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Virulence mechanisms of *Streptococcus suis*:
Molecular characterisation of the biological activities of suilysin

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Meiner Familie
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Δ
Delta
%
Percent
A. bidest.
Aqua bidestillata
A. dest.
Aqua destillata
ANOVA
ANalysis Of VAriance
ATCC
American Type Culture Collection, Manassas, USA
BALB/c
Bagg ALBino; color locus c/c
BBB
Blood Brain Barrier
BMEC
Brain Microvascular Endothelial Cells
bp
Base pair(s)
BSA
Bovine Serum Albumin
°C
Degree Celsius
CcpA
Catabolite control protein A
CDC
Cholesterol-Dependent pore-forming Cytolysins
Cdc42
Cell division control protein 42 homolog
cf.
Conferre
CFSE
CarboxyFluorescein Succinimidyl Ester
CFU
Colony Forming Units
cm
Centimetre
CNS
Central Nervous System
CO2
Carbon dioxide
CPS
Capsule PolySaccharide
Crl:CD1 (ICR)
Charles River:CD1 (Institute for Cancer Research)
CRM
Cholesterol Recognition Motif
C3
C3 toxin of Clostridium limosum
Da
Dalton
DAPI
4',6-DiA midino-2-PhenylIndole
CD
Cluster of Differentiation
DMEM
Dulbecco’s Modified Eagle’s Medium
DNA
DeoxyriboNucleic Acid
DTT
DiThioThreitol
EDTA
EthyleneDiamineTetraacetic Acid
EF
Extracellular Factor
e.g.
Exampli gratia
ECM
ExtraCellular Matrix
et al.
Et alii
etc.
Et cetera
FBPS
Fibronectin and Fibrinogen Binding Protein of S. suis
FCS
Fetal Calf Serum
FESEM
Field Emission Scanning Electron Microscopy
Fig.
Figure
FITC
Fluorescein IsoThioCyanate
FL
FLuorescent
FSC
Forward SCatter
g
Gravitational constant
pH  Power of Hydrogen
PLY  Pneumolysin of Streptococcus pneumoniae
PMN  Polymorphonuclear Neutrophil
PTS  PhosphoTransferase System
PVDF PolyVinylDene Fluoride
Rac Ras-related C3 botulinum toxin substrate
Rho Ras homolog gene family
RNA RiboNucleic Acid
rSLY Recombinant suilysin
rSVD Recombinant RGD-SVD mutant of suilysin
rW461F Recombinant W461F mutant of suilysin
rpm Rounds per minute
RT Room Temperature
RT-PCR Reverse Transcriptase PCR
® Registered trademark
s Second(s)
SDS Sodium Dodecyl Sulphate
SLY Suilysin
SSC Sideward SCatter
ST Sequence Type
STSS Streptococcal Toxic Shock like Syndrome
TACY Thiol-Activated CYtolysin
TcdB Toxin B of Clostridium difficile
TcdB-F Toxin B of Clostridium difficile Serotype F strain 1470
THB Todd Hewitt Broth
Tig Trigger factor from S. suis
TLR Toll-Like Receptor
™ Trade mark
TMH TransMembrane Hairpin
TNL Tracheo-Nasal Lavage
TNF-α Tumor Necrosis Factor alpha
Tris Tris-(hydroxymethyl-) aminomethane
TRITC TetramethylRhodamine-IsoThioCyanate
U Unit
V Volt
wt wild type
× Multiply

Abbreviations of bacterial strains mentioned

C. limosum  Clostridium limosum
C. difficile  Clostridium difficile
E. coli  Escherichia coli
L. lactis  Lactococcus lactis
St. aureus  Staphylococcus aureus
S. suis  Streptococcus suis
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Chapter 1

General introduction
1. **Streptococcus (S.) suis**

*Streptococcus suis* is a gram-positive, facultative anaerobic bacterium causing invasive diseases in swine worldwide, associated with meningitis, septicaemia, arthritis, endocarditis and bronchopneumonia. In the last years *S. suis* has been considered as an important human pathogen leading to bacterial meningitis and the life-threatening streptococcal toxic shock like syndrome (STSS) in humans (Gottschalk *et al.*, 2010; Tang *et al.*, 2006). The bacterium shows α-haemolysis on sheep blood agar plates and α- and β-haemolysis on horse blood agar plates.

On the basis of the capsule polysaccharides, 33 different serotypes have been described so far, of which serotype 2 is worldwide most frequently isolated from diseased pigs and humans in Europe and Asia (Gottschalk *et al.*, 2010; Silva *et al.*, 2006; Wei *et al.*, 2009). Distribution of serotypes differs between geographical regions. Serotype 9 has emerged as the most common pig isolate in Germany and The Netherlands, serotype 7 is most prevalent in Scandinavia and Germany, and serotype 1 and 14 in the United Kingdom (Baums and Valentin-Weigand, 2009; Perch *et al.*, 1983; Tian *et al.*, 2004; Wisselink *et al.*, 2000). In contrast, in Canada and the USA serotypes 2, 1/2 and 3 are most frequently associated with disease (Messier *et al.*, 2008). Noteworthy, a specific sequence type (ST), namely ST7, evolved from the highly pathogenic ST1 type of a serotype 2 strain, which was found to be responsible for human outbreaks in china and directly associated with the STSS (Ye *et al.*, 2006; Ye *et al.*, 2009). The ST7 carries a putative pathogenicity island (designated 89K), possibly involved in development of STSS (Chen *et al.*, 2007; Zhao *et al.*, 2011).

1.1. **S. suis** infections

*S. suis* can infect pigs of each age group, including suckling and weaning piglets as well as growers. The natural habitat of *S. suis* is the upper respiratory tract. *S. suis* colonizes the nasopharynx, in particular the tonsils and the nasal cavities, as well as other mucosal surfaces like the intestinal and genital tract asymptomatically, resulting in a high carrier rate of healthy pigs of up to 100% (Arends *et al.*, 1984; Clifton-Hadley *et al.*, 1986; Higgins and Gottschalk, 1990; Lowe *et al.*, 2011; O'Sullivan *et
Such carrier-pigs are the most important source of infection (Clifton-Hadley and Alexander, 1980). Horizontal transmission of disease occurs most frequently via the direct 'nose-to-nose' contact between healthy and infected pigs, besides airborne transmission of \textit{S. suis} has also been shown in experimental infected piglets (Berthelot-Herault et al., 2001). Oral infection (feed) and transmission via skin wounds is also possible. Vertical transmission of \textit{S. suis} via the navel or the genital tract is another relevant route of infection (Amass et al., 1997; Robertson et al., 1991; Staats et al., 1997). Furthermore, insects (houseflies) as potential vectors have been discussed (Enright et al., 1987).

Although the morbidity rate of pigs is less than 5% due to prophylaxis with antibiotics (Clifton-Hadley et al., 1986), in the case of disease the mortality rate can reach 20% in the absence of treatment. Several forms of streptococcal disease in pigs are known. The occurrence of sudden death due to a peracute septicaemia as well as the development of severe meningitis, polyarthritis and bronchopneumonia can be observed. Acute infections are indicated by high fever (>40°C), persistent anorexia followed by lameness and central nervous failure (Sanford and Tilker, 1982; Windsor and Elliott, 1975). Typical histopathological lesions are characterized by an acute fibrinosuppurative inflammation of the respective tissue (Beineke et al., 2008; Williams and Blakemore, 1990).

As an important emerging zoonotic agent \textit{S. suis} has gained public interest due to increased reports on human infections (Gottschalk et al., 2007; Trottier et al., 1991; Wertheim et al., 2009). To date human cases of \textit{S. suis} infections are mainly reported in Asia. In Vietnam \textit{S. suis} is considered as the most common causative pathogen of bacterial meningitis (second most common in Thailand and third most frequent in Hong Kong) (Mai et al., 2008; Petersen et al., 2011; Sriskandan and Slater, 2006; Wangkaew et al., 2006). Particularly humans exposed to infected pigs or contaminated pig-products are at risk (Arends and Zanen, 1988). An additional potential source of infection for humans are wild boars (Baums et al., 2007). Therefore, \textit{S. suis} is an occupational disease of people in close contact with swine, like farmers, butcher, hunters and veterinarians (high-risk group). Hence, the nasopharyngeal carrier rate of \textit{S. suis} serotype 2 strains of persons belonging to the
high-risk group in Germany was 5.3% (Strangmann et al., 2002). For humans without swine contact oral transmission via raw pork or contaminated pig-products is possible, whereas a 'human-to-human' infection has not been proven so far (Wertheim et al., 2009). After an incubation period ranging from a few hours to five days post infectionem, purulent meningitis, septicaemia and arthritis associated with leukocytosis and neutrophilia are the most common manifestations in humans (Arends and Zanen, 1988; Fongcom et al., 2001). A serious consequence following *S. suis* meningitis is chronic deafness (Navacharoen et al., 2009). Two human outbreaks in China in 1998 and 2005 were associated with increased severeness of clinical symptoms. A noticeable high incidence of the STSS, which is characterized by high fever, erythoderma and multi organ failure (liver, heart, kidney, CNS) was observed, resulting in a high mortality rate of more than 20% (Tang et al., 2006; Yu et al., 2006).

### 1.2. Pathogenesis and virulence mechanisms

The mechanisms underlying pathogenesis of *S. suis* infections are only poorly known. A hypothetical model of pathogenesis includes three main steps. Firstly, after colonisation the mucosal surface of the upper respiratory tract, *S. suis* invades into deeper tissues of the epithelium. Secondly, the bacterium disseminates within the bloodstream to finally cross the endothelium of target tissues, such as the blood brain barrier (BBB) of the central nervous system (CNS) to cause meningitis (Chanter et al., 1993; Gottschalk and Segura, 2000). *S. suis* produces a wide array of virulence and virulence-associated factors, either secreted or surface-associated, involved in this process. A comprehensive review of bacterial factors expressed by *S. suis* was recently published (Baums and Valentin-Weigand, 2009).

Bacterial factors such as the fibronectin and fibrinogen binding protein (FBPS) (de Greeff et al., 2002) or the cell wall component lipoteichonic acid (LTA) (Fittipaldi et al., 2007; Vanier et al., 2007) mediate adherence of bacteria to target cells for initial colonisation. Another bacterial mechanism for sufficient colonisation is the formation of biofilms probably enhancing bacterial resistance to innate and adaptive host defence mechanisms and treatment with antibiotics (Bonifait et al., 2008). To get
access into deeper tissues bacteria might invade the respiratory epithelium. Suilysin, the haemolysin of *S. suis*, is discussed to play a role in interaction of *S. suis* with epithelial cells and disruption of these cells due to its cytolytic function (cf. 2.4.2.). Furthermore, the capsule is assumed to be involved in host cell interaction. Since its main function is protection against phagocytosis after entering the bloodstream (Benga et al., 2008; Chabot-Roy et al., 2006; Charland et al., 1998; Segura and Gottschalk, 2002), it has been proposed that the capsule is down-regulated during colonisation of the mucosal epithelium to allow adherence and invasion of the bacterium to overcome this first barrier within the host (Gottschalk and Segura, 2000; Okamoto et al., 2004; Willenborg et al., 2011). In accordance, unencapsulated *S. suis* stains showed higher adhesion and invasion rates, indicating a negative correlation between encapsulation and interaction with host cells (Benga et al., 2004; Gottschalk et al., 1991). A possible explanation for this phenotype is the masking effect of the capsule, whereby potentially involved surface associated proteins or cell wall components might be hidden (Lalonde et al., 2000; Tenenbaum et al., 2008; Vanier et al., 2007). Moreover, a direct uptake of *S. suis* by monocytes for crossing the epithelium as well as for entering the bloodstream within circulating cells, known as the 'Trojan horse theory', is controversially discussed due to the protecting effect of the capsule. A 'travelling' of either free bacteria or monocyte-associated bacteria ('modified Trojan horse theory') is more likely (Gottschalk and Segura, 2000). Circulation of bacteria within the bloodstream may lead to onset of acute bacteraemia or septicaemia and the release of several pro-inflammatory cytokines by cells of the innate immune system to control acute infection or to contribute to immunopathology (Segura et al., 1999; Segura et al., 2002; Segura et al., 2006). Nevertheless, for induction of meningitis *S. suis* has to penetrate the BBB to reach the CNS. *S. suis* has the ability to adhere to and invade into brain microvascular endothelial cells (BMEC) and porcine choroid plexus epithelial cells (PCPEC), the main components of the BBB (Benga et al., 2005; Charland et al., 2000; Tenenbaum et al., 2005; Tenenbaum et al., 2008; Vanier et al., 2004). Moreover, an increase in tight junction permeability and loss of barrier function is ascribed to direct cytotoxic effects of suilysin (Charland et al., 1998; Vanier et al., 2004). Apart from suilysin
S. suis can stimulate the production of pro-inflammatory cytokines like interleukin-6 (IL-6), IL-8 and monocyte chemotactic protein-1 (MCP-1) by BMEC, which in turn alters BBB permeability (Vadeboncoeur and Pelletier, 1997). However, Tenenbaum et al. (2009) described the entry S. suis into the CNS as a transcellular translocation without destruction of PCPEC lining of the BBB.

2. Cholesterol-dependent pore-forming cytolysins (CDC)
Suilysin, the secreted haemolysin of S. suis, belongs to the family of cholesterol-dependent pore-forming cytolysins (CDC) and was considered as a virulence-associated factor, which contributes to pathogenesis of S. suis. CDC are a large family of membrane-damaging toxins produced by more than 20 gram-positive bacteria (Tweten, 2005), including the genera Streptococcus, Listeria, Bacillus, Clostridum, Paenibacillus, Arcanobacterium and most recently Gardnerella (Gelber et al., 2008) and Lactobacillus (Rampersaud et al., 2011).

2.1. The structure of CDC
All common CDC show a high similarity in primary amino acid sequence varying between 40-70% identity. CDC are single stranded peptides showing an elongated rod-like three-dimensional structure, which was first described for the prototype of CDC, perfringolysin O of Clostridium perfringens.

CDC were previously named thiol-activated cytolysins (TACY) due to the fact, that most members carry a single cysteine residue within the undecapeptide at the carboxy-terminus of the molecule. This cysteine residue is believed to be essential for membrane binding and the cytolytic function. It is known that oxidation of carbon-bonded sulfhydryl groups (formation of disulfide bonds) inhibits CDC. The name 'thiol-activated' derives from the properties of thiols, which are able to reactivate the toxin via the cleavage of disulfide bonds of the cysteine residue. However, two members of the toxin family, pyolysin (Arcanobacterium pyogenes) (Billington et al., 1997) and intermedilysin (Streptococcus intermedius) carry an alanin residue instead of the cysteine without any deficiency in lysis activity (Herbert and Todd, 1941). Moreover, mutation studies targeting the cysteine residue did not alter the cytolysis
In contrast to the cytolytic activity, cysteine substitution of listeriolysin O decreased invasion of *Listeria monocytogenes* (Stachowiak *et al.*, 2009). Nevertheless, these findings clearly show, that the cysteine residue is not required for cytolytic function of the toxin, therefore the name 'thiol-activated' is no longer appropriate.

