morphology lysozyme resistant strains.*** *Strain 10 (wild type), 10-LysR-1, 10-LysR-2, 10-Δ0475, 10-Δcps2EF and 10-LysR-2-cps2E were grown exponentially in THB and visualised using crystal violet and light microscopy (A) and TEM (B).*

Discussion

In this study we showed lysozyme resistance levels in the *S. suis* species are highly variable. Furthermore with the use of two different approaches we identified and characterized factors involved in lysozyme resistance in *S. suis*. The first approach was based on investigating homologues of well-known peptidoglycan modifying enzymes present in other Gram-positive species, and the second (unbiased) approach involved comparative whole genome analysis of a lysozyme sensitive strain and selected lysozyme resistant derivatives. With the use of isogenic mutants we provided convincing evidence that the OatA enzyme

of *S. suis* is involved in increasing lysozyme resistance and that autolysin activity and capsule production may also be linked to variation in lysozyme resistance.

The OatA protein of *S. suis* serotype 2 strain 10 (relatively lysozyme sensitive) and serotype 9 strain 8067 (relatively lysozyme resistant) used in this study share >95% amino acid sequence identity with homologous proteins in other *S. suis* isolates present in the NCBI database. Furthermore, *S. suis* OatA proteins share around 50% protein sequence identity with the Adr proteins (OatA homologous) of *S. pneumoniae*. The *oatA* gene is widely distributed in the *S. suis* species as was evidenced by PCR analysis. Here we provided evidence that OatA, in contrast to PgdA [18], is partly involved in lysozyme resistance in *S. suis* in both relatively lysozyme resistant isolates and in relatively sensitive isolates. The contribution of *S. suis* OatA to lysozyme resistance is in agreement with observations in *Staphylococcus aureus* [30,31], *Listeria monocytogenes* [32], and *S. pneumoniae* [12,14], emphasizing OatA is an important factor involved in lysozyme resistance in Gram-positive species.

In our experiments, no changes in lysozyme resistance due to the presence of the *murMN* operon was observed in *S. suis*, in contrast to observations in *S. pneumoniae* [15,16]. Possibly, genetic differences in *murMN* sequence of *S. suis* compared to *S. pneumoniae* (45% protein sequence homology) or the bacterial background in which *murMN* is expressed might influence lysozyme resistance phenotypes in *S. suis*.

Using a comparative genome sequence analysis approach, based on specifically selected strains, we were successful with the identification of genetic factors contributing to lysozyme resistance. With this approach we identified and characterized autolytic activity and capsule as important mediators reducing lysozyme resistance in *S. suis*. To our knowledge this kind of approach is the first described to identify factors involved in lysozyme resistance. Besides investigating factors involved in lysozyme resistance, similar approaches may be useful to identify factors and understand mechanisms involved in other phenotypic characteristics like bacteriophage resistance, and antimicrobial peptide resistance.

The effect of increased lysozyme resistance in the presence of reduced autolytic activity strongly suggests *S. suis* autolysins acts in synergy with lysozyme to cause bacterial lysis. Similar to lysozyme, autolysins are able to

break down the (1,4)-β bond between the NAM and NAG residues, facilitating daughter cell separation. Synergistic effects of autolysin and lysozyme have been reported in *S. pneumoniae* [33]. Interestingly, autolysin activity depends on bacterial growth phase and is tightly regulated by the presence of teichoic acids. Therefore the level and the structure of teichoic acids might influence lysozyme resistance as well, as has been shown in *S. aureus* [34].

The increased resistance to lysozyme of capsule-deficient *S. suis* strains was unexpected. It may be assumed that in the absence of capsule the bacterial peptidoglycan is more easily accessible for lysozyme. One possible explanation for our unexpected finding is that in the absence of capsule there might be increased activity of peptidoglycan modifying enzymes (such as OatA), resulting in increased lysozyme resistant phenotypes. This theory is in agreement with a previous observation in *S. pneumoniae* in which *pgdA* and *adr* mutants displayed thicker capsules [12]. It can be speculated that modifications of the peptidoglycan structure negatively affect the amount of capsule produced and *vice versa*.

Since increased resistance to lysozyme can be acquired by just a few SNPs as evidenced in this study, it is expected that persistence of lysozyme-sensitive isolates in host-environments containing high levels of lysozyme, such as the upper respiratory tract, would be low. On the other hand, the observed differences in bacterial morphology and growth between the lysozyme resistant derivatives: strain 10-LysR-1 and 10-LysR-2, compared to the parent strain suggest acquiring lysozyme resistance reduces overall bacterial fitness. Non-encapsulated *S. suis* mutants are nonvirulent [29] and autolysin mutants are attenuated in virulence in other streptococci [35,36]. Possibly, acquiring lysozyme resistance benefits the ability to colonize, but decreases the ability to cause systemic disease.

Although our experiments clearly showed involvement of OatA, autolysin and capsule in lysozyme resistance, some other uncharacterised factors are most probably involved in lysozyme resistance in *S. suis* as well. Specific candidates include the additional identified genes in the lysozyme resistance selection procedure (which also contained SNPs), besides the autolysin and the capsule transferase. Especially gene SSU0383, encoding a protein phosphatase, is of increased interest since a homologue of the protein has recently been described to affect bacterial morphology in a serotype 9 isolate [37]. Furthermore, we

cannot exclude that additional selection procedures will result in the identification of other unknown factors involved in lysozyme resistance.

Overall, this study has gained conclusive evidence that the lysozyme resistant phenotype of *S. suis* involves multiple factors, including those responsible for increased resistance and those responsible for reduced resistance. As displayed in a model (Fig. 7) we hypothesize that, peptidoglycan modification, autolysin activity, and the level of peptidoglycan associated structures such as capsule, are closely related and as a whole determine the level of lysozyme resistance. For example, bacteria with low capsule expression, low activity of autolysins and expressing peptidoglycan modifying enzymes such as OatA are most capable to resist high levels of lysozyme, nevertheless acquiring lysozyme resistance might affect bacterial morphology and overall bacterial fitness and/or virulence.

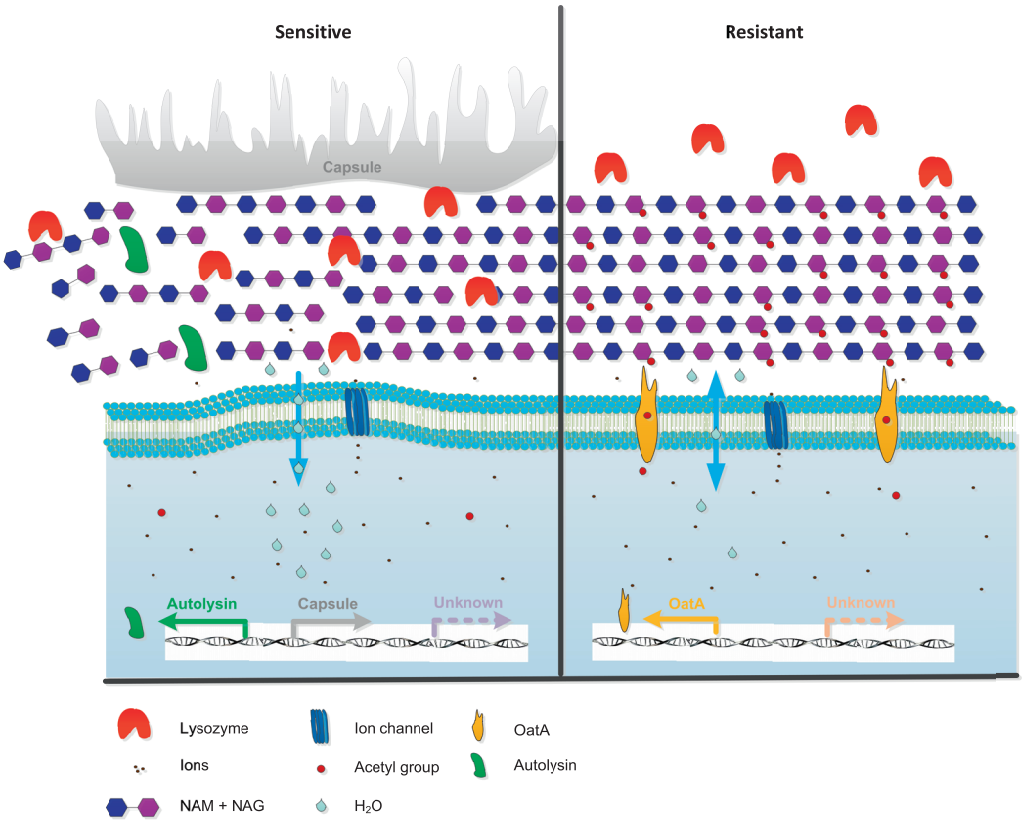

FIG. 7. ***Lysozyme resistance model of S. suis.*** *S. suis bacteria are expected to resist the antimicrobial activity of lysozyme efficiently in the absence or reduced expression of capsule, in the presence of the peptidoglycan modifying enzyme OatA, and during reduced activity of autolysins. Loss of peptidoglycan stability, due to lysozyme digestion, makes S. suis increased vulnerable for osmotic pressure resulting in the flow of water into the bacterium's cytoplasm finally resulting in bacterial lysis.*

Acknowledgements

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Supplementary information

Table S1. Primer sequences

Primer nr.	Application	Primer name	Sequence (5'-3')
1	Δ*oatA* mutant	OatA F part A2	CACAGCAAATGAGCCAGACAG
2	Δ*oatA* mutant	OataR-partA_SalI	ATGTCGACTACCCGAGCAGCAAAAATCATAGT
3	Δ*oatA* mutant	OataF-partB_XmaI	TCCCCGGGGATCTGCTGCTGAAAACGCTAAAG
4	Δ*oatA* mutant	OatA R Part B2	CCTAGTACCCTAGCAGGTTTTGATTC
5	Δ*oatA* mutant	OataF-over spec	GGCTTTGTGGGGGTTGAC
6	Δ*oatA* mutant	OataR-over spec	GACATCTTCGGTTTTCTCGTGA
7	Δ*oatA* mutant	OataF-falling out	ATTGGCCCTTGCTCATTATCTT
8	Δ*oatA* mutant	OataR-falling out	CGTGTTGTATTTAGTTGGGTCTGA
9	Δ*murMN* mutant	MurMN-F-partA_ApaI	TGGGGCCCCACTACGGGCAAGCCCTTCCTAAT
10	Δ*murMN* mutant	MurMN-R-partA_SalI	ACGTCGACGTGGAGGTGAAAGTCATGGCTTCT
11	Δ*murMN* mutant	MurMN-F-partB_XmaI	TTCCCGGGAAGGCGTACTTGAATTGGTGGTCA
12	Δ*murMN* mutant	MurMN-R-partB_SacII	ccCCGCGGGGGGCGACTACCTTCTAGCGACAG
13	Δ*murMN* mutant	Over MurMN heen F	TATGACTTGGAGGTACATTGCTAA
14	Δ*murMN* mutant	Over MurMN heen R	TTCAAAGAATTCGTAAGGCTATTT
15	Δ*murMN* mutant	Falling out MurMN F	CCTGCTTTACTAACATGGGTGGAG
16	Δ*murMN* mutant	Falling out MurMN R	TTAAAACCAGCATAGCGAACATCA
17	Spectinomysin	SpecF_SalI	GC*GTCGAC*GCAGGTCGATTTTCGTTCGT
18	Spectinomysin	SpecR_XmaI	ATC*CCGGG*ATGCAAGGGTTTATTGTTTTCTAA
19	ΔSSU0475 mutant	SSU0475-1	GAAGTCCCAAACTGTGTAGCAGAT
20	ΔSSU0475 mutant	SSU0475-2	AAATCGACCTGCACGGTCTCTGCCAAGCAGAAA
21	ΔSSU0475 mutant	SSU0475-3	TTGGCAGAGACCGTGCAGGTCGATTTTCGTTCGTGAATA
22	ΔSSU0475 mutant	SSU0475-4	CCGAAACTCGTGTCGGTACCCTATGCAAGGGTTT
23	ΔSSU0475 mutant	SSU0475-5	GCATAGGGTACCGACGGCTTTGAGCACCACCTAGA
24	ΔSSU0475 mutant	SSU0475-6	GTTTGGATAGGGTGGGTGCCA
25	ΔSSU0475 mutant	SSU0475-7	TCGTTCGTATTCAAAGTGGATCACAC
26	ΔSSU0475 mutant	SSU0475-8	ATTGGTGAAGATCGTAGTTCAAGTCAG
27	ΔSSU0475 mutant	SSU0475-9	CTAAACCATTAAGCCAAAAGACGATAGG
28	ΔSSU0475 mutant	SSU0475-10	ATGCTTCCTCCTAGAAAACTAGCCA
29	pSET5 vector	RepA-F	GGGCGTATCTATGGCTGTCA
30	pSET5 vector	RepA-R	CTCCCCTAAGGCGAATAAAAG
31	pJet1.2 vector	Pjet primer 1F	ATCCTTTGATCTTTTCTACGGGGTCT
32	pJet1.2 vector	Pjet primer 1R	CTCAACAGCGGTAAGATCCTTGAG
33	Expression *murMN*	Expr-Murmn-F-BamHI	TGGGATCCCAATTTAATTATGACTTGGAGGTACATTGC
34	Expression *murMN*	Expr-Murmn-R1-SalI	CGGTCGACCGCAGGCTCAGACTTCTGC
35	Expression *murMN*	Chloramp-F-SalI	AGGTCGACCTTGGTCTGACAGTTACCAATGC
36	Expression *murMN*	Chloramp-R-SalI	GGGTCGACCCGAGGCTCAACGTCAATAAAGC
37	Complementation SSU0519	SSU0513-F-2	ATCCGATACTGATCTCAGCTTTAGACG
38	Complementation SSU0519	SSU0514-promotor-R-SSU0519-2	ATCCTATTTCAATATTCATAGAGTAGCTTAATTCCTGAACC
39	Complementation SSU0519	SSU0519-F-Promotor-513-2	GGAATTAAGCTACTCTATGAATATTGAAATAGGATATCGCC
40	Complementation SSU0519	SSU0519-R-SPEC	CGAAAATCGACCTGCTTACTTACTTCCCTCTCTCAACAAT
41	Complementation SSU0519	SSU0519-F-SPEC	GAGAGGGAAGTAAGTAAGCAGGTCGATTTTCGTTCGTGAATA
42	Complementation SSU0519	Spec-R	TCGGTACCCTATGCAAGGGTTTA
43	MurM gene detection	MurM F	GTCAGTGGGCAACCGTAAAAAGTG
44	MurM gene detection	MurM R	CCAAATACCTCCCAAATCAACAAT
45	MurN gene detection	MurN F	AGGCGTACTTGAATTGGTGGTCA
46	MurN gene detection	MurN R	GGCTTTCGGGTCAAGTTCTGC
47	OatA gene detection	OatA rt PCR Frag A Forward	AGTATTACCTTGTCTGGGCTGGTT
48	OatA gene detection	OatA rt PCR Frag B Reverse	CCATCGATCTGTGCATCTGGTA
49	PgdA gene dectection	PgdA rt PCR frag A Forward	TGTATGGTTATTTGCAGGACTTCAA
50	PgdA gene dectection	PgdA rt PCR falling out R	GAGCGACCTGTCTCAAATGTTTC
51	Real time PCR MurMN	MurMN real-time falling out primer F	CGAGGTGTGAAATGGCAAAA
52	Real time PCR MurMN	MurMN real-time falling out primer R	AAAGTGATAAAGGCCTCCGTCTAA