The monomeric protein contains four domains (D1-D4, Figure 1-1A): D1 and D3 are located at the N-terminus of the protein, linked to D4 via the connecting domain D2 (Rossjohn *et al.*, 1997). The main part of the protein is formed by β-strands (43%), whereas in particular D3 also contains a couple of α-helixes (14% α-helices of the whole structure), playing a special role in pore-formation (Xu *et al.*, 2010). The most conserved regions are the C-terminal tryptophan-rich undecapeptide (Figure 1-1A), consisting of eleven amino acids, (ECTGLAWEWWR; c.f. Chapter 4, Figure 4-2) of D4 and the hydrophobic core of D1. The undecapeptide is considered to be responsible for the initial membrane binding and triggers the formation of functional pores (Rossjohn *et al.*, 1997). Supportively, it has been shown, that recombinant D4 alone is still able to bind to membranes of erythrocytes (Weis and Palmer, 2001). In a recent study the crystal structure and D4-folding of CDC, including suilysin, intermedilysin, perfringolysin O and anthrolysin (*Bacillus anthracis*), was analyzed in more detail. Bending-degree of the angle between D1 and D4 as well as the conformations of the tryptophan-rich loop on the tip of the undecapeptide were compared. Predictions of intermedilysin and suilysin revealed a more extended structure (Figure 1-1A), whereas the undecapeptide of perfringolysin O and anthrolysin was folded back into a hydrophobic pocket (Xu *et al.*, 2010).
2.2. The mechanism of pore-formation by CDC

All members of the CDC family are secreted as water soluble monomers due to a signal sequence at the N-terminus of the protein, with the exception of pneumolysin (Streptococcus pneumoniae), which is not released until lysis of the bacterial cell (Walker et al., 1987). A common, but still putative, mechanism of pore-formation was described by Tweten et al., 2005. Generation of channels is a 4-step process (Figure 1-1B) (Tweten, 2005). (1) Initially several monomers bind vertically to the target cell membrane. Interaction between the CDC molecule and the cell is mediated via D4, forming a hydrophobic 'danger' consisting of the undecapeptide, which superficially
inserts into the membrane (Rossjohn et al., 1998). Binding of D4 to the membrane is necessary for subsequent structural changes within the not directly connected D3, called 'conformational coupling' (Rossjohn et al., 2007). Subsequently, cell-bound monomers undergo a lateral shift to form an oligomer. (2) Further structural changes within D3 lead to the formation of the oligomeric ring- or arc-shaped prepore complex (Figure 1-1C). (3) The N-terminal D3 builds the inner structure of the transmembrane pore. Therefore, six α-helices form two β-hairpins (Figure 1-1A; TMH1 and TMH2). This ultimate β-barrel structure consists of several β-hairpins of agminated monomers, which are connected through an intermolecular interaction of the β1-strand and β4-strand of two individual molecules (Ramachandran et al., 2004). (4) In the final step, the connecting domain D2 serves as a 'hinge-joint' to bring D3 in close contact with the cell surface. After the 'collapse' of D2 the β-barrel structure inserts into the membrane and forms the transmembrane pore. This pore, 30 nm in size, consists of 35-50 monomers and is permeable for ions and macro molecules (Figure 1-1C).

2.3. The functional role of cholesterol and membrane recognition

The role of cholesterol in CDC function is not finally clarified (Hotze and Tweten, 2011). Nevertheless, a general observation is that cholesterol is required for pore-formation and free cholesterol inhibits haemolysis of CDC (Alouf, 2000; Jacobs et al., 1994; Watson et al., 1972). Furthermore, cholesterol-depletion studies using cholesterol-containing liposomes showed that >30mol% of total membrane lipids has to be cholesterol for efficient membrane binding (Flanagan et al., 2009; Heuck et al., 2000). However, not all CDC use cholesterol as a membrane receptor. It has been shown that the human complement regulator molecule CD59 is required for membrane binding of intermedilysin and vaginolysin, thereby conferring a species specificity. However, cell lysis activity of intermedilysin and vaginolysin depends on cholesterol (Gelber et al., 2008; Giddings et al., 2004).

The cholesterol recognition motif (CRM) is most likely located within D4. Several studies concerning the CRM defined the undecapeptide to be responsible for cholesterol binding (Rossjohn et al., 1997; Rossjohn et al., 2007). In contrast, more
recent studies revealed three highly conserved loops (Figure 1-1A; L1-3) located next to the undecapeptide to mediate recognition of cholesterol (Soltani et al., 2007; Soltani et al., 2007b). Furthermore, it has been suggested that CDC present only a single binding side due to the fact that cholesterol binding activity is linear to CDC concentration (Johnson et al., 1980).

2.4. Suilysin

Suilysin was identified as a haemolysin of S. suis several years ago (Jacobs et al., 1994) and confirmed to be a member of CDC by sequence analysis. The protein has a molecular weight of 54 kDa and possesses an N-terminal signal sequence, thus considered as a secreted exotoxin. Suilysin is most related to pneumolysin, sharing 52% amino acid identity (Segers et al., 1998).

2.4.1. Prevalence, diversity, and regulation of the sly gene

Suilysin is expressed by many but not all S. suis strains. The sly gene has been detected in 95% of European and Asian invasive serotype 2 strains, but only in 7% of the North American strains (Segers et al., 1998). In various studies concerning the prevalence of the sly gene in isolates, belonging to different S. suis serotypes, obtained from diseased pigs in European countries, including Germany, Italy, Spain, Poland, France, The Netherlands and The United Kingdom, the sly gene was detectable in a range between 33.7% and 100% (Berthelot-Herault et al., 2000; Blume et al., 2009; de Greeff et al., 2011; Fabisiak et al., 2005; King et al., 2001; Princivali et al., 2009; Silva et al., 2006; Tarradas et al., 2001). Approximately 80% of these clinical isolates were obtained from porcine cases of invasive disease associated with meningitis, septicaemia, and arthritis. Isolates derived from lung samples (pneumonia) were positive tested for suilysin to a lesser degree of approximately 50% (King et al., 2001; Silva et al., 2006). Furthermore, the sly gene is prevalent in up to 90% of colonising S. suis strains isolated from the tonsils of healthy pigs (Fabisiak et al., 2005; King et al., 2001). Besides, suilysin is present in wild boars and domestic swine S. suis strains of Northwestern Germany in 39% and 66%, respectively (Baums et al., 2007). In Asian S. suis strains, isolated from healthy
(slaughtered) as well as from diseased swine, the prevalence of suilysin reaches a maximum of 100% (Hoa et al., 2011; Kim et al., 2010; Padungtod et al., 2010; Wei et al., 2009; Xiong et al., 2007). Equally, almost all isolates from human cases of a S. suis infection carry a sly gene (de Greeff et al., 2011; King et al., 2001; Princivalli et al., 2009). The situation is different for North American isolates, in which suilysin is less frequently present (Fittipaldi et al., 2009; Gottschalk et al., 1998).

However, presence of the sly gene has been used for characterisation and differentiation of S. suis strains (Gottschalk et al., 2007; Vecht et al., 1991), but presence of the sly gene does not necessarily result in protein expression (de Greeff et al., 2011).

Suilysin was detected as a highly conserved single copy gene within the S. suis genome (Okwumabua et al., 1999). Furthermore, the genetic diversity as well as the allelic variance of the sly gene appears to be low (King et al., 2001). The flanking genes (2 open reading frames (ORF) upstream, orf100 and orf101; and 2 ORF downstream, nanE and pstG) are also highly conserved. Sly-negative strains exhibit an alternative gene (orf102) at the same position instead of the sly gene. Therefore, genetic exchange via homologous recombination between different S. suis strain is most likely (Takamatsu et al., 2002). Moreover, it is conceivable that the sly gene is not organised as an operon and under the control of its own promoter, because of large non-coding region upstream and downstream of the sly gene.

Little is known about the regulation of the sly gene. The protein is expressed at late logarithmic growth phase, possibly dependent on nutrient availability, pH and bacterial density (Gottschalk et al., 1995). A hyper-haemolytic phenotype is described for a manN-negative S. suis mutant strain, suggesting that the mannose phosphotransferase system affects the suilysin promoter activity (Lun et al., 2003). The global orphen response regulator CovR (control of virulence regulator) controls the expression of about 200 genes, including the capsule biosynthesis and suilysin. Inactivation of covR led to the production of a thicker capsule and slightly higher haemolytic activity associated with increased adhesion to epithelial cells and reduced phagocytosis and killing by polymorphonuclear neutrophils (PMN) (Pan et al., 2009). In contrast to other CDC, like intermediylin, which is under the transcriptional
catabolite control protein A (CcpA), the homologous global regulator of *S. suis* is most likely not involved in regulation of suilysin (Tomoyasu *et al.*, 2010; Willenborg *et al.*, 2011). Besides, expression of the *sly* gene is influenced by the trigger factor from *S. suis* (*Tig*) and the orphan response regulator RevSC21. Both, deletion of the *tig* gene and the RevSC21 gene degraded expression of suilysin and resulted in a lack of haemolytic activity of the respective *S. suis* strain (Wu *et al.*, 2009; Wu *et al.*, 2011).

### 2.4.2. Role of suilysin in host-pathogen interaction

Similar to other members of the CDC family suilysin can damage different types of host cells by its cytolytic activity (Benga *et al.*, 2004; Charland *et al.*, 2000; Jacobs *et al.*, 1994; Lalonde *et al.*, 2000; Norton *et al.*, 1999; Segura and Gottschalk, 2002; Tenenbaum *et al.*, 2005; Tenenbaum *et al.*, 2006; Vanier *et al.*, 2004). A suilysin-induced cell injury is associated with loss of cytoplasmic density, disruption of cytoplasmic membranes and the release of cellular contents (Allen *et al.*, 2001; Segura and Gottschalk, 2002). Haemolysis of erythrocytes was first described by Jacobs *et al.* (1994), whereas human red blood cells were the most susceptible, followed by horse, sheep, and cow erythrocytes (Gottschalk *et al.*, 1995). A multifunctional role in pathogenesis was described for other members of the CDC-family. These biological effects can be observed even at subcytolytic concentration of the respective toxins (Billington *et al.*, 2000). For pneumolysin, listeriolysin O, and intermedilysin was reported that they may contribute to bacterial adherence and invasion (Cockeran *et al.*, 2002; Krawczyk-Balska and Bielecki, 2005; Rubins *et al.*, 1998; Sukeno *et al.*, 2005). Likewise, suilysin has been described to be involved in the modulation of *S. suis* host cell interaction, including endothelial cells, epithelial cells, PMN and macrophages (Baums and Valentin-Weigand, 2009; Gottschalk and Segura, 2000). In particular, it has been suggested that suilysin plays a role in pathogenesis of *S. suis* (Norton *et al.*, 1999) such as the crossing of the BBB by interruption of intracellular junctions for entering the CNS (Charland *et al.*, 2000; Tenenbaum *et al.*, 2005; Tenenbaum *et al.*, 2008). However, the ability of several
S. suis strains to interact with PBMEC does not correlate with suilysin production (Vanier et al., 2004; Vanier et al., 2007).

More recently, it has been found that the toxin may be involved in cytokine release and protection against opsonophagocytosis. Thus, a reduced survival time of a sly-deficient mutant strain after co-incubation with polymorphonuclear neutrophils (PMN) was observed by Benga et al. (2008). This was confirmed by using antisera raised against purified suilysin, which increased the uptake of the wild type strain by PMN. In addition, adding recombinant suilysin at subcytolytic concentration increased the growth capacity of the sly-deficient mutant (Benga et al., 2008). Furthermore, suilysin contributes to resistance of complement-dependent killing of S. suis by neutrophils (Chabot-Roy et al., 2006), perhaps by impairing complement factor C3 deposition on the surface of S. suis (Lecours et al., 2011). For pneumolysin, reduction of serum complement levels and decreased opsonisation of pneumococci has been described as well (Alcantara et al., 2001). Although, S. suis was found to be resistant to phagocytosis by murine astrocytes, suilysin was mainly responsible for pro-inflammatory cytokine production and partially involved in toll-like receptor 2 (TLR2) expression of these cells (Zheng et al., 2011). Similarly, recognition of pneumolysin via the TLR4 is critically involved in the innate immune response to pneumococci (Dessing et al., 2009; Malley et al., 2003).

In general, interference of suilysin with different types of immune cells and induction of cytokine release suggests the important role of suilysin in host innate defence response. Suilysin is responsible for the release of IL-6 and IL-8 by BMEC (Vadeboncoeur et al., 2003) and PBMEC (Vanier et al., 2008) as well as for the production of tumour necrosis factor α (TNF-α) by human monocytes and IL-6 by porcine pig pulmonary alveolar macrophages and monocytes (Lun et al., 2003). In contrast, suilysin did not have a major impact on phagocytosis as well as on TNF-α and MCP-1 production by murine microglia (Dominguez-Punaro et al., 2010). Accordingly, suilysin failed to induce TNF-α and IL-6 in murine macrophage line J774 (Segura et al., 1999) and plays a limited role in modulation of cytokines and chemokine response in a whole-blood system (Segura et al., 2006). An involvement in cytokine production by bone marrow–derived dendritic cells was recently described.
by (Lecours et al., 2011). In inflammatory events increased recruitment of leucocytes is linked to adhesion molecules. Stimulation of THP-1 monocytes with suilysin led to an up-regulation of CD11a/CD18 and CD11c/CD18 (Al Numani et al., 2003). Most of the other CDC have also been shown to display a role in modulation of immune response mechanisms (Cockeran et al., 2002; Ratner et al., 2006; Tsuchiya et al., 2005).

2.4.3 Role of suilysin in virulence and pathogenesis
The importance of suilysin in the pathogenesis of S. suis is not finally clarified. While suilysin has been shown to be associated with virulent strains, there are also virulent strains that do not produce suilysin (Staats et al., 1999). Only few experimental infections of mice and piglets addressing the role of suilysin were performed so far. Intraperitonally infection of BALB/c mice demonstrated attenuation of a sly-deficient mutant in comparison to the highly virulent S. suis serotype 2 strain P1/7. In contrast, the sly knock-out strain was only slightly attenuated (reduced severeness of clinical signs and pathological findings) in an intravenous porcine infection model (Allen et al., 2001), indicating that suilysin does not play a major role in disease development after systemic administration. Similar results were obtained by Lun et al. (2003) using three different sly-deficient strains in an intrapharyngeal piglet infection model. All swine developed clinical symptoms associated with septicaemia, arthritis, and meningitis regardless of the challenge strain. However, vaccination containing purified suilysin was capable to induce protection in BALB/c mice and piglets after challenge with a homologous strain (S. suis P1/7). Furthermore, immunisation led to an increase in haemolysin neutralisation antibody titre (Jacobs et al., 1994; Jacobs et al., 1996). Additionally, intranasal immunisation of piglets with a S. suis live vaccine elicited most prominently serum immunoglobulin G responses against suilysin (Kock et al., 2009). Likewise, the highly related pneumolysin protects mice against homologues challenge and moreover a pneumolysin-deficient mutant was attenuated in a BALB/c infection model (Alexander et al., 1994; Orihuela et al., 2004).
3. **Outline of the present study**

As pointed out in the general introduction, knowledge on pathogenesis of *S. suis* infection and potentially involved bacterial factors is still limited. The aim of this study was to evaluate suilysin-mediated effects on *S. suis*-epithelial cell interaction including investigations on the underlying mechanisms and the phenotypical characterisation of putatively involved functional domains of the suilysin molecule. Furthermore, the study focussed on the development of an intranasal mouse infection model to further elucidate the role of suilysin in colonisation and invasion of the upper respiratory tract *in vivo*.

According to these objectives, the results are divided into 3 chapters. In **chapter 3** the role of suilysin in *S. suis* invasion in epithelial cells is investigated, revealing a subcytolytic activity of suilysin in a Rac-dependent activation of the actin cytoskeleton promoting invasion of *S. suis*. **Chapter 4** comprises the characterisation of two (functional) domains within the suilysin molecule. Site-directed amino acid substitution and comparative functional analysis of the tryptophan-rich undecapeptide and a putative integrin-binding RGD-motif indicate that both domains are required for cytolytic function. Additionally, a functional RGD-motif is essential for membrane-binding and activation of Rac. Finally, in **chapter 5** the establishment of an intranasal *S. suis* mouse infection model is described. The results are generally discussed in **chapter 6** with regard to relevance for *S. suis* pathogenesis. A short summary of this thesis is provided in **chapter 7** (English) and **chapter 8** (German).
References


Chapter 2

Material and methods
If not stated otherwise all materials were purchased from Sigma (Muenchen, Germany).

1. **Bacterial strains and growth conditions**

In this study *Streptococcus (S.) suis* serotype 2 wild type strain 10, kindly provided by H. Smith (Lelystad, NL), and its isogenic mutants were used. This strain expresses EF, MRP, SLY, FBPS and OFS. It has been used by different groups successfully for mutagenesis and experimental intranasal infections of pigs (Baums *et al.*, 2006; Smith *et al.*, 1999; Vecht *et al.*, 1997). The unencapsulated isogenic mutant strain 10cpsΔEF of the virulent *Streptococcus (S.) suis* serotype 2 wild type strain 10 (designated strain 10cpsΔEF) was kindly provided by H. Smith (Lelystad, NL). Strain 10cpsΔEF has been generated by insertional mutagenesis of the genes cps2E and cps2F involved in biosynthesis of the capsule and has been demonstrated to be severely attenuated in virulence, most likely due to increased opsonophagocytosis (Smith *et al.*, 1999). The corresponding suilysin deficient mutants of wild type strain 10 (designed 10Δsly) and 10cpsΔEF (designed 10cpsΔEFΔsly) were constructed by insertion of an erythromycin cassette in the sly-gene of the unencapsulated strain 10cpsΔEF using the plasmid pBlue/sly/erm as previously described for generation of strain 10Δsly (Benga *et al.*, 2008). Mutagenesis was confirmed by PCR and Southern Blot analysis. Streptococci were grown on Columbia agar supplemented with 7% sheep blood (Oxoid, Wesel, Germany) or in Bacto Todd Hewitt broth (THB) overnight under aerobic conditions at 37°C. If required, antibiotics were added to the media at the following concentrations: spectinomycin 100 µg/ml for *S. suis*; erythromycin 1 µg/ml for *S. suis*.

*Lactococcus lactis* subspecies cremoris MG1363 (*L. lactis*), *L. lactis* heterologously expressing SLY (*L. lactis* pORI-SLY, *L. lactis* pORI-W461F, *L. lactis* pORI-SVD) and *L. lactis* carrying the shuttle vector pORI23 (*L. lactis* pORI23) were kindly provided by D. Reinscheid (Fachhochschule Bonn-Rhein-Sieg, Germany). All *L. lactis* strains were cultured on M17 agar plates (Oxoid) supplemented with 5% glucose (GM17) overnight under aerobic conditions at 30°C. If required, 5 µg/ml of erythromycin was added to the GM17 medium or plates.
Escherichia (E.) coli strains BL21, BL21 (DE3) and DH5α were used for molecular cloning and protein expression experiments. E. coli were grown in Luria Bertani (LB) medium overnight under aerobic conditions at 37°C with vigorous shaking. In appropriate cases, 100 μg/ml of ampicillin was added to the LB medium or plates. For infection of HEp-2 cells, streptococci were grown in Todd-Hewitt broth (THB, Difco, Detroit, USA) overnight at 37°C under aerobic conditions, adjusted to an optical density (OD600) of 0.02 in pre-warmed media the next day and grown to late exponential growth phase (OD600 0.8).

2. Molecular biology and protein biochemical methods
Routine molecular biology techniques including restriction endonuclease digestion, DNA ligations, agarose gel electrophoresis, transformation of E. coli and plasmid isolation were performed according to standard procedures (Sambrook et al., 1989). Restriction enzymes were purchased from New England Biolabs (Frankfurt am Main, Germany). Plasmid preparations were performed with kits from Machery-Nagel (Dueren, Germany).

2.1. Construction of mutated suilysin W461F und SVD
W461F and RGD-SVD substitutions were constructed using site-directed-mutagenesis according to the instruction manual of QuikChange® Site-Directed Mutagenesis Kit (Stratagene, Ja Jolla, CA). As template DNA pET45bslynew was used (Kock et al., 2009). Oligonucleotide primers encoding the W461 to F461 substitution, namely slyTrp-Phefor new (TACAGGATTAGCATTTGAGTGGTGGAGAAC) and slyTrp-Pherevnew (GTTCTCCACCACCAATGCTAATCCTGTAC), and oligonucleotide primers encoding R124- G125-D126 to S124-V125-D126, namely slyRGDMutfor (CAGTATTGCGTCGGTAGATCTGACGCTTAG) and slyRGDMutrev (CTAAGCGTCAGATCTACCGACGCAATACTG) were used. After digestion of the methylated non-mutated parental DNA template with DpnI, mutated plasmid pET45bslynewW461F was electroporated (2.5 kV, 200 Ω, 25 mF) into E. coli BL21 (DE3) and then purified. Mutation sites of each suilysin derivate were sequenced.
using the primers slyseqfor (GGATCATTCAGTGCTTATG) and pET45seqrev (TGCTGGCGTCAAAATTTCGC).

2.2. Expression of recombinant proteins
Recombinant His-tagged suilysin (rSLY) was expressed in *E. coli* BL21 pET45bslynew (Kock *et al*., 2009). In accordance, recombinant point-mutated suilysin W461F (rW461F) was expressed in *E. coli* BL21 pET45bslynewW461F and recombinant RGD-SVD (rSVD) in *E. coli* BL21 pET45bslynewRGD-SVD. Proteins were expressed after induction with 0.4 mM isopropyl-β-D-thiogalactoside for 4 h. His-tagged proteins were purified under native conditions with Protino® Ni-TED 2000 packed columns as recommended by the manufacturer (Macherey-Nagel) with some modification. rSLY and rW461F were eluted with a 1:10 dilution of the elution buffer (containing 10 mM imidazole). The eluted proteins were dialysed against 100 mM TrisHCl [pH 7.0] with 1 mM DTT. Protein concentrations were measured according to microplate assay protocol (Bio-Rad, Muenchen, Germany) and the haemolytic activity was tested as described by Takamatsu *et al.* (2001). Purification of proteins were verified by immunoblot analysis with anti-Penta His antibodies (Qiagen, Hilden, Germany) as recommended. Silver staining was used to control the purity grade. The protein was stored at -20°C.

Suilysin was completely sequenced with standard oligonucleotide primers T7 (TAATACGACTCACTATAGGG) and T7 term (CTAGTTATTGCTCAGCGGT; Eurofins MWG operon, Ebersberg, Germany).

2.3. Heterologous expression of SLY, W461F and SVD in *L. lactis*
The *sly* gene was amplified from chromosomal DNA of *S. suis* by PCR with oligonucleotide primers slyPstI (TAGTCTGCAGCTCCTAGCCTCTCTGCTGCTAA) and slyBamH1optRBS (CAGAGGATCCAGGAAGAAAACTTATGAGAAAAG). After digestion of the PCR product with BamHI and PstI the fragment containing the whole *sly*-gene was cloned into the BamHI/PstI-digested shuttle vector pOri23 (Que *et al*., 2000). The resulting plasmid was named pOri23-SLY and transferred in *L. lactis* as described previously (Holo and Nes, 1989). Transformants were screened by plating
on GM17 agar plates containing 5 µg/ml erythromycin. For heterologous expression of W461F and SVD site-directed mutagenesis was performed as described above using purified plasmid DNA pOri23sly and the respective oligonucleotide primers slyTrp-Phe for new and slyTrp-Phe rev new as well as slyRGDMut for and slyRGDMut rev new. Resulting constructs pOri23-W461F and pOri23-SVD were electroporated in L. lactis.

2.4. Immunoblot analysis
Recombinant proteins or supernatants of infected cells were separated by SDS-polyacrylamid gel electrophoresis with a 4% stacking and a 10% separating gel under denaturing conditions and transferred to a PVDF-membrane (Serva, Heidelberg, Germany). For suilysin immunoblot, membrane-blocking was performed overnight with 3% milk powder in TBS with 0.5% Tween. Polyclonal antiserum raised against rSLY (Benga et al., 2008) diluted 1:1300 in 1% milk powder was used to detect either rSLY, rW461F, rSVD or suilysin and its mutated derivates expressed by the respective L. lactis strains (L. lactis pORI-SLY, L. lactis pORI-W461F and L. lactis pORI-SVD) present in culture supernatants. For detection of His-tagged recombinant proteins membranes were blocked with 3% BSA in TBS with 0.5% Tween overnight and incubated with a monoclonal anti-Penta His antibody diluted 1:2000 in 3% BSA. Membranes were developed with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antiserum diluted 1:10000 in 1% milk powder (Amersham, Freiburg, Germany) followed by using SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, USA) according to the manufactures protocol.

3. Cell culture methods
3.1. Epithelial cells
The human laryngeal epithelial cell line HEp-2 (ATCC CCL 23) was used. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco-Invitrogen, Groningen, The Netherlands) supplemented with 10% fetal calf serum (FCS) and 5 mM glutamine at 37°C and 8% CO₂. The cells were subcultured every 2-3 days after detachment with 0.25% trypsin and 1 mM Na-EDTA (trypsin-EDTA, Gibco-
Invitrogen). For antibiotic protection assays, approximately $1.8 \times 10^5$ cells per well were seeded on 24 well tissue culture plates, for immunofluorescence and electronmicroscopy $0.5 \times 10^5$ and $1.5 \times 10^5$ cells per well were seeded on a 12 mm diameter glass cover slips placed in 24 well plates. For flow cytometry analysis approximately $6 \times 10^5$ cells per well were seeded on a 6 well plate and for cellular cytotoxicity assay $0.2 \times 10^5$ cells were seeded on a 96 well plate. The cells were grown over-night and then used for experiments.

3.2. Antibiotic protection assay

The number of adherent and invasive streptococci was quantified using a gentamicin protection assay as described with the following modifications (Valentin-Weigand et al., 1996). Adhesion and invasion assays were performed with fresh grown streptococci in THB harvested at an OD$_{600}$ of 0.8 by centrifugation. Subsequently, bacteria were resuspended in PBS containing 0.9 mM CaCl$_2$/0.5 mM MgCl$_2$6H$_2$O [pH 7.3] (PBS$^+$; Gibco-Invitrogen) and adjusted to an OD$_{600}$ 0.6. Then, diluted 1:10 in PBS$^+$ and used for adherence and invasion assay. Confluent HEp-2 cell monolayers were inoculated with 100 bacteria per cell. The number of colony forming units (CFU) inoculated per well was determined by serial platings on Columbia agar supplemented with 7% sheep blood. The cells were incubated for 2 h at 37°C with 8% CO$_2$ in order to allow adherence and invasion of the bacteria. Supernatants of infected cells were collected 2 hours post infection for immunoblot analysis of suilysin. To determine adherence rates cells were washed three times with PBS immediately after supernatant collection, and 100 μl trypsin-EDTA solution was added to each well. After 5 min, cells were lysed by adding 400 μl 0.025% Triton X-100. Serial dilutions of these lysates were plated in triplicates on Columbia agar and incubated at 37°C for 24 h. For invasion assay, cells were washed 3 times with PBS 2 h post infection and incubated with 500 μl per well of DMEM containing 100 μg gentamicin and 5 μg penicillin per ml for 2 additional hours at 37°C with 8% CO$_2$ to kill extracellular bacteria. The cells were washed and lysed as described for adherence and lysates were plated for viable counts of streptococci. Results for
adherence and invasion were expressed as percentage of the inoculum used for infection.

In some adherence and invasion assays, HEp-2 cells were treated before infection with the following inhibitors: 1 µg/ml Latrunculin B for 30 min, 10 ng/ml toxin B of Clostridium (C.) difficile (TcdB), 10 ng/ml toxin B of C. difficile serotype F strain 1470 (TcdB-F) and 1 µg/ml C3 toxin of C. limosum for 3 h. C3 toxin was always reconstituted freshly by mixing the binding domain (C2IIa) with the catalytic domain (FT) in a ratio of 1:1 as described (Just et al., 1995). After preincubation with inhibitors cells were washed and adherence and invasion assays were performed as described above. Latrunculin B was left on to the cells during the whole infection experiment because its effect is reversible up to 1 h.

3.3. Labelling of bacteria for flow cytometry experiments

CellTrace™ CFSE Cell Proliferation Kit (Invitrogen) was used for fluorescent staining of bacteria. Labelling of bacteria with CFSE was done as described by Logan et al. (1998) with some modifications. Streptococcal strains 10cpsΔEF and 10cpsΔEFΔsly were grown to an OD600 of 0.8, pelleted by centrifugation, washed once with PBS (Gibco-Invitrogen) and resuspended in PBS containing 5 µM carboxyfluorescein succinimidyl ester (CFSE)-reagent (prepared from a CFSE stock solution as recommended by the manufacturer). Staining procedures were carried out at 37°C for 20 min on an end-over-end rotator. After washing (removal of unbound fluorescent dye) culture stocks were prepared as described above. Labelling efficiency (>90%) was confirmed by fluorescent microscopy and measurement of fluorescent intensity by flow cytometry (side-ward-scatter [ssc] versus fluorescence channel FL-1) in comparison to unlabeled streptococci (data not shown).

3.4. Bacteria-cell association

HEp-2 cell monolayers were infected with CFSE-labelled streptococci at a multiplicity of infection [moi] of 125:1 bacteria per cell, followed by centrifugation (250 x g for 5 min) and incubation at 37°C for 2 h. After incubation supernatants of infected cell were collected for determination of sulysin expression by immunoblot analysis. Cells
were detached with trypsin-EDTA solution, washed with PBS and fixed by resuspension in PBS with 0.375% formaldehyde in PBS. In complementation experiments, rSLY and rW461F were added to strain 10cpsΔEFΔsly in a protein concentration of 100 ng/ml during the experiment. Bacteria-cell association was measured using FACScan® (Becton Dickinson, 488 nm Argon laser). Further analysis was performed with the software WinMDI (version 2.9.). For each determination at least 10,000 events were acquired and initial analysis of infected cells was carried out by dot plot analysis (forward scatter [fsc] versus sideward scatter [ssc]) to define the cell population of interest (data not shown). Subsequently, fluorescent cells were detected at channel FL-1. The numerical results were expressed as mean fluorescent intensity [mfi] values of cell population. Noninfected cells served as background control. Means and standard deviations refer to eight independent experiments. To exclude cytotoxic effects of SLY during the experiment cells were stained afterwards with propidium iodid (4 µg/ml, DNA-intercalating dye) for 2 min at room temperature to discriminate between viable and nonviable cells. Red fluorescent cells (dead cells) were determined by dot plot analysis (ssc versus FL-2). In some assays, HEp-2 cells were treated before infection with 1µg/ml latrunculin B for 30 min and latrunculin B was also added to the cells during the whole experiment because its effect is reversible during 1 h. After preincubation with latrunculin B cells were washed and flow cytometry was performed as described above.