Table S2. Characteristics wild type *S. suis* strains

(lysozyme MICs; presence *oatA*, *pgdA* and *murMN*)

				Gene presence (PCR level)		
Isolate	**Serotype**	**MIC Lysozyme (μg/ml)**	**CGH cluster**	***oatA***	***pgdA***	***murMN***
6388	1	626	A	+	+	-
6112	1	626	A	+	+	-
NCTC / 428	1	313	A	+	+	-
C160	1	1250	A	+	+	-
C187	1	625	A	+	+	-
3995	2	625	A	+	+	-
3988	2	625	A	+	+	-
17	2	625	A	+	+	-
S735	2	625	A	+	+	-
1890	2	625	A	+	+	-
3	2	625	A	+	+	-
10	2	313	A	+	+	-
22	2	625	A	+	+	-
D282	2	625	A	+	+	-
7696	2	625	A	+	+	-
P1/7	2	625	A	+	+	-
BM190	2	625	A	+	+	-
BM191	2	625	A	+	+	-
BM334 (B)	2	625	A	+	+	-
BM407	2	625	A	+	+	-
FX59 (4)	2	625	A	+	+	-
95-8242	2	625	A	+	+	-
R75 / S2	2	625	A	+	+	-
98HAH12	2	1250	A	+	+	-
12	2	1250	B	+	+	+
16/T129	2	1250	B	+	+	+
25	2	2500	B	+	+	+
T15	2	1250	B	+	+	+
89/1591	2	2500	B	+	+	ND
FX125 (2)	2	1250	B	+	+	-
89-999	2	2500	B	+	+	ND
21997	2	625	ND	+	+	-
7711	7	2500	B	+	+	-
7917	7	2500	B	+	+	-
8039	7	5000	B	+	+	-
C126	7	2500	B	+	+	-
15009	7	2500	B	+	+	+
87	7	2500	B	+	+	-
106	7	2500	B	+	+	-
8074	7	2500	B	+	+	-
7997	9	2500	B	+	+	+
8067	9	2500	B	+	+	+
8017	9	2500	B	+	+	+
7709	9	5000	B	+	+	+
C132	9	2500	B	+	+	+
5973	9	2500	B	+	+	+
22083 (ref)	9	2500	B	+	+	-
7998	9	2500	B	+	+	+
21946	9	2500	ND	+	+	+
21947	9	2500	ND	+	+	+
21948	9	10000	ND	+	+	ND
21949	9	2500	ND	+	+	+
21950	9	2500	ND	+	+	+
21951	9	2500	ND	+	+	+
21952	9	2500	ND	+	+	+
21953	9	5000	ND	+	+	+
21954	9	2500	ND	+	+	+
21955	9	10000	ND	+	+	+
21956	9	2500	ND	+	+	+
21957	9	5000	ND	+	+	+
21958	9	2500	ND	+	+	+
21959	9	2500	ND	+	+	+
21960	9	2500	ND	+	+	+
21961	9	2500	ND	+	+	+
21962	9	2500	ND	+	+	+
21963	9	2500	ND	+	+	+
21964	9	2500	ND	+	+	+
21965	9	5000	ND	+	+	+
21966	9	5000	ND	+	+	+
21967	9	2500	ND	+	+	+
21968	9	5000	ND	+	+	+
21969	9	5000	ND	+	+	+
21970	9	2500	ND	+	+	+
21971	9	2500	ND	+	+	+
21972	9	2500	ND	+	+	+
21973	9	1250	ND	+	+	+
21974	9	5000	ND	+	+	+
21975	9	5000	ND	+	+	+
21976	9	5000	ND	+	+	+
21977	9	10000	ND	+	+	+
21978	9	1250	ND	+	+	+
21979	9	2500	ND	+	+	+
21980	9	10000	ND	+	+	-
21981	9	5000	ND	+	+	+
21982	9	2500	ND	+	+	+
21983	9	2500	ND	+	+	+
21984	9	2500	ND	+	+	+
21985	9	5000	ND	+	+	+
21986	9	2500	ND	+	+	+
21987	9	2500	ND	+	+	+
21988	9	2500	ND	+	+	+
21989	9	2500	ND	+	+	+
21990	9	1250	ND	+	+	+
21991	9	5000	ND	+	+	+
21992	9	5000	ND	+	+	+

CGH cluster: strains were separated into two (A and B) different genetic clusters based on CGH data. ND: not determined. +: fragment of expected size amplified in PCR, -: no fragment of expected size amplified in PCR.

Chapter 6

Summarizing Discussion

Aim of the study and major findings

Streptococcus suis is an important pig pathogen able to cause systemic disease and also able to asymptomatically colonize the upper respiratory tract. In almost all countries, *S. suis* has become an endemic pathogen and a substantial economic burden due to loss of production and expensive control measurements. Especially the increase in antibiotic resistant *S. suis* isolates is expected to further complicate the *S. suis* problem [1,2,3,4]. Besides being a pig pathogen, *S. suis* is a zoonotic agent. The fast majority of *S. suis* bacteria isolated from diseased pigs and humans worldwide belong to serotype 2, however, serotype 9 isolates are currently spreading very efficiently in the European and Asian pig population, despite or maybe due to their reduced virulence [5,6,7]. The ability to colonize and invade host tissues and to cause disease is expected to partially depend on the reactivity of the host innate immune system. The aim of the study described in this thesis was to better understand the interactions of *S. suis* with the host innate immune system.

The research presented in this thesis led to the identification and characterization of *S. suis* factors that are able to activate the innate immune system. In addition, we discovered strategies that are used by *S. suis* to evade host innate immune defense mechanisms. The results show that lipoproteins of *S. suis* are dominant activators of the human TLR2/6 complex and that lipoproteins significantly contribute to porcine PBMC activation. The lipid-protein structure of lipoproteins was demonstrated to be required for lipoprotein mediated innate immune system activation. We also found that one of the identified lipoproteins, designated TroA, is dominantly present in an innate activating fraction of *S. suis* and has an important function in manganese acquisition and virulence in mice. Regarding the effector function of the innate immune system, we showed that the resistance of different isolates/serotypes of *S. suis* to the antimicrobial protein lysozyme is highly variable and that *S. suis* can adapt to lysozyme pressure by genomic alterations. We discovered that peptidoglycan modification, capsule, and autolysin activity are important factors involved in lysozyme resistance in *S. suis*. The information obtained in this study may help to improve the development of future vaccines and/or therapeutics to control *S. suis* infections in the pig industry.