3.5. Immunofluorescence microscopy
Membrane binding of rSLY, rW461F and rSVD was determined by immunofluorescent microscopy. HEp-2 cells were incubated with the recombinant suilysin derivates at subcytolytic (100 ng/ml) or lytic (400 ng/ml) concentrations for 30 min at 37°C. Cells were washed three times to remove unbound suilysin, fixed with 2% paraformaldehyde and permeabilised with 0.1% Triton X-100 in PBS (Bio-Rad, Muenchen, Germany). Blocking was performed for 30 min at room temperature with PBS containing 10% FCS. Suilysin was stained using a polyclonal antiserum raised against rSLY (Benga et al., 2008) diluted 1:1,300 in 1% milk powder for 1 h at room temperature followed by an incubation with FITC-conjugated goat anti-rabbit antibody
(1:1,000 in 1% FCS in PBS, Dianova, Hamburg, Germany). F-actin was labelled with TRITC-conjugated Phalloidin (20 µg/ml, Invitrogen) for 40 min at room temperature. After final washing DAPI (Invitrogen) was used for staining the nuclei. Antifading reagent DABCO (Sigma) was used for sealing of the samples. Mounted samples were examined using inverted immunofluorescence microscope Nikon Eclipse Ti-S equipped with a 40×, 0.6 S Plan Fluor objective (Nikon, Duesseldorf, Germany) driven by NIS Elements software BR 3.2..

3.6. **Double immunofluorescence microscopy (DIF)**

Determination of adherence and invasion of streptococci by DIF was done as described by Benga *et al.* (2004) with the following modifications. Semiconfluent HEp-2 cells were incubated with streptococci at a moi of 100 bacteria per epithelial cell. ProLong® Gold antifade reagent with DAPI (Invitrogen) was used for sealing and staining of the nucleus. Mounted samples were examined using inverted immunofluorescence microscope Nikon Eclipse Ti-S. equipped with a 40×, 0.6 S Plan Fluor objective (Nikon, Duesseldorf, Germany) driven by NIS Elements software BR 3.2..

3.7. **Colocalisation experiments**

For suilysin localization experiments GFP-Rac1-expressing HEp-2 cells were fixed after incubation with rSLY (protein concentration of 100 ng/ml) for 30 min at 37°C with 3% PFA in modified cytoskeleton buffer (10 mM MES [pH 7.0], 150 mM NaCl, 5 mM EGTA, 5 mM MgCl₂, 5 mM sucrose) for 20 min and quenched with 10 mM glycine in PBS and permeabilised with 0.1% Triton X-100 in PBS. Permeabilised cells were blocked in PBS with 5% horse serum and 1% BSA (blocking buffer) for 30 min at room temperature and subsequently incubated with polyclonal antiserum raised against rSLY (Benga *et al.*, 2008) diluted 1:1,200 in blocking buffer for 45 min at room temperature. Samples were washed twice in PBS and incubated with Cy5-conjugated goat anti-rabbit IgG (Millipore, Schwalbach/Ts, Germany) for 30 min at room temperature. F-actin was labelled with Alexa® Fluor 568-conjugated Phalloidin (Invitrogen) for 20 min at room temperature. Coverslips were washed three times in
PBS and then mounted using ProLong® Gold antifade reagent with DAPI (Invitrogen). Mounted samples were examined using a LSM 510 Meta confocal microscope equipped with a 63×, 1.2NA Plan-NEOFLUAR oil immersion objective (Zeiss, Jena, Germany) driven by LSM software v3.2. For 3D z-stack acquisition the pinholes were set to one Airy unit and confocal planes were acquired every 340 nm. Images were deconvolved and maximum intensity projection were rendered using Huygens® Essential (Hilversum, The Netherlands).

3.8. **Field emission scanning electron microscopy (FESEM)**

Association of HEp-2 cell monolayers with either 10cpsΔEF or 10cpsΔEFΔsly at 100 bacteria per cell were investigated with FESEM as described previously (Benga *et al.*, 2004).

3.9. **Cell-permeability and macropore-formation assay**

Experiments were performed in PBS containing 0.9 mM CaCl$_2$/0.5 mM MgCl$_2$6H$_2$O [pH 7.3] (Gibco-Invitrogen). Cell permeability was determined after co-incubation of HEp-2 cells (4 x 10$^5$ cells/ml) with recombinant proteins (rSLY, rW461F, and rSVD) at subcytolytic (100 ng/ml) or lytic (400 ng/ml) concentrations and the fluorescent marker calcein (2 µg/ml). Incubation was performed for 2 h at 37°C on an end-over-end rotator. After two washing steps with PBS to remove extracellular calcein, cells were fixed with 0.37% formaldehyde (for 10 min at room temperature and afterwards resuspended in 500 µl PBS for measurement of fluorescent cells using FACScan®. Further analysis was performed with the software WinMDI. For each determination at least 10,000 events were measured. Initial analysis of fluorescent cells was carried out by dot plot analysis (forward scatter [fsc] versus sideward scatter [ssc]) to define the cell population of interest (data not shown). Subsequently, fluorescent cells were detected at channel FL-1. Data were expressed in comparison to cells only treated with calcein (background control).
3.10. Detection of $\alpha_5\beta_1$ integrin expression on HEp-2 cells

HEp-2 cell-suspension was incubated with either a monoclonal anti-human CD29 antibody or a monoclonal anti-human CD49e (Bio Legend) both used at a concentration of 500 ng per $1.5 \times 10^6$ cells (Bio Legend, Fell, Germany). All antibodies were diluted in blocking buffer (PBS [pH 7.3] containing 3% BSA) and incubation was performed for 30 min at room temperature with gently agitation. After three washing steps with blocking buffer, cells were incubated with a TRITC-conjugated goat anti-mouse antibody (1:1,000, Dianova, Hamburg, Germany). Stained cells were washed three times, fixed with 0.37% formaldehyde and resuspended in blocking buffer for subsequent measurement. Flow cytometry measurement was performed as described above. Fluorescent cells were counted at channel FL-1 and results were expressed in comparison to unstained cells (background control).

3.11. Haemolysis assay

The haemolytic activities of rSLY, rW461F or rSVD were determined by a haemolysis assay, performed essentially as described by Takamatsu et al. (2001). Titration of haemolytic activity was performed in a 96-well vee bottom microplate. Twofold dilutions of the toxins were incubated with a 2% sheep erythrocyte suspension in 0.9% NaCl for 2 h at 37°C mixed gently. Unlysed red blood cells were allowed to pellet by centrifugation and 100 µl of the supernatant was transferred into a new flat bottom microplate. Subsequently, absorption was measured at 550 nm in a microplate reader (GENios Pro, TECAN AUSTRIA GMBH). Determination of haemolytic activity was done in three independent experiments. One haemolytic unit (HU) was defined as the concentration of toxin causing 50% haemolysis of a 2% sheep blood suspension.

3.12. Cytotoxicity assays

Cytotoxicity was detected by an LDH-release assay as described previously (Benga et al., 2004). HEp-2 cells were either incubated with rSLY or rW461F. Supernatants were removed 2 h after treatment and their LDH activities were determined using the
Cytotox® 96 assay kit (Promega, Mannheim, Germany). The experiments were performed in triplicates and repeated at least three times. To quantify relative cellular damage, results were expressed relative to LDH release observed after Triton X-100 (Biorad, Muenchen, Germany) lysis of non-infected cells.

To determine haemolytic and cytotoxic activity of heterologous expressed SLY, W461F or SVD in L. lactis, respective lactococci (L. lactis pORI-SLY, -W461F and -SVD) were grown in GM17 medium overnight, adjusted to an optical density (OD$_{600}$) of 0.02 in pre-warmed media the next day and then grown to late exponential growth phase (OD$_{600}$ 0.8). Subsequently bacteria were removed by centrifugation and culture supernatants were concentrated and purified using Amicon centrifugal filter devices 30 kDa (Millipore, Schwalbach/Ts., Germany) to remove remaining medium components. Concentrated culture supernatants diluted 1:5 in 0.9% NaCl were used as test samples.

3.13. Pull down experiments

Experiments were done as described previously with some modifications (Reid et al., 1996). HEp-2 cells were incubated with rSLY or rW461F (100 ng/ml) for indicated time periods. Then cells were rinsed twice with ice cold PBS to stop incubation. Lysis of cells was done by addition of 1 ml of ice cold lysis buffer (50 mM NaCl, 20 mM Tris-HCl [pH 7.4], 3 mM MgCl$_2$, 1% Nonidet-P 40, 0.25% Triton X-100, 5 mM dithiothreitol, 100 µM PMSF). Cells were scraped off, and the lysates were centrifuged at 14,000 rpm. The supernatant was split and used for pull down experiments of activated RhoA and Rac1 in parallel. For this, 20 µl of beads slurry bearing approximately 20 µg protein of the GST-fusion protein of either the Rho-binding domain C21 or the PAK-GBD, respectively, were added to 500 µl sample and incubated on a rotator at 4°C for 30 min. Beads were collected by centrifugation at 10,000 rpm, washed twice with lysis buffer and subjected to immunoblot analysis using monoclonal antibodies against Rac-1 (clone 102; BD Pharmingen, Heidelberg, Germany) and against RhoA (clone 26C4; Santa Cruz, Heidelberg, Germany), respectively. Amounts of (activated and total) GTPases were quantified by densitometric measurement of three independent immunoblot analyses.
3.14. GLISA

HEp-2 cells were treated with rSLY, rW461F or rSVD at subcytolytic concentration (100 ng/ml) for 15 min and rinsed twice with ice cold PBS to stop incubation. Lysis of cells was achieved by addition of 1 ml of ice cold lysis buffer (50 mM NaCl, 20 mM Tris-HCl, pH 7.4, 3 mM MgCl$_2$, 1% Nonidet-P 40, 0.25% Triton X-100, 5 mM dithiothreitol, 100 µM PMSF). Cells were scraped off, and the lysates were centrifuged at 14,000 rpm. The supernatant was used for a pull down-based GLISA (Cytoskeleton, CO, USA) to determine activation of Rac1, RhoA, and Cdc42 according to the protocol by the supplier.

4. Mouse infection model

4.1. Preparation of infection culture

For infection of mice, streptococci were grown in Todd-Hewitt broth (THB, Difco, Detroit, USA) overnight at 37°C under aerobic conditions, adjusted to an optical density (OD$_{600}$) of 0.02 in pre-warmed media the next day and then grown to late exponential growth phase (OD$_{600}$ 0.8). Streptococci were harvested by centrifugation, resuspended in sterile PBS (pH 7.4) and adjusted to the final concentration of $5 \times 10^{11}$ CFU/ml for intranasal infection. Inoculum concentrations were verified by plating 10-fold serial dilutions on Colombia agar plates with 7% sheep blood after infection.

4.2. Intranasal infection of mice

Four (expt. 1 to 4) or six (expt. 5) week old specific-pathogen-free female mice of the outbreed strain Crl:CD1 (ICR) were obtained from Charles River Laboratories (Sulzfeld, Germany). Mice of the *Staphylococcus (St.) aureus* free outbred strain Hsd:ICR (CD1®) were purchased from Harlan Laboratories (AN Venray, The Netherlands). Animals were randomly divided into groups consisting of five to six animals each. Mice were allowed to acclimate for one week and cared for in accordance with the principles outlined in the European Convention for the Protection of Vertebrate Animal Used for Experimental and Other Scientific Purposes [European Treaty Series, no. 123; http://conventions.coe.int/treaty/EN/MenuPrincipal.htm; permit
Before infection mice were anesthetized via inhalation of isofluran (IsoFlo®, Albrecht, Germany). In experiment 2 to 5 mice were pre-treated with 12.5 µl 1% acetic acid [pH 4.0] placed in each nostril 1 h prior intranasal infection. After a controlled recovery phase and re-anaesthesia per isofluran inhalation, mice were infected with $5 \times 10^9$ CFU of either S. suis wild type strain 10, 10Δsly, 10cpsΔEF or 10cpsΔEFΔsly. The dose was applied in two drops of 12.5 µl volume placed in front of the nostrils.

### 4.3. Intravenous infection of mice

Five- to seven-week-old specific-pathogen-free female inbreed strain C57/BL6 mice and outbreed strain Crl:CD1 (ICR) mice were purchased from Charles River (Sulzfeld, Germany), randomly divided into groups (five to six mice each) and kept in conformity with the European conventions. After an adjustment period of one week, mice were infected via the lateral tail vein with ~6 x $10^5$ CFU, 1x$10^6$ CFU, 5 x $10^8$ CFU, and 1 x $10^9$ CFU per individual (total volume of 100 µl per ventral tail vein).

### 4.4. Clinical score

Animals were clinically scored every 8 h. The health status was rated using a clinical score sheet (Table 5-1), including weight development, clinical signs of general sickness (rough coat, rapid breathing, dehydration), clinical signs indicating meningitis (apathy, apraxia), and septicaemia (swollen eyes, depression), developed on the basis of mouse clinical monitoring score described by the Research office of the Australian National University [http://www.anu.edu.au/ro/ORI/Animal/AEEC001_MouseMonitoringSOP.doc]. A cumulative score of 3 to 4 indicated mild clinical symptoms, a score of 5 to 6 moderate clinical symptoms and a score greater than 6 severe clinical symptoms, in particular persistent anorexia, apathy, and/or neural disorder. Mice with a cumulative score equal or greater than 3 were classified as diseased (calculation of morbidity). In the case of severe weight loss (> 20%) and/or enduring severe clinical signs, mice were euthanized for reasons of animal welfare by inhalation of CO$_2$ and cervical dislocation.
4.5. Histological screening
Immediately after euthanasia, necropsy was conducted and the organs were aseptically removed and split for histological and bacteriological screenings, including spleen, liver, kidney, heart, lung, brain, spinal cord, and nose. The histological screenings were carried out as blind experiments. Findings were scored as described for piglets (Baums et al., 2006). In contrast to piglets, in addition to fibrinosuppurative lesions, purulent necrotizing lesions associated with the challenge strain were scored as well (see results). Rhinitis was not included in the general score designed to reflect lesions caused by invading streptococci.

4.6. Reisolation of S. suis strains from tissue and tracheo-nasal lavage (TNL)
One half of each organ was suspended in 5 ml PBS [pH 7.4] and weighed. All organs were homogenized with an Ultra Turrax (IKA, Staufen, Germany). Ten-fold serial dilutions of samples were plated on blood agar plates. Colony forming units (CFU) were counted the next day after incubation at 37°C for 24 h and CFU per mg organ was determined.

For sampling TNL the trachea was opened and a retrograde irrigation of the nasal cavity with 300 µl PBS was collected. Number of typical α-haemolytic streptococci per µl TNL was determined by serial plating on blood agar. Isolated α-haemolytic streptococci were investigated in a S. suis multiplex PCR for the detection of mrp, epf, sly, arcA, gdh, cps1, cps2, cps7, and cps9 (Silva et al., 2006). Isolates received from mice challenged either with strain 10Δsly, 10cpsΔEF or 10cpsΔsly were additionally tested in a cps2E-specific PCR (with oligonucleotide primers cps2Efor (TTTCGCACTTTTCAAGACGTG) and cps2Erev (GGACGGGTACCGACTAGACTC) and sly-specific PCR using primers slyAgefor (TGTACCGGTGATTCCAAACAAGATATTAA) and slyAge3new (TTACCGTTACTCTATCCTCATCCG). Based on CFU per mg organ or per µl TNL, bacterial loads were classified as mild (+; <100), moderate (++; ≥100 - <1000) or severe (+++; >1000).
5. **Statistical analyses**

If not stated otherwise, experiments were performed at least three times and results were expressed as means and standard deviations. Data were analyzed by \( t \)-test. *Pull down* analysis was carried out using an unpaired \( t \)-test with no assumption of Gaussian distribution (Mann-Whitney). A \( P \)-value <0.05 was considered significant.
References


Chapter 3

Results, part I:

Subcytolytic suilysin promotes invasion of *Streptococcus suis* in HEp-2 epithelial cells by Rac1-dependent activation of the actin cytoskeleton


Running title: Subcytoytic suilysin promotes invasion of *S. suis*

(Manuscript submitted)
Abstract

Suilysin is a cholesterol-dependent pore-forming cytolysin secreted by *Streptococcus suis*, an important swine and zoonotic pathogen. The role of suilysin in *S. suis*-host cell interaction is still unclear. In the present study we found a higher invasion rate in epithelial cells of an unencapsulated *sly*-positive strain compared to its *sly*-negative mutant. Electron microscopy revealed that uptake-accompanying membrane ruffling was abolished in the *sly*-negative mutant. Since invasion of *S. suis* depended on actin polymerization, we used specific inhibitors of Rho-GTPases and could show that Rac, but not Rho was involved in suilysin-mediated uptake. Accordingly, *pull down* analysis with specific ligands demonstrated activation of Rac, but not Rho in suilysin-treated cells. Furthermore, confocal microscopy revealed colocalisation of suilysin with F-actin and Rac1. To further dissect the relevance of cytolytic pore-formation we produced recombinant suilysin in which the protein domain responsible for membrane binding and pore-formation was inactivated via substitution of a tryptophan by phenylalanine. The mutated suilysin had lost haemolytic and cytolytic activity but retained its ability to mediate *S. suis* invasion and activation of Rac. Concluding, our results indicate that subcytolytic suilysin promotes *S. suis* invasion in epithelial cells by Rac-dependent activation of the actin cytoskeleton.
Introduction

*Streptococcus (S.) suis* is one of the most important swine pathogens worldwide causing meningitis, arthritis, septicemia, bronchopneumonia, and other pathologies. Furthermore, *S. suis* colonizes the nasopharynx and other mucosal surfaces, resulting in high carrier rates of healthy pigs (Arends *et al.*, 1984; O'Sullivan *et al.*, 2011). *S. suis* is also an important zoonotic agent (Trottier *et al.*, 1991; Gottschalk *et al.*, 2007). Meningitis and septicemia are the most common manifestations in humans, occurring particularly in people exposed to pigs or pig-products (Arends and Zanen, 1988). Two human outbreaks in China in 1998 and 2005 were associated with increased severeness of clinical symptoms, a high rate of mortality, and *streptococcal toxic shock-like syndrome* (Tang *et al.*, 2006). Among the 33 serotypes of *S. suis*, serotype 2 is worldwide most frequently isolated from diseased pigs (Wisselink *et al.*, 2000) and humans (Gottschalk *et al.*, 2010). Mechanisms underlying pathogenesis of *S. suis* infections, however, are only poorly known.

Suilysin was identified as haemolysin of *S. suis* several years ago (Jacobs *et al.*, 1994). It is a member of the cholesterol-dependent pore-forming cytolysins (CDC) family and expressed by many *S. suis* strains. The *sly* gene has been detected in 95% of European and Asian invasive serotype 2 strains (Segers *et al.*, 1998), and it was found in 69.4% of isolates from 10 different serotypes. Though these isolates were mainly obtained from porcine cases of meningitis and septicemia (King *et al.*, 2001), experimental infections demonstrated attenuation of a *sly* knock-out mutant only in mice, but not in piglets (Allen *et al.*, 2001; Lun *et al.*, 2003).

Similar to other members of the CDC family suilysin can damage host cells by its cytolytic activity (Norton *et al.*, 1999; Charland *et al.*, 2000; Lalonde *et al.*, 2000; Segura and Gottschalk, 2002; Vanier *et al.*, 2004; Tenenbaum *et al.*, 2005; Tenenbaum *et al.*, 2006). It has also been suggested that suilysin plays a role in invasion and pathogenesis of *S. suis* (Norton *et al.*, 1999). More recently, it has been found that the toxin may be involved in cytokine release and protection against opsonophagocytosis (Benga *et al.*, 2008; Lecours *et al.*, 2011). Some of the other CDC have been shown to display biological effects at sublytic concentrations, e. g.
phosphorylation of p38 mitogen-activated protein kinase (MAPK) in epithelial cells, which is crucial for local production of IL-8 and subsequent recruitment of neutrophils to the site of infection (Ratner et al., 2006). For the CDC of *S. pneumoniae* (pneumolysin), *Listeria monocytogenes* (listeriolysin O), and *S. intermedius* (intermedilysin) it was suggested that they contribute to bacterial adherence and invasion (Rubins et al., 1998; Sukeno et al., 2005; Krawczyk-Balska and Bielecki, 2005). These findings indicate that CDC express subcytolytic activities that may modify host cell responses to infection. For suilysin, however, such activities and their possible biological relevance still await to be elucidated in detail.

The objective of this study was to evaluate the possible role of suilysin in host cell interaction of *S. suis*, in particular its impact on association of *S. suis* with respiratory epithelial cells, i.e. adherence and invasion. Using respective mutant strains and HEp-2 epithelial cells we identified a suilysin-dependent invasive phenotype of an unencapsulated *S. suis* serotype 2 strain and determined host cell GTPases involved in suilysin-mediated effects. Furthermore, we showed that these effects did not require formation of a cytolytic pore.
Results

Suilysin promotes adherence and invasion of *S. suis*. The present study was undertaken to investigate the possible role of the cytolytic toxin suilysin, since its role in virulence of *S. suis* is poorly understood. For this, we constructed a suilysin-negative mutant of the unencapsulated strain 10cpsΔEF (kindly provided by H. E. Smith, Lelystad, NL). We used this strain because it originated from a highly virulent serotype 2 strain and displays a much better epithelial cell adherence and invasion than encapsulated strains, as observed in our previous studies (Benga *et al.*, 2004). We compared the *sly*-positive strain with its *sly*-negative mutant (10cpsΔEFΔsly) in adherence and invasion using the respiratory epithelial cell line HEp-2. Under our experimental conditions we could exclude cytotoxic effects of suilysin, as confirmed by viability staining of HEp-2 cells with propidium iodide (data not shown). First we determined bacterial association with HEp-2 cells by FACS of CFSE-labelled streptococci, which had been incubated with epithelial cells for 2 h. The unencapsulated mutant showed a high association with HEp-2 cells (mean fluorescence intensity [mfi] of 10.4 as compared to 3.1 of uninfected cells). The *sly*-negative mutant of this strain demonstrated significantly lower association (mfi of 7.2, *P* = 0.0092; Figure 3-1A). Secondly, we performed antibiotic protection assays to differentiate adherence and invasion of both *S. suis* strains. Results revealed that the *sly*-negative strain adhered to a much lower degree and showed a significantly reduced invasion as compared to the *sly*-positive strain (Figure 3-1B and C). Notably, differences in adherence were not significant due to high standard deviations, but were significant with respect to invasion. Thus, we assume that differences seen in FACS analyses (Figure 3-1A) reflect differences in invasion capacity rather than in adherence. Detection of suilysin by immunoblot analysis confirmed that suilysin was expressed under these conditions only by strain 10cpsΔEF, but not by its *sly*-negative mutant (Figure 3-1C, inlay). Thirdly, we compared both strains by double immunofluorescent microscopy (DIF) for differentiation of extra- and intracellular streptococci. Results confirmed that the *sly*-positive strain adhered and invaded HEp-2 cells much better than the *sly*-negative mutant, which could be detected only
extracellularly (Figure 3-1D and E). Quantification of microscopic examinations by counting of intracellular bacteria per HEP-2 cell confirmed that these differences were significant ($P = 0.042$; Figure 3-1F). Finally, we analysed adherence and invasion process of both strains by field emission scanning electron microscopy (FESEM). Similar to what we have found in previous studies (Benga et al., 2004), uptake of unencapsulated sly-positive S. suis was accompanied by formation of membrane ruffles in close vicinity to the streptococci (Figure 3-1G and H). Interestingly, this was observed only for the sly-positive strain, but not for the sly-negative strain, which showed adherence to cells but no association with ruffles or other cell morphologies reminiscent of invasion (Figure 3-1I). These findings suggest that suilysin contributes to S. suis induced membrane ruffling and uptake by epithelial cells.
Figure 3-1: Suilysin promotes invasion of *S. suis* in HEp-2 epithelial cells.
Comparison of HEp-2 cell association, adherence and invasion of *S. suis* strain 10Δcps and its sly-negative mutant 10ΔcpsΔsly by microscopic analyses. (A) FACS cytometry analysis of HEp-2-cell association of *S. suis* strains. Cells were incubated with CFSE-labelled bacteria for 2 h and then fluorescence intensity of epithelial cells was measured by FACS. Results are expressed as mean fluorescent intensity (mfi) values. Uninfected cells served as background control. Mean and SD of eight independent experiments are shown. Significance is indicated by ** (*P*-value < 0.01). (B, C). Determination of adherence (B) and invasion (C) of *S. suis* strains by antibiotic protection assay after their incubation with HEp-2 cells for 2 h. Results are expressed as percentage of CFU recovered as compared to CFU used for infection. Mean and SD of three independent experiments are shown. Inlay in C shows immunoblot analysis for detection of suilysin expression in the supernatant of infected cells. The respective Coomassie stained SDS gel shown below indicates an unrelated protein used for loading control. Significance is indicated by * (*P*-value < 0.05).
Determination of extra- and intracellular bacteria by double immunofluorescence (DIF) microscopy after incubation of S. suis strains with HEp-2 cells. In (D) cells were infected with strain 10Δcps, in (E) strain 10ΔcpsΔsly was used for infection. Extracellular bacteria are shown in orange-red and intracellular bacteria are stained in green. The nucleus was labelled by DAPI (blue). Bars represent 15 µm. (F) shows results of quantification of intracellular streptococci per cell by counting green (intracellular) bacteria in 50 epithelial cells (F). Mean and SD of two independent experiments are shown. Significance is indicated by * (P-value < 0.05). (G-I). Analysis of association of S. suis strains with HEp-2 cells by field emission scanning electron microscopy (FESEM). In (G) and (H) strain 10Δcps is shown to be associated with the formation of membrane ruffles. Strain 10ΔcpsΔsly adhered to the cell surface, but did not induce membrane ruffling or uptake by HEp-2 cells (I). Bars represent 1 µm.

Invasion-promoting activity of suilysin involves Rac-dependent activation of the actin cytoskeleton. Based on our results described above we hypothesized that suilysin promotes invasion of S. suis by manipulating the host cell cytoskeleton. Since actin is the major component involved in formation of lamellipodia and membrane ruffles, we first performed experiments with latrunculin B, a specific inhibitor of actin polymerisation (Coue et al., 1987; Greenwood et al., 2006). As shown in Figure 3-2A, FACS analysis revealed that bacteria-cell association of the sly-positive strain was significantly reduced in the presence of latrunculin B as compared to control cells without inhibitor. Reduced cell-association reached a level which was almost similar to that of the sly-negative mutant (Figure 3-2A).

Next we studied the role of the small GTPases Rho, Rac, and Cdc42. These belong to the family of Rho-GTPases which are molecular switches signalling to the actin cytoskeleton, thereby translating environmental signals into cellular morphological responses (Hall, 1998). We performed bacterial invasion experiments with HEp-2 cells, which were pretreated with different inhibitors of Rho-GTPases. First we used toxin B of Clostridium (C.) difficile (TcdB), which inhibits all Rho-GTPases by monoglycosylation (Just et al., 1995). The treatment of cells with TcdB resulted in a significant reduction of invasion of strain 10cpsΔEF as determined by gentamicin protection assays (Figure 3-2B). In contrast, the invasion of the isogenic sly-negative strain 10cpsΔEFΔsly was not affected. Next we tested invasion of HEp-2 cells pretreated with C3 exoenzyme, an ADP-ribosyltransferase of C. limosum, which specifically inactivates Rho-, but not Rac- and Cdc42-GTPase. This treatment had no effect on invasion of either of the two S. suis strains (Figure 3-2C). Thirdly, we used a specific inhibitor of Rac1-GTPase, TcdB toxin of C. difficile serotype F strain 1470 (TcdB-F). TcdB-F pretreatment of epithelial cells resulted in a significant inhibition of
invasion of the sly-positive S. suis strain, which was as low as that of the isogenic sly-mutant (Figure 3-2D). Taken together, these findings suggest that the actin cytoskeleton and Rac-GTPases are involved in suilysin-mediated invasion of S. suis.

Figure 3-2: Suilysin-mediated invasion of S. suis in epithelial cells involves the actin cytoskeleton and Rho-GTPases.
Effects of specific inhibitors of the actin cytoskeleton and small Rho-GTPases on HEp-2 cell association and invasion of S. suis strain 10cps and its sly-negative mutant 10ΔcpsΔsly. (A) FACS analysis of association of S. suis strains 10Δcps and 10ΔcpsΔsly with HEp-2 cells that were either incubated in the presence of latrunculin A (Lat A, 1 µg/ml) or left untreated. Results are expressed as means and SD of three independent experiments. Significance is indicated by * (P-value < 0.05). (C-D). Determination of invasion of S. suis strains by antibiotic protection assay after their incubation with HEp-2 cells that were pre-treated either with 10 ng/ml TcdB (B), 1 µg/ml C3 (C) or 10 ng/ml TcdB-F (D). Untreated cells served as controls (shown in black or white bars, respectively). Results are expressed as means and SD of three independent experiments. Significance is indicated by * (P-value < 0.05) or ** (P-value < 0.01).

To further prove that suilysin can lead to activation of Rac-GTPase, we performed pull down assays with specific ligands of activated Rac1 and RhoA. HEp-2 cells were treated with recombinant suilysin (rSLY) at subcytolytic concentration (100 ng/ml),
lysed, and supernatants were precipitated with specific ligands coupled to agarose beads. Amounts of bound GTPases were then detected by immunoblot analysis and densitometric quantification of detected GTPase. Four independent analyses were performed and results expressed as the relative quotient of active and total GTPase. Results revealed a time-dependent increase of the amount of activated Rac1, whereas level of activated RhoA remained unchanged (Figure 3-3A and B). The relative amount of active Rac1 increased significantly (1.4 fold) reaching a maximum after 15 min of HEp-2 cell treatment with rSLY, whereas activated RhoA remained unchanged, even at longer times of rSLY treatment (data not shown).