Lipoproteins of *S. suis* are dominant activators of innate immunity via TLR2 mediated signaling

The Toll-like receptor (TLR) family of pattern recognition receptors (PRRs) senses various conserved structures of microbes and plays a central role in initiating a pro-inflammatory immune response [8,9,10]. At the start of this study, knowledge on PRRs able to sense *S. suis* was limited. Therefore, we investigated the interaction of *S. suis* with some well-defined human TLR complexes (Chapter 2). We decided to use a well-established *in vitro* reporter system to evaluate the ability of *S. suis* to activate the human TLR1/2 complex, the human TLR2/6 complex and the human TLR4 complex [11,12,13]. These receptors and receptor combinations were selected because it had been described that they are able to sense various Streptococcal components like LTA [14], lipoproteins [15], peptidoglycan [16] and the Pneumococcal toxin pneumolysin [17]. Because *S. suis* does not produce a flagellin protein, the dominant ligand of TLR5 [18], we did not investigate the interaction of *S. suis* with TLR5. The results show that the human TLR2/6 complex is important for sensing lipoproteins of *S. suis*. The results also suggest that *S. suis* LTA and peptidoglycan are not/less able to activate TLR2 complexes. This observation is in agreement with recent reports that describe that previously observed TLR2 activation by LTA and peptidoglycan are, at least partially, due to contaminating lipopeptides/lipoproteins [19,20]. The lipoprotein mediated activation of the TLR2/6 complex rather than the TLR1/2 complex, suggests that lipoproteins of *S. suis* are di-acylated and not tri-acylated. This is in line with our observation that the thus far characterized genomes of *S. suis* are devoid of a gene encoding a homologue of the Lnt protein, which normally adds a third acyl chain towards the cysteine residue present in the lipobox of lipoproteins [21,22].

The N-terminal lipid-protein structure of lipoproteins is required for human TLR2/6 complex activation, as evidenced by the Lgt-deficient strain which had lost the ability to activate HeLa cells expressing human TLR2/6 (Chapter 3). Thus direct linkage of the lipid moiety to the protein backbone of lipoproteins is required for TLR2/6 activation. As all lipoproteins contain this lipid-protein structure, it is expected that all *S. suis* lipoproteins, rather than specific lipoproteins, are able to activate the human TLR2/6 complex, although differences in binding affinity cannot be excluded. Furthermore based on

differences in the level of expression of lipoproteins, some lipoproteins are expected to be more involved in innate immune activation than others. It is known that the bacterial environment influences the level of expression of certain lipoproteins [23,24]. Therefore, the nine lipoproteins identified in our study (Chapter 3) are likely the lipoproteins that are most abundantly expressed or released from *S. suis* under the conditions tested.

The lipoprotein mediated activation of TLR2 by *S. suis* resembles observations in *S. agalactiae* [15] and strongly suggests that lipoproteins are the main TLR2 activating components of Streptococcal species. The apparent inability of *S. suis* to activate TLR4 suggests that the excreted suilysin, the homologue of pneumolysin of *S. pneumoniae* is unable to activate TLR4. Sequence differences between suilysin and pneumolysin (50% amino acid sequence identity) might be responsible for this [17], however there is growing evidence that pneumolysin may not be a TLR4 ligand either. Recent studies show that pneumolysin activates the NLRP3 inflammasome and not TLR4 [25,26]. Therefore, it may be worthwhile to investigate the ability of suilysin to activate host inflammasomes.

Besides activating the human TLR2/6 complex, *S. suis* lipoproteins are also very potent activators of the porcine innate immune system, as evidenced by the reduced IL-1β and IL-8 expression in porcine PBMCs upon stimulation with the Lgt-deficient strain (Chapter 3). We hypothesize that *S. suis* lipoproteins stimulate IL-1β and IL-8 expression in porcine PBMCs by activating porcine TLR2/6. Porcine TLR2/6 and human TLR2/6 show about 70% sequence homology (NCBI database). However, we could not provide direct evidence for the involvement of porcine TLR2/6 in recognizing lipoproteins of *S. suis*, since porcine TLR ligand specificity has not been validated in transfection systems. As the Lgt-deficient *S. suis* strain showed reduced rather than totally abrogated induction of IL-1β and IL-8 expression in porcine PBMCs (Chapter 3), we expect that other components than lipoproteins are also able to activate PBMCs. A recent study provides evidence that *S. suis* DNA is able to activate PBMCs via the intracellular TLR9 complex [27], as observed for *S. pneumoniae* [28,29]. It is assumed that non-specific endocytosis facilitates TLR9 mediated sensing of *S. suis* DNA [30,31]. Knowledge on the role of the intracellular NLRs in sensing other *S. suis* components (e.g. peptidoglycan) is still limited and awaits further study.

The levels of NF-κB activation in TLR2/6 expressing HeLa cells and the levels of cytokine induction in porcine PBMCs (Chapters 2 and 3) were consistently higher after stimulation with penicillin-treated *S. suis* bacteria compared to live logarithmic-phase grown *S. suis* bacteria. Apparently, the loss of bacterial cell wall integrity, due to penicillin treatment, results in an increased availability of PRR ligands such as lipoproteins. The effect of increased innate sensing after penicillin treatment has also been reported for *S. pneumoniae* [32]. This intriguing observation suggests that the lipid-protein structure of lipoproteins is not accessible for innate immune system activation once buried in the cell wall of live bacteria. In addition, we observed that the surrounding capsule further prevents lipoprotein mediated innate immune system activation, since capsular-deficient *S. suis* strains showed increased activation of the human TLR2/6 complex and an increased cytokine induction in porcine PBMCs (unpublished data). In the host, independently of penicillin but due to various other causes such as nutrient limitation and activity of immune effector molecules, bacterial integrity may also become compromised, resulting in increased activation of the innate immune system by the increased availability/release of PAMPs including lipoproteins.

Dual role of lipoproteins

Lipoproteins are an important target for the host innate immune system but are also important for the behavior of bacteria, usually related to adaptation to particular environmental conditions [33,34,35]. Due to this dual role, interpretation of *in vivo* experimental data with regard to the biological function of lipoproteins and of Lgt processing of lipoproteins is complicated. For example, inactivation of Lgt is expected to result in evasion of lipoprotein-mediated innate recognition favoring bacterial growth. On the other hand, immature (non-lipidated) lipoproteins may not be (fully) functional active which may negatively affect bacterial survival and/or bacterial growth in the host. Thus, it may be hard to evaluate the role of lipoproteins in innate activation in the host due to these bilateral effects. Nevertheless the effect of Lgt-inactivation on virulence has been studied. Lgt-deficient strains of *S. sanguinis* [36] and *S. pneumoniae* [37] have been reported to display reduced virulence in rabbits and mice, respectively, whereas in *S. agalactiae* and *S. aureus* inactivation of Lgt resulted in hyper-

virulent phenotypes at low infection doses [15,38]. The Lgt-deficient *S. suis* strain, described in Chapter 3, was tested in mice and showed slightly reduced virulence characteristics compared to wild type bacteria (unpublished results). This suggests that Lgt-mediated lipoprotein processing in *S. suis* is, in one way or another, involved in virulence but not absolutely required for causing disease. In this respect it was surprising that inactivation of a specific lipoprotein of *S. suis* (TroA) resulted in an avirulent phenotype in mice (Chapter 4). Apparently, the absence of a particular lipoprotein may more severely reduce virulence than the absence of Lgt-mediated lipidation of lipoproteins. We hypothesize that lipoproteins are still (partly) functional without the presence of a lipid moiety. Our observations with regard to the virulence of Lgt and TroA mutants are in agreement with observations for other Streptococci such as *S. equi* [39] and *S. sanguinis* [36] and emphasize that lipoproteins fulfill important bacterial functions *in vivo* and that lipid modification is involved but not essential for lipoprotein functionality.

Role of lipoproteins in bacterial pathogenesis

Analysis of the *S. suis* genome sequences available so far predicts the presence of >40 different lipoproteins per genome [2,40,41,42,43,44]. Based on sequence homologies, these lipoproteins are expected to fulfill various functions in bacterial homeostasis and pathogenesis varying from nutrient acquisition to host-attachment and antibiotic resistance [33,34,35]. Furthermore, compared to the sortase anchored proteins, *S. suis* lipoproteins are highly conserved and widespread among *S. suis* isolates and serotypes making them promising vaccine candidates. The more so because several lipoproteins have been shown to be highly immunogenic [23,24,45,46,47] and are involved in activating the host innate immune system (this thesis). So far, only one study has been published showing that a single lipoprotein (SsuiDRAFT_0103), which is induced under di-valent cation deprivation, is able to provide a significant protective immune response in mice against a homologous *S. suis* strain [23]. In this thesis we provide compelling evidence that an immunogenic lipoprotein of *S. suis* (TroA) is involved in manganese acquisition and essential for virulence in mice (Chapter 4). Whether TroA is also required for virulence in the natural porcine host awaits further study. Based on the reduced growth of the mutant in porcine serum we

expect TroA to be a virulence factor in pigs as well, although observed virulence phenotypes of different *S. suis* strains in pigs and mice are not always similar [48]. In contrast to manganese concentrations in nutrient rich media used for *in vitro* growth, the concentration of manganese in biological fluids like blood is relatively low (<20 μM) [49]. Apparently, *S. suis* requires the expression of TroA to sustain in blood. Based on bioinformatics analysis, we assume that several other lipoproteins identified in the innate activating fraction (Chapter 3) are involved in nutrient acquisition as well.

The *S. suis* TroA protein is most similar to the PsaA protein of *S. pneumonia* with amino acid sequence identity levels of about 30%. Despite this relatively low level of amino acid sequence identity, the functional similarities between *S. suis* TroA and pneumococcal PsaA are relatively high. Like PsaA, TroA is involved in manganese acquisition, is required for virulence, is recognized by convalescent sera and its expression is induced *in vivo* [50,51,52,53,54]. Interestingly, PsaA is currently considered as one of the most promising vaccine candidates of *S. pneumoniae* facilitating protection against multiple serotypes [55,56,57,58,59,60]. We consider TroA of *S. suis*, due to its wide spread and conserved nature, requirement for *in vivo* growth, location at the bacterial surface, and its immunogenicity as a promising vaccine candidate providing protection towards multiple *S. suis* serotypes. In addition, we believe that our strategy of identifying lipoproteins in an innate activating fraction by mass spectrometry and subsequently generating defined lipoprotein mutants is a very suitable approach for the identification of virulence factors and/or vaccine candidates.

Lysozyme resistance

The innate immune system not only shows PRR-dependent responses initiating an inflammatory response but also produces bioactive effector molecules able to kill bacteria directly. Lysozyme is one of the most important effector molecules of the innate immune system and is able to lyse bacteria by hydrolyzing the bond between NAM and NAG residues in peptidoglycan. Lysozyme is present in variable concentrations in bodily secretions and is also a main constituent of neutrophil granules [61,62,63,64]. We observed that lysozyme resistance levels strongly vary between *S. suis* serotypes and isolates (Chapter 5). Interestingly,

most lysozyme sensitive isolates belong to the generally more invasive serotypes 1 and 2 [6,65,66], while most of the lysozyme resistant isolates belong to serotype 9, which are in general effective colonizers of the upper respiratory tract [5,6,7,65]. This may indicate a relationship between the natural habitat of a particular strain and the level of resistance towards lysozyme. It can be speculated that *S. suis* strains, that acquired increased lysozyme resistance are better able to sustain at the upper respiratory tract, while lysozyme-sensitive isolates may more efficiently invade and cause disease. In Chapter 5 we provide evidence that the acquisition of increasing lysozyme resistance is accompanied by morphologic changes which are likely to reduce the invading abilities of *S. suis*. A similar negative correlation between lysozyme resistance and virulence has been suggested for *S. pneumoniae* [67]. Although lysozyme resistance may be a relatively stable trait of a strain, we expect that transcriptional regulation of factors involved in lysozyme resistance still allows *S. suis* to adapt to minor fluctuations in lysozyme concentration.

The successful approach of combining targeted mutant selection and comparative whole genome analysis of selected lysozyme derivatives enabled us to identify several determinants of lysozyme resistance in *S. suis*, particularly peptidoglycan modification, capsule expression and autolysin activity (Chapter 5). The observation that lysozyme resistance can depend on only three SNPs is intriguing. However, as the SNPs might have additional negative effects on the bacterial characteristics such as growth or resistances to other hostile factors, development of lysozyme resistance *in vivo* may be more complex. Our results clearly indicate that lysozyme resistance in *S. suis* is not simply linked to the presence or absence of a distinct genetic factor but involves multiple mechanisms and multiple genetic loci. The list of factors involved in lysozyme resistance in other Gram-positive bacteria is also growing and contains various cell wall modifying enzymes [67,68,69,70,71,72], regulatory mechanisms [73,74,75], specific inhibitors [76], autolysins [77] and structures attached to the cell wall [78]. Why bacteria, including *S. suis*, have multiple ways to influence lysozyme resistance is unknown, but may be related to the associated different bacterial phenotypes that are beneficial in distinct host environments. That each of the identified mechanisms confers only small changes in resistance against lysozyme suggests that only limited modification of peptidoglycan is possible, probably

because peptidoglycan is an essential bacterial structure. The finding that the majority of field strains, either sensitive or resistant to lysozyme, contain peptidoglycan modifying enzymes, autolysins as well as capsule is consistent with the need to fine-tune lysozyme resistance with other environmental needs.