![Figure 3-3: Treatment of HEp-2 cells with recombinant suilysin (rSLY) leads to activation of Rac1, but not RhoA.](image)

HEp-2 cells were treated with rSLY (100 ng/ml) for indicated time periods and were then analysed by pull down assay using specific ligands for activated Rac1 and RhoA, respectively, as described in Experimental procedures. A representative immunoblot is shown in (A). Amounts of (activated and total) GTPases were quantified by immunoblot analysis and densitometry (B). Results are expressed as ratio of activated compared to total GTPase. Mean and SD of three independent experiments are shown. Significance is indicated by * ($P$-value < 0.05).
Assuming that actin and Rac seemed to play crucial roles in suilysin-mediated effects we then performed colocalisation studies of actin, Rac1 and suilysin by confocal immunofluorescence microscopy. For this, we used transiently Rac1-eGFP expressing HEp-2 cells and treated them with rSLY at subcytolytic concentration (100 ng/ml). Results revealed that rSLY bound to the cell plasma membrane, as indicated by blue spots shown in Figure 3-4. The spots varied in size suggesting different number of suilysin-molecules and stages of oligomerisation. Bound rSLY appeared to be scattered over the entire surface of the HEp-2 cell and was frequently located in close vicinity to or colocalised with F-actin (stained in red) and Rac1 (stained in green) (Figure 3-4).

**Figure 3-4:** Recombinant suilysin (rSLY) binds to HEp-2 cell membrane and is located in association with F-actin and Rac1. Confocal laser scanning micrograph of GFP-Rac1-expressing HEp-2 cells which were treated with rSLY for 30 min and then processed for confocal microscopy as described in Experimental procedures. F-actin is stained in red, Rac1 in green, and rSLY in blue. The optical slice of the region indicated by two vertical white lines in (A) is shown in (B). The right part shows an enlargement of (B). Arrows indicate colocalisation of rSLY, F-actin and Rac1. Bar represents 15 µm.

**Suilysin-mediated promotion of bacteria-cell association and activation of Rac does not require formation of a cytolytic pore.** The suilysin-mediated effects observed in our study occurred at subcytolytic conditions, and they seemed to be associated with binding and oligomerisation of suilysin, as suggested by colocalisation experiments (see Figure 3-4). Therefore, we were interested to find out
whether formation of a cytolytic pore was required for suilysin-mediated bacteria-cell association and Rac-activation. In some CDC it has been shown by structural or functional analyses that the tryptophan (Trp)-rich motif at the tip of the C-terminal of domain 4 is crucial for cytolytic activity (Billington et al., 2000). Hence, we introduced a point mutation in the sly gene by site-directed mutagenesis, resulting in a single amino acid substitution of a conserved Trp residue at position 461 (W was replaced by F). An alignment of the homologous Trp-rich motif of suilysin, the mutated suilysin derivative W461F, and pneumolysin (PLY) is shown in Figure 3-5A. We first compared haemolytic and cytolytic activities of rSLY and rW461F. Determination of haemolytic activities showed that the point-mutated toxin rW461F almost completely lost its capacity to lyse sheep erythrocytes. Compared to rSLY, of which 256 (2^8) ng/ml were sufficient for 50 % haemolysis, rW461F had to be applied at 4096 (2^{12}) ng/ml to cause 50 % haemolysis (Figure 3-5B). Calculation of haemolytic units (HU) revealed 0.53 x 10^5 HU per mg of rSLY as compared to 0.25 x 10^4 HU/mg for rW461F. In good agreement, cytotoxic activities determined by LDH release assay using HEp-2 cells were abolished in rW461F, reaching only 2.1% of that of rSLY. Figure 3-5C shows the respective dose-response curve and estimation of concentrations needed for 50% cytotoxicity. Based on these findings, for the following experiments we used rSLY and rW461F at 100 ng/ml, which was clearly below the (rSLY) protein concentrations causing 50% cytotoxicity. These data indicate that substitution of one amino acid in the conserved Trp-rich motif of domain 4 completely inactivates haemolytic and cytolytic activity of suilysin. To our knowledge, this is the first experimental proof that the Trp-rich motif of suilysin is crucial for formation of a functional (i.e. cytolytic) pore, which corresponds well to the high similarities of this region within the CDC family (as shown for pneumolysin in Figure 3-5A) as well as to predictions based on the crystal structure (Xu et al., 2010).
Figure 3-5: Substitution of an amino acid in the Trp-rich undecapeptide of domain 4 of recombinant suilysin leads to abolishment of its haemolytic and cytolytic activity.

(A) Primary sequence alignment of suilysin (SLY), suilysin with a point mutation resulting in replacement of a tryptophan by phenylalanine at position 461 (W461F) and pneumolysin (PLY). The highly conserved N-terminal undecapeptide of domain 4 within the CDC family is highlighted in grey.

(B) Determination of haemolytic activity of rSLY and rW461F proteins by standard haemolysis assay using sheep erythrocytes. Proteins were added to erythrocytes in microtiter plates at indicated concentrations and haemolysis was determined by measuring the absorbance of cell-free supernatants at 550 nm. Results are expressed as % haemolysis compared to H2O-lysed erythrocytes. The horizontal line indicates 50% haemolysis. The inlay shows representative effects of rSLY (left, haemolysis) and rW461F (right, no haemolysis).

(C) Determination of cytotoxic activity of rSLY and rW461F proteins by standard LDH release assay using HEp-2 cells. Proteins were added to cells in microtiter plates at indicated concentrations and LDH release as an indicator of cytotoxicity was determined in the supernatants as described in Experimental procedures. Results are expressed as % cytotoxicity compared to Triton X-100-lysed cells. The horizontal line indicates 50% cytotoxicity.
Next we tested whether addition of both recombinant proteins to the sly-negative S. suis mutant could reconstitute the phenotype of the sly-positive parental strain. For this, we determined HEp-2 cell-association of S. suis strains by FACS in the presence and absence of both recombinant proteins. Results revealed that addition of rSLY and rW461F both resulted in regain of function of the sly-negative strain, i.e. the mutant reached cell-association levels comparable to that of the sly-positive strain when proteins were present (Figure 3-6A). Immunoblot analysis for detection of recombinant suilysin protein in the supernatants of infected cells showed that rSLY and rW461F were present in equal amounts and were comparable to suilysin expressed by the sly-positive strain (Figure 3-6A, lower part). Finally, we tested whether rW461F was still able to activate host cell GTPase Rac. For this, we performed pull down experiments as described above. Results clearly showed that rW461F treatment of HEp-2 cells led to an increase of the amount of activated Rac1 comparable to that induced by rSLY (Figure 3-6B).

Concluding, our results revealed that suilysin can promote invasion of S. suis in epithelial cells by Rac-dependent activation of the actin cytoskeleton. Furthermore, we showed that these effects occur at subcytolytic concentrations and do not require formation of a functional (cytolytic) pore. Whether this can be considered as a common mechanism of CDC producing streptococci remains to be an interesting question for future studies.
Figure 3-6: Substitution of an amino acid in the Trp-rich undecapeptide of domain 4 of recombinant suilysin does not affect its ability to promote HEp-2 cell association of \textit{S. suis} or to activate Rac1.

(A) FACS determination of HEp-2 cell association of \textit{S. suis} strains in the presence of rSLY or rW461F. Cells were incubated with CFSE-labelled bacteria for 2 h and then fluorescence intensity of epithelial cells was measured by FACS. Results are expressed as mean fluorescent intensity (mfi) values. Uninfected cells served as background control. Mean and SD of three independent experiments are shown. Significance is indicated by * ($P$-value $< 0.05$) or ** ($P$-value $< 0.01$).

Detection of suilysin in the respective supernatants by immunoblot analysis is shown underneath the graph. The respective Coomassie stained SDS gel shown below indicates an unrelated protein used for loading control. (B, C) Pull down analysis of Rac1-activation in HEp-2 cells treated with rSLY or rW461F for 15 min. (B) Representative immunoblot, (C) quantification of amounts of activated Rac1 as described in Figure 3-3.
Discussion

Suilysin has been identified as a secreted toxin produced by many virulent *S. suis* strains (Jacobs *et al.*, 1995; Segers *et al.*, 1998). It belongs to the pore-forming cholesterol-dependent cytolysin (CDC) family, as recently confirmed by determination of its crystal structure (Xu *et al.*, 2010). *In vitro* and *in vivo* experiments suggest that suilysin, though it is not essential for virulence of *S. suis*, most likely contributes to pathogenesis by modification of host-pathogen interactions (Norton *et al.*, 1999; Allen *et al.*, 2001; Vadeboncoeur *et al.*, 2003; Lun *et al.*, 2003; Benga *et al.*, 2008). The current concept of the biological role of suilysin is a matter of ongoing discussions. A possible role of suilysin in host cell invasion of *S. suis* has been suggested by Norton *et al.* (1999). Furthermore, we and others have observed that suilysin may be involved in protection against opsonophagocytosis and cytokine release by host cells (Benga *et al.*, 2008; Lecours *et al.*, 2011). However, the molecular mechanisms of these effects are poorly understood. Considering that suilysin may *in vivo* often not reach concentrations sufficient for lysis of host cell membranes, it is plausible to speculate that the toxin expresses biological activities below cytotoxicity. Subcytolytic effects of suilysin have not been investigated in detail, but for some other CDC such activities have been shown to modulate host cell responses (Tsuchiya *et al.*, 2005; Krawczyk-Balska and Bielecki, 2005; Ratner *et al.*, 2006; Iliev *et al.*, 2009).

This prompted us to analyse effects of suilysin on epithelial cell-interactions of *S. suis* at subcytolytic conditions. Based on preliminary studies to establish such conditions, we then used 100 ng/ml (recombinant) suilysin in all experiments, which are clearly below cytolytic effects (see also Figure 3-5). Accordingly, in studies on other CDC nanomolar concentrations were also found to be far beyond that needed for cytolytic pore-formation (Ratner *et al.*, 2006).

An unencapsulated *S. suis* serotype 2 strain was used since in our previous studies unencapsulated *S. suis* adhered to and invaded epithelial cells much better than encapsulated strains (Benga *et al.*, 2004). One might argue that an unencapsulated strain does not represent a virulent phenotype. However, capsule expression is highly regulated, most likely in response to environmental signals such as glucose.
availability (Willenborg et al., 2011). Thus, by using this strain we mimic an *in vivo* situation of low capsule expression which is relevant mainly during adherence and invasion of host cells. Our comparison of this strain with its *sly*-negative mutant revealed that inactivation of the *sly* gene resulted in a significant reduction of bacteria-cell association, as determined by FACS analysis, and of invasion, as determined by gentamicin-protection assay and microscopy. Furthermore, we observed that cholesterol-depletion of epithelial cells significantly reduced uptake of streptococci (data not shown). These results suggest that suilysin contributes to invasion of *S. suis*.

Our findings in this and a previous study (Benga et al., 2004) revealed that *S. suis* invasion of epithelial cells was accompanied by formation of membrane ruffles. A major component of ruffles is actin. Thus, we hypothesized that the invasion-mediating effects of suilysin may be due to activation of the actin host cell cytoskeleton after binding of the (secreted) toxin to the host cell membrane. Involvement of the actin cytoskeleton in invasion process could be shown using latrunculin B as a specific inhibitor. It is known to complex G-actin and prevents actin polymerisation (Couë et al., 1987), and, in our study, caused a significantly reduced HEp-2 cell association of *S. suis*. Membrane ruffling and involvement of actin is also seen in the trigger-like uptake mechanism described for other pathogenic bacteria, e. g. *Salmonella* (Finlay and Cossart, 1997). Noteworthy, our results suggest that in *S. suis* a toxin can promote membrane ruffling and uptake of bacteria.

It has been shown for other pathogens that activation of GTPases is involved in bacterial invasion of host cells (Finlay, 2005). The underlying mechanisms seem to be rather complex and differ between pathogenic bacteria. For instance, Rho-GTPases Rac, Rho and Cdc42 are necessary for invasion of group A streptococci (Burnham et al., 2007). In contrast, invasion of *Salmonella* Typhimurium requires only Rac1 and Cdc42 (Criss and Casanova, 2003). Hence, to analyse the molecular basis of suilysin-mediated effects on actin remodelling and streptococcal uptake in more depth, we focussed on involvement of small Rho-GTPases. These proteins are known to signal to the actin cytoskeleton, thereby regulating morphological responses of the cell to environmental signals (Mackay and Hall,
Using clostridial toxins as specific inhibitors we could show that the recruitment of Rac-GTPase, but not Rho is crucial for invasion-mediating effects of suilysin. This corresponds to the well-established role of Rac in polymerization of actin fibres involved in formation of lamellipodia and membrane ruffles (Ridley et al., 1992), whereas Rho is considered a regulator mainly of stress fibre formation. Accordingly, our colocalisation experiments revealed that suilysin bound to the epithelial cell membranes and was spatially associated with Rac1 and F-actin. This supports our assumption that suilysin can bind to epithelial cells and activate Rac1, which leads to activation of the actin cytoskeleton and formation of membrane ruffles. To prove that activated GTP-ases were involved we treated HEp-2 cells with suilysin and then detected activated Rac1 and RhoA by pull down analysis using specific ligands coupled to agarose beads. Results showed that Rac1 was activated within 15 min after addition of suilysin, whereas activation of RhoA seemed not to be affected. Involvement of Cdc42 in streptococcal internalisation is unlikely, because activation of Cdc42 triggers mainly the formation of filopodia and microspikes (Mackay and Hall, 1998). Accordingly, in preliminary experiments we found that Cdc42 is not significantly activated by suilysin (data not shown).

Among other members of CDC an interaction with G-proteins has been described for pneumolysin. Iliev et al. (2007) reported formation of stress fibres, filopodia, and lamellipodia as a consequence of RhoA and Rac1 recruitment after treatment of human neuroblastoma cells with pneumolysin. Strikingly, suilysin seems to activate only Rac1, but not RhoA. However, it has to be considered that we used respiratory epithelial cells, and that both host cells play different roles during streptococcal infection process.

The CDC share two basic functions, which is reflected by more than 40% sequence identity over their common domain regions 1-4 (Xu et al., 2010). First, CDC bind to host cell membrane via specific cholesterol-dependent interaction between the C-terminal region of domain 4 and the cell membrane. Second, after binding toxin monomers oligomerise and change conformation in the N-terminal region, which finally leads to formation of transmembrane pores and lysis of the target cell (Tweten, 2005). Structural-functional analyses revealed that domain 4 carries a highly
conserved tryptophan-rich motif consisting of 11 amino acids, which is crucial for cytolytic activity of CDC (Billington et al., 2000). An alignment of the homologous region of pneumolysin and suilysin is shown in Figure 3-5A. Point mutations within this undecapeptide of pneumolysin (Feldman et al., 1990), perfringolysin O of Clostridium perfringens (Polekhina et al., 2005) or listeriolysin O of Listeria monocytogenes (Michel et al., 1990) lead to an attenuated ability to cause membrane damage. It is known that such mutated toxins are still able to bind to the cell plasma membrane, but the formation of a functional permeable transmembrane pore is inhibited (Korchev et al., 1998). For suilysin, predictions based on its crystal structure suggest that it shares common features with other CDC and, concerning the undecapeptide, particularly with intermedilysin (Xu et al., 2010). However, these predictions have yet to be proven by functional experiments.