Recent observations show that lysozyme not only directly helps the host to clear bacterial infections by hydrolyzing the bacterial cell wall, but also acts indirectly via increased release of bacterial PAMPs that stimulate the inflammatory response. In *S. aureus*, *Listeria monocytogenes* and *S. pneumoniae* lysozyme based digestion of peptidoglycan results in increased cytokine production by the host innate immune system [70,79,80]. The relationship between lysozyme digestion and PRR-mediated sensing of *S. suis* is expected to be complex because of the variation in lysozyme resistance phenotypes. Furthermore, we expect that peptidoglycan modifications, involved in increasing lysozyme resistance, might also affect the affinity of the peptidoglycan monomers to activate innate immunity by, for example, the NLRs, as evidenced for *L. monocytogenesis* [81]. The link between lysozyme digestion and PRR sensing of PAMPs illustrates that different constituents of the innate immune system work in close collaboration to eliminate an infection.

Balance between innate defense and evasion

To increase survival in hostile environments, some bacterial species have evolved mechanisms that facilitate evasion of the host innate immune system. Such mechanisms include modification of components of the innate immune system, killing of innate immune cells by bacterial toxins, reducing accessibility or affinity of PRR ligands, and the production of components interfering in signaling cascades of the innate immune system. The knowledge about immune evasion strategies used by *S. suis* was very limited at the beginning of this study. We identified and characterized two new evasion mechanisms. One involves the shielding of the lipid-protein structure of lipoproteins responsible for activation of human TLR2/6 and porcine PBMCs by a thick peptidoglycan and capsule layer. The other involves the adaptations to lysozyme pressure by modification of peptidoglycan and by reducing autolytic activity and the amount of capsule.

To counteract the ability of bacteria to evade the action of the innate immune system, the host has developed multiple defense strategies, all acting at

different levels, to eliminate infections. The resulting battle between host and pathogen becomes a complex and dynamic process which even varies between locations in a single host. For example, the pig expresses factors that bind free circulating nutrients, like trace metals, resulting in an environment in which *S. suis* bacteria have more trouble to sustain [82,83,84]. To counteract this so called "nutritional immunity" *S. suis* bacteria increase their expression of high affinity metal binding lipoproteins, like TroA. Subsequently, the increased expression of these lipoproteins results in increased recognition of *S. suis*, mediated by the TLR2/6 complex, which in turn increases the inflammatory response involved in bacterial killing.

Overall, the innate immune system plays an important role, at various levels, in protecting the host against *S. suis* infections. However, the effectiveness of the innate immune system may vary between different hosts and different environments within a host but also largely depends on the characteristics of the invading *S. suis* strain. Differences in the expression of virulence factors, innate immune evasion strategies, and metabolic capacities have significant effects on the outcome of an infection. Since nutrient availability and pressures within a single host may differ completely at various sites of infection, it is expected that certain strains have acquired specialization in colonization and others are more prone to cause systemic disease. The increased resistance of serotype 9 strains towards lysozyme in combination with the increased recognition by the innate immune system (Chapter 2) suggests that serotype 9 strains, in contrast to serotype 2 strains, are more specialized in colonization rather than invasion. Altogether, the existence of differences in the level of PRR-mediated activation by various *S. suis* strains and the existence of differences in lysozyme resistance between strains strongly suggests that *S. suis* is widely exposed to and has adapted to the host innate immune system.

Implications of this study for the development of future vaccines and control strategies

In this study we obtained new insights into the mechanisms involved in the interaction of *S. suis* with the innate immune system. These and future new insights in host-pathogen interactions form the basis for the development of new intervention strategies. Currently, no vaccines are available that protect against

multiple *S. suis* serotypes. Sub-unit vaccines based on surface proteins are expected to have the best potential to confer protection against more than one *S. suis* serotype. The highly conserved lipoproteins of *S. suis* and especially lipoproteins like TroA are interesting candidate vaccine antigens. Besides as antigen, *S. suis* lipoproteins are probably also potent adjuvants [85]. The combination of antigen and adjuvant in a single molecule is considered most effective in initiating, strengthening and directing humoral and cell-mediated protection. Unfortunately, the large scale production of recombinant lipoproteins containing the lipid modification, essential for innate immune activation, is currently difficult to accomplish. Lipoproteins with signal sequences, over-expressed in heterologous expression systems are often toxic [86,87]. Nevertheless, strategies have successfully been used to produce lipoprotein antigens with intrinsic adjuvant characteristics [88,89,90,91]. To improve future *S. suis* vaccines it may be worthwhile to initially add synthetic lipopeptides as adjuvants, to protein antigens present in vaccines, thereby directing and enhancing the adaptive immune response of future vaccines. The use of lipopeptides as adjuvant has already been shown to be effective with various other protein antigens [92,93,94]. Alternatively, synthetic lipopeptides may be produced which express protein epitopes in the same molecule [85]. Besides improving future vaccines, the knowledge obtained in this study can be helpful in the identification of therapeutic targets. Especially, therapeutics interfering with capsule production or autolytic activity may have potential as an alternative for current used antibiotics.

Conclusions

With the use of state of the art techniques like mass spectrometry and comparative whole genome analysis, we generated new insights in the *S. suis* components able to activate the innate immune system and the strategies used by *S. suis* to evade the action of the innate immune system. *S. suis* lipoproteins were identified as potent activators of the human TLR2/6 complex and of porcine PBMCs. Furthermore, a specific group of *S. suis* strains was shown to be efficiently killed *in vitro* by the anti-microbial protein lysozyme. The results also indicate that *S. suis* evades lipoprotein-mediated recognition by the innate immune system by burying the active protein-lipid structure of lipoproteins in the cell

membrane under a thick peptidoglycan and capsule layer. We also showed that *S. suis* is able to increase the resistance to lysozyme by modification of the bacterial peptidoglycan and by reducing autolytic activity and the amount of capsule. Last but not least we identified and characterised a novel promising candidate vaccine antigen (TroA) involved in manganese acquisition and virulence in mice. The work described in this thesis thus provides an interesting basis for future vaccine development needed to reduce the burden of *S. suis* to human and animal health and the economy.

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Nederlandse samenvatting

Dankwoord

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Nederlandse Samenvatting

Aanleiding van het onderzoek

Streptococcus suis veroorzaakt hersenvliesontsteking, gewrichtsontsteking, longontsteking en sepsis in varkens. Vooral biggen in het begin van de speenperiode zijn erg gevoelig voor een infectie. Op nagenoeg alle varkensbedrijven wereldwijd zijn één of meerdere van de in totaal 33 bekende *S. suis* serotypen aanwezig. Serotype 2 isolaten zijn verantwoordelijk voor het merendeel van de *S. suis* problemen, echter de afgelopen jaren worden in Europa en Azië ook steeds meer serotype 9 isolaten geassocieerd met ziekte. Serotype 9 isolaten veroorzaken over het algemeen minder klinische verschijnselen dan serotype 2 isolaten. Daarentegen lijken serotype 9 isolaten zich gemakkelijker te verspreiden binnen de varkenspopulatie. *S. suis* kan ook ziekte bij mensen veroorzaken. In de westerse wereld betreft dit vooral mensen die als gevolg van hun beroep in contact komen met besmette varkens en/of besmette varkensproducten. In Zuidoost-Azië zijn recent twee grote humane *S. suis* uitbraken geweest, waarbij patiënten vaak toxische-shock-achtige symptomen vertoonden. Tot nog toe worden *S. suis* infecties voornamelijk tegengegaan door preventieve of curatieve behandelingen met antibiotica. Het grootschalige gebruik van antibiotica in de varkenshouderij heeft echter tot gevolg dat het aantal antibioticum resistente isolaten aan het toenemen is. Een effectief vaccin kan uitkomst bieden om *S. suis* gerelateerde problemen te reduceren. De huidige vaccinatie strategie, gebaseerd op immunisatie met formaline afgedode bacteriën, biedt echter alleen bescherming tegen *S. suis* stammen behorende tot hetzelfde serotype dat is gebruikt voor vaccinproductie.

Om ziekte te kunnen veroorzaken in een gastheer, in dit geval het varken of de mens, dient *S. suis* diverse barrières te doorbreken. Eén van de grootste obstakels voor *S. suis* is het aangeboren immuunsysteem. Het aangeboren immuunsysteem speelt een cruciale rol in de herkenning, het uitschakelen en het opruimen van ziekteverwekkers zoals bacteriën, virussen en parasieten. Op diverse cellen van het aangeboren immuunsysteem, zoals antigeen presenterende cellen (APC), endotheel cellen, epitheel cellen en B- en T-cellen, zitten receptoren die in staat zijn geconserveerde structuren van micro-

organismen te herkennen. Een belangrijke groep van deze “pattern recognition receptors”, is de Toll-like receptor (TLR) familie. In mensen en varkens zijn tot dusver 10 verschillende TLR’s geïdentificeerd. Het merendeel van deze TLR’s functioneert als homodimeer, echter TLR2 functioneert als heterodimeer met TLR1 of TLR6. Activatie van TLR’s door bijvoorbeeld lipopolysacharide of flagelline resulteert in de productie van o.a. cytokinen, chemokinen en antimicrobiële peptiden. Deze eiwitten/peptiden dirigeren vervolgens het opruimen van pathogenen door bijvoorbeeld fagocyterende cellen naar de plaats van infectie te sturen. Daarnaast wordt ook de ontwikkeling van een specifieke afweerreactie geïnitieerd, gebaseerd op B- en T-cellen. Er wordt verondersteld dat meer kennis over de aangeboren immuunrespons tegen *S. suis* en verbeterde inzichten in de pathogenese van *S. suis* infecties de ontwikkeling van serotype-overkoepelende vaccins zal bevorderen. Zoals beschreven in **Hoofdstuk 1** waren aan het begin van deze studie de componenten van *S. suis* die het aangeboren immuunsysteem activeren vrijwel onbekend. Daarnaast was er ook relatief weinig informatie beschikbaar over de mechanismen die *S. suis* gebruikt om de activatie van het aangeboren immuunsysteem te voorkomen en/of de effecten ervan te omzeilen.

Doel van het onderzoek

Het doel van het onderzoek was om meer inzicht te verkrijgen in de interacties tussen *S. suis* en het aangeboren immuunsysteem om zodoende meer inzicht in de pathogenese van *S. suis* infecties te verkrijgen en om de mogelijkheden voor de ontwikkeling van een serotype-overkoepelend vaccin te vergroten. Het onderzoek heeft zich vooral gericht op de karakterisatie van de componenten van *S. suis* die in staat zijn het aangeboren immuunsysteem te activeren en op de identificatie van de specifieke gastheer receptoren betrokken bij de herkenning van deze componenten. Ook is de invloed van een belangrijk antimicrobieel eiwit van de gastheer, lysozym, op *S. suis* onderzocht. Daarnaast zijn mechanismen geïdentificeerd die *S. suis* gebruikt voor het ontduiken van het aangeboren immuunsysteem.

Resultaten van het onderzoek

Met behulp van een transfectie systeem, waarbij specifieke receptoren tot expressie worden gebracht in een cellijn, is onderzocht of *S. suis* in staat is individuele TLR's en TLR combinaties te activeren. De resultaten, beschreven in **Hoofdstuk 2**, laten zien dat *S. suis* in staat is het humane TLR2/6 complex te activeren terwijl het humane TLR1/2 complex en het humane TLR4 complex niet werden geactiveerd. Door gebruik te maken van verschillende technieken, waaronder zuiveringen, enzymatische behandelingen, en inactivatie van specifieke bacteriële genen is vervolgens vastgesteld dat het de lipoproteïnen van *S. suis* zijn die TLR2/6 activeren. De lipide-eiwit structuur bleek essentieel voor de TLR2/6 activatie te zijn, mede omdat een *S. suis* stam met een defect in de lipide modificatie van eiwitten (Δ*lgt* mutant) niet meer in staat was om het TLR2/6 complex te activeren.

In het vervolgonderzoek, zoals grotendeels beschreven in **Hoofdstuk 3,** is het aandeel van lipoproteïnen in de activatie van het aangeboren immuunsysteem van het varken verder uitgezocht. De resultaten laten zien dat lipoproteïnen van *S. suis* in grote mate verantwoordelijk zijn voor de aanmaak van cytokinen, waaronder interleukine-1β en interleukine-8, in witte bloedcellen van varkens. Waarschijnlijk worden de lipoproteïnen van *S. suis* herkend door het TLR2/6 complex van varkens.

De activatie van het humane TLR2/6 complex en van witte bloedcellen van varkens nam sterk toe wanneer gestimuleerd werd met *S. suis* bacteriën waarvan de integriteit van de celwand was aangetast door een behandeling met penicilline. Penicilline remt de aanmaak van peptidoglycaan, de belangrijkste structuur van de celwand van *S. suis*. Waarschijnlijk zorgt een penicilline behandeling voor een toename in het vrijkomen van lipoproteïnen die het immuunsysteem activeren. *In vivo* kan, als gevolg van perioden van onvoldoende beschikbaarheid van voedingstoffen of door de activiteit van antimicrobiële peptiden/eiwitten, de integriteit van de bacteriële celwand eveneens worden aangetast, met als gevolg een toename in het vrijkomen van *S. suis* lipoproteïnen en een verhoogde activatie van het aangeboren immuunsysteem.

Een interessante waarneming was dat *S. suis* in de afwezigheid van het polysacharide kapsel, dat normaal gesproken *S. suis* behoedt voor fagocytose,

beter in staat is tot het activeren van het aangeboren immuunsysteem dan in aanwezigheid van het polysacharide kapsel. Kapsel speelt dus een rol in het ontwijken van lipoproteïne-gemedieerde herkenning door het aangeboren immuunsysteem.

Al met al heeft het onderzoek, beschreven in **Hoofdstukken 2 en 3** laten zien dat lipoproteïnen van *S. suis* componenten zijn die door het aangeboren immuunsysteem worden herkend en dat *S. suis* probeert deze herkenning te minimaliseren door het lipide-eiwit gedeelte van de lipoproteïnen te verbergen in de bacteriële celwand onder een dikke laag peptidoglycaan en kapsel.