Interactions of pneumolysin with Rho-GTPases (see above) seem to occur independent of (macro) pore-formation (Iliev et al., 2007). This prompted us to further dissect the relevance of pore-formation in suilysin-mediated effects on epithelial cells. For this, we took advantage of the high similarities in primary amino acid sequence and three-dimensional secondary structure among members of CDC family (Segers et al., 1998b; Xu et al., 2010) as described above. Hence we produced recombinant suilysin in which the undecapeptide was inactivated by substitution of the first tryptophan (W461) by phenylalanine (see also alignment in Figure 3-5A). Haemolytic and cytolytic activity of the mutated recombinant suilysin (rW461F) were almost completely abolished in comparison with the suilysin (rSLY), indicating a loss in transmembrane pore-forming activity. This was further confirmed in experiments using fluorescent calcein to prove macropore-formation (data not shown). Remarkably, the mutated suilysin had retained its ability to promote epithelial cell invasion of S. suis and to activate Rac1 comparable to rSLY.

To our knowledge, this is the first experimental evidence for the function of this domain in suilysin-mediated cytolysis according to the predictions based on the crystal structure (Segers et al., 1998b; Xu et al., 2010). Furthermore, our results indicate that a functional undecapeptide motif in domain 4 is not required for CDC-mediated GTPase-activation and invasion of bacteria.
Concluding, this study revealed that suilysin can promote invasion of S. suis in epithelial cells by Rac-dependent activation of the actin cytoskeleton at subcytolytic conditions. Thus, we propose that subcytolytic activities of suilysin play a role in host-pathogen interactions by modulating different host cell functions, such as morphological responses, as shown here, or release of cytokines, as shown by others (Lun et al., 2003; Chen et al., 2007; Vanier et al., 2009; Zheng et al., 2011; Lecours et al., 2011). Further studies will show which domains of suilysin are involved in this process and which significance this might have for virulence and pathogenesis.
Experimental procedures

If not stated otherwise all materials were purchased from Sigma (Muenchen, Germany).

**Bacterial strains and growth conditions.** The unencapsulated isogenic mutant strain 10cpsΔEF was generated by insertional mutagenesis of the virulent *Streptococcus (S.) suis* serotype 2 strain 10 (both strains were kindly provided by H. Smith, Lelystad, NL) as described previously (Smith *et al.*, 1999). The corresponding suilysin deficient mutant of 10cpsΔEF, strain 10cpsΔEFΔsly, was constructed as described below. *Escherichia (E.) coli* strains BL21, BL21 (DE3) and DH5α were used for molecular cloning and protein expression experiments. Streptococci were grown on Columbia agar supplemented with 7% sheep blood (Oxoid, Wesel, Germany) overnight under aerobic conditions at 37°C. For infection of HEP-2 cells, streptococci were grown in Todd-Hewitt broth (THB, Difco, Detroit, USA) overnight at 37°C under aerobic conditions, adjusted to an optical density (OD<sub>600</sub>) of 0.02 in pre-warmed media the next day and grown to late exponential growth phase (OD<sub>600</sub> 0.8). *E. coli* strains were cultured on Luria Bertani (LB) agar overnight at 37°C under aerobic conditions. When necessary, antibiotics were added to the media at the following concentrations: spectinomycin 100 µg/ml for *S. suis*; erythromycin 1 µg/ml for *S. suis*; ampicillin 100 µg/ml for *E. coli*.

**DNA techniques.** Routine molecular biology techniques including restriction endonuclease digestion, DNA ligations, agarose gel electrophoresis, Southern Blot analysis, transformation of *E. coli* and plasmid isolation were performed according to standard procedures (Sambrook *et al.*, 1989). Restriction enzymes were purchased from New England Biolabs (Frankfurt am Main, Germany). Plasmid preparations were performed with kits from Machery-Nagel (Dueren, Germany).

**Construction of the suilysin deficient mutant strain 10cpsΔEFΔsly.** The mutant 10cpsΔEFΔsly was constructed by insertion of an erythromycin cassette in the *sly*
gene of the unencapsulated strain 10cpsΔEF using the plasmid pBlue/sly/erm as previously described (Benga et al., 2008). Mutants were controlled by PCR and Southern Blot analysis.

**Expression of recombinant wild type suilysin proteins.** Recombinant His-tagged suilysin (rSLY) and point-mutated suilysin W461F (rW461F) were expressed in BL21 pET45bslynew (Kock et al., 2009) and BL21 pET45bslynewW461F, respectively. The plasmid pET45bslynewW461F was constructed by site-directed-mutagenesis according to the instruction manual of QuikChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) with some modifications. Purified plasmid DNA carrying the sly gene (pET45bslynew) was amplified using oligonucleotide primers encoding the W461 to F461 substitution (slyTrp-PheF1: [TACAGGATTAGCAT TTGAGTGGTGGAGAAC], slyTrp-PheR1: [GTTCTCCACCACTCAAATGCTAATC CTGTAC]). The resulting plasmid pET45bslynewW461F was electroporated into *E. coli* BL21 (DE3), purified and controlled by sequencing. Expression and purification of rSLY and rW461F were performed as described previously (Willenborg et al., 2011). The quality of purified proteins was controlled by separation on SDS-polyacrylamide gels and immunoblot analysis using with anti-Penta His antibodies (Qiagen, Hilden, Germany).

**Immunoblot analysis.** Recombinant proteins or supernatants of infected cells were separated by SDS-polyacrylamide gel electrophoresis with a 4% stacking and a 10% separating gel under denaturing conditions and transferred to a PVDF-membrane (Serva, Heidelberg, Germany). Membrane-blocking was performed overnight with 1% milk powder in TBS with 0.5% Tween. For detection of His-tagged recombinant proteins membranes were blocked with 3% BSA and incubated with a monoclonal anti-Penta His antibody (Qiagen) diluted 1:2,000 in 3% BSA. Polyclonal antiserum raised against rSLY (Benga et al., 2008) diluted 1:1,300 in 1% milk powder was used to detect either rSLY, rW461F or secreted suilysin in supernatants of infected cells. Membranes were developed with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antiserum diluted 1:10,000 in 1% milk powder (Amersham, Freiburg,
Germany) followed by using SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, USA) according to the manufactures protocol.

**HEp-2 epithelial cell culture.** The human laryngeal epithelial cell line HEp-2 (ATCC CCL 23) was used as described previously (Benga et al., 2004). For adherence and invasion assays, approximately $1.8 \times 10^5$ cells per well were seeded on 24 well tissue culture plates. For immunofluorescence and electron microscopy $0.5 \times 10^5$ and $1.5 \times 10^5$ cells per well were seeded on 12 mm diameter glass cover slips placed in 24 well plates. For FACS analysis approximately $6 \times 10^5$ cells per well were seeded on a 6 well plate, and for cellular cytotoxicity assay $0.2 \times 10^5$ cells per well were seeded on a 96 well plate. The cells were grown overnight and then used for experiments. For colocalisation experiments HEp-2 cells were transfected with GFP-tagged wild type Rac1 (GFP-Rac1) using the Amaxa nucleofection system (Amaxa, Cologne, Germany) applying the standard protocol as specified by the manufacturer. Plasmids were purified with the EndoFree™ plasmid purification kit from Qiagen.

In some assays, HEp-2 cells were treated before infection with the following inhibitors: $1 \mu g/ml$ latrunculin B for 30 min, $10 ng/ml$ toxin B of *Clostridium* (*C.* difficile) (TcdB), $10 ng/ml$ toxin B of *C. difficile* serotype F strain 1470 (TcdB-F) and $1 \mu g/ml$ C3 toxin of *C. limosum* for 3 h. Toxins were purified as described previously (Huelsenbeck et al., 2007). C3 toxin was always reconstituted freshly by mixing the binding domain (C2IIa) with the catalytic domain (FT) in a ratio of 1:1 as described (Just et al., 1995). After preincubation with inhibitors cells were washed and used for further experiments as described below, except for latrunculin B, which was left on to the cells during the whole infection experiment because its effect is reversible up to 1 h.

**Determination of haemolytic activity and cytotoxicity.** Haemolytic activities of rSLY and rW461F were determined in 96-well vee bottom microplates as described by Takamatsu et al. (2001) with some modifications. Briefly, twofold dilutions of the toxins were incubated with a 2% sheep erythrocyte suspension in 0.9% NaCl for 2 h.
at 37°C. Unlysed red blood cells were allowed to pellet by centrifugation, and 100 µl of the supernatant was transferred into a new flat bottom microplate. Subsequently, absorption was measured at 550 nm in a microplate reader (GENios Pro, TECAN AUSTRIA GMBH). Determination of haemolytic activity was done in three independent experiments. One haemolytic unit (HU) was defined as the concentration of toxin causing 50% haemolysis of a 2% sheep blood suspension (Takamatsu et al., 2001).

Cytotoxicity was detected by LDH-release assay as described previously (Benga et al., 2004). Briefly, HEp-2 cells were either incubated with rSLY or rW461F at indicated concentrations. Supernatants were removed 2 h after treatment and LDH activities were determined using the Cytotox® 96 assay kit (Promega, Mannheim, Germany). All experiments were performed in triplicates and repeated at least three times. Results were expressed as % LDH-release compared that of Triton X-100 lysed non-infected cells.

**Antibiotic protection assay.** The number of adherent and invasive streptococci was quantified using a gentamicin protection assay as described earlier (Valentin-Weigand et al., 1996) with the following modifications. Adherence and invasion assays were performed with streptococci grown in THB to an OD$_{600}$ of 0.8. Subsequently, bacteria were resuspended in Dulbecco’s PBS containing 0.9 mM CaCl$_2$ and 0.5 mM MgCl$_2$6H$_2$O, pH 7.3 (PBS$^+$; Gibco-Invitrogen) and adjusted to an OD$_{600}$ 0.6. Then, streptococcal suspensions were diluted 1:10 in PBS$^+$ and used for adherence and invasion assay. Confluent HEp-2 cell monolayers were inoculated with 100 bacteria per cell. The number of colony forming units (CFU) inoculated per well was determined by serial platings. After incubation infected cells for 2 h the supernatants were collected for detection of suilysin expression by immunoblot analysis. To determine adherence cells were washed three times with PBS immediately after supernatant collection and lysed by 0.025% Triton X-100. Serial dilutions of these lysates were plated in triplicates on Columbia agar for determination of CFU. For invasion assay, after PBS washes cells were incubated for 2 h with 100 µg gentamicin and 5 µg penicillin per ml to kill extracellular bacteria.
Then lysates were prepared and plated as described for adherence. Results were expressed as % adherence/invasion compared to the inoculum used for infection.

**FACS analysis of bacteria-cell association.** For this, streptococcal strains were labelled with the carboxyfluorescein succinimidyl ester (CFSE) using CellTrace™ CFSE Cell Proliferation Kit (Invitrogen). Labelling was performed as described by (Logan et al., 1998) with some modifications. Briefly, *S. suis* strains were grown to an OD\textsubscript{600} of 0.8, pelleted by centrifugation, washed once with PBS\textsuperscript{+} and resuspended in PBS\textsuperscript{+} containing 5 µM CFSE-reagent (prepared from a CFSE stock solution as recommended by the manufacturer). Staining procedures were carried out at 37°C for 20 min on an end-over-end rotator. After removal of unbound fluorescent dye by washing labelling efficiency was confirmed to be > 90%.

HEp-2 cell monolayers were infected with CFSE-labelled streptococci at a multiplicity of infection [moi] of 125:1 bacteria per cell. For this, streptococci were centrifuged on the epithelial cells (250 x g for 5 min) and then incubated with the cells at 37°C for 2 h. Then supernatants were collected for determination of suilysin expression by immunoblot analysis. Epithelial cells were then detached with trypsin-EDTA, washed with PBS and fixed with 0.375% formaldehyde in PBS. In some experiments, rSLY or rW461F were added to epithelial cells together with streptococcal strains (100 ng/ml, final concentration). To exclude cytotoxic effects of suilysin during the experiment cells were stained afterwards with the DNA-intercalating dye propidium iodide (4 µg/ml) for 2 min at room temperature to discriminate between viable and nonviable cells. Bacteria-cell association was measured using FACScan® (Becton Dickinson, 488 nm Argon laser) and analysed using the software WinMDI (version 2.9.). For each determination at least 10,000 events were measured. Results were expressed as mean fluorescent intensity [mfi] values of the cell population. Uninfected cells served as background control. Means and standard deviations were determined from eight independent experiments.

**Fluorescence and electron microscopy.** Determination of adherence and invasion of streptococci by double immunofluorescence microscopy (DIF) was performed as
described previously (Benga et al., 2004) with the following modifications. Semiconfluent HEp-2 cells were incubated with streptococci at a moi of 100 bacteria per epithelial cell. ProLong® Gold antifade reagent with DAPI (Invitrogen) was used for sealing and staining of the nucleus. Mounted samples were examined using inverted immunofluorescence microscope Nikon Eclipse Ti-S equipped with a 40×, 0.6 S Plan Fluor objective (Nikon, Duesseldorf, Germany) driven by NIS Elements software BR 3.2.. Intracellular streptococci were quantified by counting green (intracellular) bacteria in at least 50 cells, and results were expressed as intracellular bacteria per epithelial cell.

For colocalisation experiments HEp-2 cells transiently expressing GFP-Rac1 were fixed after incubation with rSLY (100 ng/ml) for 30 min at 37°C with 3% paraformaldehyde in modified cytoskeleton buffer (10 mM MES [pH 7.0], 150 mM NaCl, 5 mM EGTA, 5 mM MgCl₂, 5 mM sucrose) for 20 min, quenched with 10 mM glycin in PBS and then permeabilised with 0.1% Triton X-100 in PBS. Permeabilised cells were blocked in PBS with 5% horse serum and 1% BSA (blocking buffer) for 30 min at room temperature and subsequently incubated with polyclonal antiserum raised against rSLY (Benga et al., 2008) diluted 1:1,200 in blocking buffer for 45 min at room temperature. Samples were washed twice with PBS and incubated with Cy5-conjugated goat anti-rabbit IgG (Millipore, Schwalbach/Ts, Germany) for 30 min at room temperature. F-actin was labelled with Alexa® Fluor 568-conjugated Phalloidin (Invitrogen) for 20 min at room temperature. Coverslips were washed three times in PBS and then mounted using ProLong® Gold antifade reagent with DAPI (Invitrogen). Mounted samples were examined using a LSM 510 Meta confocal microscope equipped with a 63×, 1.2NA Plan-NEOFLUAR oil immersion objective (Zeiss, Jena, Germany) driven by LSM software v3.2. For 3D z-stack acquisition the pinholes were set to one Airy unit and confocal planes were acquired every 340 nm. Images were deconvolved and maximum intensity projection were rendered using Huygens® Essential (Hilversum, The Netherlands).