In een eiwit fractie van *S. suis*, die witte bloedcellen van varkens efficiënt activeerde, hebben we m.b.v. massaspectrometrie negen lipoproteïnen weten te identificeren. Eén van de lipoproteïnen, aanwezig in een hoge concentratie in de fractie, genaamd TroA, is verder gekarakteriseerd in het onderzoek dat beschreven staat in **Hoofdstuk 4**. De interesse in het TroA lipoproteïne was groot omdat dit lipoproteïne al eerder was beschreven als een immuun-dominant eiwit van *S. suis*. Met behulp van een isogene *troA* mutant hebben we ontdekt dat het TroA eiwit belangrijk is voor het transporteren van extracellulair mangaan. In een omgeving met een lage mangaan concentratie, zoals serum, groeide de mutant slecht. Echter door toevoeging van een klein beetje mangaan kon de groei van de mutant hersteld worden tot het niveau van wildtype bacteriën. Dat mangaan belangrijk is voor bacteriële groei komt waarschijnlijk doordat mangaan een belangrijke co-factor is voor vele eiwitten. Naast verminderde groei in de afwezigheid van voldoende mangaan, bleek de *troA* mutant ook meer gevoelig voor oxidatieve stress. Daarnaast was de mutant niet in staat om ziekte te veroorzaken in muizen, in tegenstelling tot de wildtype stam. Het TroA lipoproteïne is waarschijnlijk onderdeel van een zogenaamd ABC transport systeem, waarbij TroA fungeert als een metaal bindingseiwit met hoge affiniteit voor mangaan. Het TroA lipoproteïne is aanwezig in alle *S. suis* isolaten die tot nu toe gesequenced zijn. Het TroA lipoproteïne is dus in staat tot het activeren van het aangeboren immuunsysteem, is sterk immunogeen, draagt bij aan de virulentie van *S. suis* in muizen en is wijd verspreid in de *S. suis* populatie. Al deze eigenschappen scheppen hoge verwachtingen van TroA als antigene component in een serotype-overkoepelend vaccin.

In **Hoofstuk 5** zijn experimenten beschreven die de variatie in de gevoeligheid van *S. suis* voor het antimicrobiële eiwit lysozym in kaart hebben gebracht. Lysozym is als antimicrobieel eiwit een belangrijk onderdeel van het aangeboren immuunsysteem. Lysozym hydrolyseert het peptidoglycaan in de celwand waardoor bacteriën lyseren. Lysozym wordt in varkens en mensen in hoge concentraties uitgescheiden in bijvoorbeeld speeksel en traanvocht. Daarnaast is lysozym één van de belangrijkste bestanddelen van granulen van neutrofiele granulocyten. We hebben gevonden dat er tussen en binnen verschillende *S. suis* serotypen een grote variatie is in de gevoeligheid voor lysozym. Opvallend was dat over het algemeen serotype 2 isolaten meer gevoelig zijn voor lysozym dan serotype 9 isolaten. Een correlatie tussen lysozym resistentie, virulentie en verspreiding is mogelijk aanwezig. Door genen te inactiveren die coderen voor celwand modificerende enzymen hebben we aangetoond dat het OatA enzym een significante bijdrage levert aan lysozym resistentie in *S. suis*. Echter, OatA bleek niet exclusief verantwoordelijk voor het lysozym resistentie fenotype. Met behulp van een systematische benadering, gebaseerd op de selectie van lysozym resistente stammen door herhaalde passage van *S. suis* bij toenemende lysozym concentraties en totale genoom analyse van de geselecteerde stammen, hebben we extra lysozym resistentie factoren weten te identificeren. De selectie procedure resulteerde in diverse puntmutaties. Twee puntmutaties resulteerden in de inactivatie van een startcodon en de introductie van een vervroegd stopcodon in respectievelijk een autolysine en een enzym betrokken bij kapsel synthese. Met behulp van specifieke isogene mutanten konden we bevestigen dat autolysine activiteit en kapsel inderdaad van invloed zijn op het niveau van lysozym resistentie. Mogelijk dat kapsel en autolysine goede targets zijn voor de ontwikkeling van nieuwe therapeutica. Lysozym resistentie in *S. suis* is dus het resultaat van een multifactorieel proces. Opvallend is dat het verkrijgen van lysozym resistentie mogelijk gepaard gaat met een verlies aan virulentie.

Conclusies

Mijn onderzoek heeft aangetoond dat lipoproteïnen een zeer belangrijke factor zijn in de herkenning van *S. suis* door het aangeboren immuunsysteem. De gastheer gebruikt hier hoogstwaarschijnlijk het TLR2/6 complex voor. Met de karakterisatie van het TroA lipoproteïne is er een veelbelovende vaccin component geïdentificeerd. Door het in kaart brengen van enkele mechanismen waarmee *S. suis* resistentie tegen lysozym ontwikkelt, zijn potentiële targets (autolysine, kapsel) gevonden voor de ontwikkeling van nieuwe therapeutica. In **Hoofdstuk 6** zijn de resultaten en conclusies van dit onderzoek in een breder perspectief geplaatst en is de voortuitgang in kennis nader uiteengezet met een nadruk op de ontwikkeling van nieuwe generatie vaccins. Het onderzoek beschreven in dit proefschrift vormt een interessante kennisbasis voor de ontwikkeling van methoden en strategieën voor de preventie en bestrijding van *S. suis* infecties bij mens en dier, waaronder de ontwikkeling van een serotype-overkoepelend vaccin.

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Curriculum vitae

Paul Wichgers Schreur werd geboren op 3 december 1982 te Diepenveen. In 2001 behaalde hij zijn HAVO diploma, waarna hij begon aan zijn bachelor opleiding Biologie en Medisch Laboratoriumonderzoek, aan de Saxion Hogeschool IJsselland te Deventer. Onder begeleiding van prof. dr. A.G.M. Tielens en dr. J.J. van Hellemond volgde hij een stage aan de Faculteit Diergeneeskunde van de Universiteit Utrecht met als onderwerp het energiemetabolisme van *Tryphanosoma theileri*. Zijn afstudeerstage verrichte hij bij de toenmalige Divisie Infectieziekten van de Animal Sciences Group van Wageningen UR onder begeleiding van dr. N. Stockhofe-Zurwieden, waar hij onderzoek deed naar het Porcine Dermatitis and Nephropathy Syndrome (PDNS). In 2005 werd het bachelor diploma behaald waarna de master opleiding Biomedische Wetenschappen aan de Universiteit Utrecht volgde. Zijn (afstudeer)stages werden wederom verricht aan de Faculteit Diergeneeskunde van de Universiteit Utrecht en bij de Divisie Infectieziekten van de Animal Sciences Group van Wageningen UR. Achtereenvolgens deed hij onderzoek naar Feline en Canine Coronavirusen en het porcine circovirus onder begeleiding van respectievelijk dr. B-J. Haijema en dr. N. Stockhofe-Zurwieden. Het master diploma werd behaald in 2008. Vanaf september 2007 is hij gestart als aio (promovendus) aan de Faculteit Diergeneeskunde van de Universiteit Utrecht, gedetacheerd bij het Centraal Veterinair Instituut van Wageningen UR. Gedurende vier jaar werd onderzoek verricht naar de interacties van *Streptococcus suis* met het aangeboren immuunsysteem, onder begeleiding van dr. H.E. Smith, dr. J.M.J. Rebel, prof. dr. M.A. Smits en prof. dr. J.P.M. van Putten. De resultaten van dat onderzoek staan beschreven in dit proefschrift. Vanaf februari 2012 is Paul werkzaam als postdoc in het Bunyaviridae veld bij de afdeling virologie van het Centraal Veterinair Instituut van Wageningen UR onder leiding van dr. J. Kortekaas en prof. dr. R.J. Moormann.

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