Field emission scanning electron microscopy (FESEM) was performed as described previously (Benga et al., 2004) using HEp-2 cells infected as described for DIF.
Pull down experiments. Experiments were done as described previously with some modifications (Reid et al., 1996). HEp-2 cells were incubated with rSLY or rW461F (100 ng/ml) for indicated time periods. Then cells were rinsed twice with ice cold PBS to stop incubation. Lysis of cells was done by addition of 1 ml of ice cold lysis buffer (50 mM NaCl, 20 mM Tris-HCl [pH 7.4], 3 mM MgCl₂, 1% Nonidet-P 40, 0.25% Triton X-100, 5 mM dithiothreitol, 100 µM PMSF). Cells were scraped off, and the lysates were centrifuged at 14,000 rpm. The supernatant was split and used for pull down experiments of activated RhoA and Rac1 in parallel. For this, 20 µl of beads slurry bearing approximately 20 µg protein of the GST-fusion protein of either the Rho-binding domain C21 or the PAK-GBD, respectively, were added to 500 µl sample and incubated on a rotator at 4°C for 30 min. Beads were collected by centrifugation at 10,000 rpm, washed twice with lysis buffer and subjected to immunoblot analysis using monoclonal antibodies against Rac-1 (clone 102; BD Pharmingen, Heidelberg, Germany) and against RhoA (clone 26C4; Santa Cruz, Heidelberg, Germany), respectively. Amounts of (activated and total) GTPases were quantified by densitometric measurement of three independent immunoblot analyses.

Statistical analyses. If not stated otherwise, experiments were performed at least three times and results were expressed as means and standard deviations. Data were analyzed by t-test. Pull down analysis was carried out using an unpaired t-test with no assumption of Gaussian distribution (Mann-Whitney). A P-value <0.05 was considered significant.

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References


Chapter 4

Results, part II:

Identification of a RGD-motif in suilysin possibly involved in host-cell binding, Rac1-activation and macropore-formation

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Running title: RGD-motif in suilysin

(Manuscript in preparation)
Abstract

Suilysin is a cholesterol-dependent pore-forming cytolysin secreted by *Streptococcus suis*, an important causative agent of meningitis in swine and human. The role of suilysin in host cell interaction is still unclear. Here, we showed that suilysin contains structural elements involved in specific functions of the toxin. Using oligonucleotide directed mutagenesis we produced recombinant suilysin in which either the highly conserved tryptophan-rich undecapeptide of the fourth domain or the RGD-motif of domain one was inactivated. Measurement of haemolytic and cytotoxic activity revealed that both domains are required for full lytic function of suilysin. In contrast to the tryptophan-mutant, the RGD-mutant showed also a deficiency in membrane binding to HEp-2 cells. To further resolve if subsequent cellular signalling in host cells is affected by amino acid substitution, we used a pull down based GLISA to study the activation of small Rho-GTPases. As anticipated from our previous studies, unmodified suilysin and the tryptophan-mutant activated Rac1. In contrast, mutation of the RGD-motif abolished ability to induce G-proteins. Furthermore, studies with the fluorescent dye calcein revealed that both mutated derivates of suilysin had lost their ability to form lytic macropores in host cells. Thus, we suggest that the RGD-motif is required for membrane binding of suilysin as well as for an outside-in signalling mediated by the G-protein kinase Rac1 and the induction of lytic macropores in HEp-2 cells.
Introduction

*Streptococcus* (*S.*) *suis* is one of the most important swine pathogens worldwide causing high economical losses in pig husbandry due to invasive diseases associated with meningitis, arthritis, septicaemia, and bronchopneumonia. Furthermore, *S. suis* is a zoonotic bacterium causing meningitis and the life-threatening *streptococcal toxic shock like syndrome* in humans (Gottschalk *et al*., 2007; Gottschalk *et al*., 2010; Tang *et al*., 2006; Trottier *et al*., 1991).

Suilysin, the secreted haemolysin of *S. suis*, belongs to the family of cholesterol-dependent pore-forming cytolysins (CDC) (Jacobs *et al*., 1994). This large family of toxins generate complex channels in target cells, causing membrane damage of host cells and lysis of erythrocytes (Lalonde *et al*., 2000; Norton *et al*., 1999; Tenenbaum *et al*., 2006; Vanier *et al*., 2004). The primary and secondary structure of CDC is highly conserved. They share the same monomeric structure, comprised of four domains (Rossjohn *et al*., 1997; Xu *et al*., 2010). A mechanism of pore-formation has been proposed by Tweten (2005). Several monomers bind vertically to the cholesterol-containing membrane of target cells via the fourth domain, followed by an oligomerisation step resulting in formation of the prepore complex. The second domain builds a 'hinge-like' region allowing the insertion of the oligomer into the membrane, whereas the third domain mainly serves as a transmembrane domain, representing the internal structure of the permeable pore (Czajkowsky *et al*., 2004; Ramachandran *et al*., 2002; Shepard *et al*., 2000). The fourth domain contains the tryptophan-rich undecapeptide, which is highly conserved and known to be responsible for formation of a functional pore (Feldman *et al*., 1990; Korchev *et al*., 1998; Michel *et al*., 1990; Polekhina *et al*., 2005). We recently confirmed the predicted 'pore-forming' function of the undecapeptide for suilysin using a point-mutated derivate of suilysin W461F (Seitz *et al*., submitted; cf. chapter 3). Furthermore, we showed that suilysin promotes invasion of *S. suis* independent of the formation of a functional pore. However, the specific molecular mechanism underlying suilysin-induced activation of host cells is still unknown.
The objective of this study was to further characterize possible functional domains of suilysin to understand the molecular mechanism underlying suilysin-induced activation of host cells. We identified a RGD (Arg-Gly-Asp)-motif within the sequence of the first domain of the molecule. This is a special feature of suilysin, since none of the other well-characterized CDC posses this sequence. Bacterial proteins containing a RGD-sequence are known to mediate adherence and entry of pathogens into host cells (Scibelli et al., 2007), most likely by binding host cell surface proteins recognizing RGD-sequences, known as integrins. Using target mutagenesis we constructed recombinant suilysin in which the putative integrin-binding side (RGD-motif) is inactivated by substitution. This abolished cytolytic and haemolytic function of suilysin. Detailed analysis revealed that the RGD-motif is most likely required for membrane binding of suilysin, subsequent activation of Rac1 as well as for the formation of lytic macropores.
Results and Discussion

Inactivation of the RGD-motif leads to almost complete loss of hemolytic and cytotoxic activity of suilysin. The purpose of this study was to characterise a second putative functional region, a RGD-motif identified within the first domain of suilysin. Hence, we introduced a point-mutation in the sly gene by site-directed mutagenesis, resulting in amino substitution of RGD by SVD. Full length suilysin lacking the signal sequence (rSLY) and the point-mutated derivates (rW461F and rSVD) were overexpressed in E. coli and purified. Silver staining of respective recombinant proteins showed no difference in size and a high purity grade, as indicated by a single clear band at approximately 58 kDa (Figure 4-1). To exclude frameshifts, rSLY was sequenced in total. Moreover, both point-mutations (rW461F and rSVD) were confirmed by sequencing of the respective regions within the molecule. Alignment of amino acid sequences of suilysin (NCBI accession # 253753945), sequenced rSLY, in silico analyzed rW461F and rSVD and four other members of the CDC-family, including pneumolysin (PLY), perfringolysin O (PFO), intermedilysin (ILY), and listeriolysin O (LLO), is shown in Figure 4-1. Sequence alignment confirmed the high similarity of the undecapeptide of the fourth domain, termed tryptophan-rich motif (coloured in light green; blue similarity bar below sequences indicates 100% homology). The RGD-motif within domain one (coloured in light red) is shown to be only present within the suilysin molecule. The substitution of the undecapeptide at position W461 (rW461F, highlighted in white) and the RGD-sequence (rSVD, highlighted in white), respectively, was confirmed.
Figure 4-1: Alignment of amino acid sequences of different CDC.
Suilysin (NCBI accession # 253753945), sequenced rSLY, in silico analyzed rW461F and rSVD and four other members of the CDC family, including pneumolysin (PLY, # 294652455), perfringolysin O (PFO, # 144884), intermedilysin (ILY, # 6729344) and listeriolysin O (LLO, # 44112) are shown. Subunits of the suilysin molecule are highlighted, including domain 1 (red; RGD-motif in light red), domain 2 (blue), domain 3 (yellow) and domain 4 (green; tryptophan-rich undecapeptide in light green). The same colour code was used for the crystal structure of the suilysin molecule modified from (Xu et al., 2010a) (cf. chapter 1). Substitutions of the RGD-motif and W461 of the undecapeptide are marked in white. The signal-sequence of SLY is marked in orange. Similarity bars (light blue) below sequences indicate a similarity of 100% of all analyzed CDC. Alignment was performed with the program Clone Manager 9. The inlay shows a silver staining of recombinant suilysin rSLY, rW461F, and rSVD (~58kDa) separated by SDS-polyacrylamid gel electrophoresis.

We have previously shown that substitution of the tryptophan-rich undecapeptide resulted in loss of lytic function (Seitz et al., submitted; cf. Chapter 3). Therefore, haemolytic and cytotoxic activity tests of the mutated suilysin rSVD were carried out in comparison to rSLY and rW461F. Determination of haemolytic capacity showed that the point-mutated toxin rSVD almost completely lost its capacity to lyse sheep erythrocytes. Compared to rSLY, of which 256 (2^8) ng/ml were sufficient for 50% haemolysis, rSVD had to applied at 2048 (2^11) ng/ml and rW461F at 4096 (2^12) ng/ml to cause 50% haemolysis (Figure 4-2A). Calculation of haemolytic units revealed 0.53 x 10^5 HU/mg for rSLY as compared to 0.3 x 10^4 HU/mg for rSVD and 0.25 x 10^4 HU/mg for rW461F. In addition, membrane damage of HEp-2 cells by suilysin and its mutated derivates was determined using a LDH release test (Figure 4-2B). Testing the specific cytotoxic activity of rSVD revealed a loss of cytotoxic function (2.1% of the rSLY), similar to the rW461F (8.1% of rSLY).
Results, part II

Chapter 4

Figure 4-2: Substitution of the RGD-motif of recombinant suilysin leads to abolishment of its haemolytic and cytolytic activity.

(A) Determination of haemolytic activity of rSLY, rW461F and rSVD proteins by standard haemolysis assay using sheep erythrocytes. Proteins were added to erythrocytes in microtiter plates at indicated concentrations and haemolysis was determined by measuring the absorbance of cell-free supernatants at 550 nm. Results are expressed as % haemolysis compared to H2O-lysed erythrocytes. The horizontal line indicates 50% haemolysis. The inlay shows representative effects of rSLY (left, haemolysis), rW461F (middle, no haemolysis) and rSVD (right, no haemolysis). (B) Determination of cytotoxic activity of rSLY, rW461F and rSVD proteins by standard LDH release assay using HEp-2 cells. Proteins were added to cells in microtiter plates at indicated concentrations and LDH release as an indicator of cytotoxicity was determined in the supernatants as described in Experimental procedures. Results are expressed as % cytotoxicity compared to Triton X-100-lysed cells. The horizontal line indicates 50% cytotoxicity.

In order to analyze whether the different suilysin derivates conferred a respective phenotype a heterologous expression system was used. Suilysin and its mutated derivates, all containing the signal sequence, were transformed into the gram-positive, non-pathogenic bacterium *Lactococcus (L.) lactis*. Protein processing and expression in *L. lactis* is very similar to *S. suis*. Due to the signal sequence, overexpressed proteins are secreted, thus present in the culture supernatant of the
respective lactococci. To verify suilysin expression and to determine haemolytic and cytotoxic activity, culture supernatants of *L. lactis* pORI23 WT, pORI23-SLY, pORI23-W461F, and pORI23-SVD obtained from late logarithmic growth phase were tested in immunoblot analysis and cytotoxic assays. By immunoblot analysis using a polyclonal antiserum raised against rSLY, we could clearly show that SLY, W461F, and SVD were present in the supernatant of respective lactococci, except for *L. lactis* pORI23 WT carrying the control plasmid (Figure 4-3A, inlay). In accordance to our findings observed using recombinant proteins (rSLY, W461F, and rSVD), only *L. lactis* pORI23-SLY was able to cause lysis of sheep red blood cells (53.3% haemolysis; Figure 4-3A) and epithelial cells (142.0% cytotoxicity; Figure 4-3B), respectively. Lactococci expressing either W461F or SVD showed no haemolytic activity and only low levels of cytotoxicity (W461F: 4.0% and SVD: 37.7%).

Taken together these data indicate that the RGD-motif is required for haemolytic and cytotoxic activity of suilysin.

Figure 4-3: Heterologous expression of SLY, but not W461F or SVD in *L. lactis*, confers a haemolytic and cytotoxic phenotype.

(A) Titration of haemolytic activity of SLY, W461F, and SVD present in culture supernatants of respective lactococci. Haemoglobin present in the supernatant caused by haemolysis was measured by absorbance at 550 nm. (B) Dose-dependent epithelial cell injury. Cytotoxicity was measured by LDH-release test. *L. lactis* pORI23 (carrying plasmid control) severed as background control for both tests. Lytic effects of distilled water (haemolysis test) or 1% Triton X-100 in PBS (cytotoxicity test) were set as 100% and depicted as controls.
The RGD-motif is required for membrane binding, Rac1-activation and macropore-formation in HEp-2 cells. Next we analysed in more detail, in which step the RGD-motif might be involved. In general, RGD-containing proteins can act as connecting molecules between pathogens and host cells to allow adherence and uptake of pathogens or in order to induce cell signalling pathways (Scibelli et al., 2005). Therefore, we first analysed the initial membrane binding capacity of suilysin and its mutated derivates using immunofluorescent staining. HEp-2 cells were treated with rSLY, rW461F or rSVD at subcytolytic (100 ng/ml; Figure 4-4B-D) or lytic (400 ng/ml; Figure 4-4E-G) concentration. Cell-bound suilysin was detected using a polyclonal antiserum raised against rSLY. Unmodified rSLY was able to bind to the cell surface at subcytolytic and lytic concentration (green spots in Figure 4-4B and E). The size of individual spots (most likely due to different numbers of molecules or oligomerisation stages) increased at higher concentration. Likewise, rW461F bound to cell membranes at lytic concentration (green spots in Figure 4-4G). This phenotype was described before for homologous tryptophan-mutants of the highly related pneumolysin as well as for intermediysin (Polekhina et al., 2005). It has been shown, that loss of lytic function did not include absence of oligomerisation and formation of the prepore complex (Boulnois et al., 1991; Korchev et al., 1998; Polekhina et al., 2005). In contrast, we showed that substitution of the RGD-motif affected membrane binding capacity drastically, so that rSVD was unable to bind to HEp-2 cells (Figure 4-4C and F).

Possible interaction partners of RGD-containing proteins are the heterodimeric transmembrane integrins. The integrin family is subdivided into groups, whereas one subgroup is specified on recognizing RGD-containing proteins (RGD-receptors), such as α5β1 integrins (Scibelli et al., 2005; Scibelli et al., 2007). Two mechanisms of integrin-bacteria interactions are known (Scibelli et al., 2007). An indirect interaction is mediated by extracellular matrix components (ECM) containing a RGD-sequence, such as fibronectin or vitronectin. These 'bridging molecules' connect bacterial ECM-binding proteins with integrins (Cue et al., 2000; Jonsson et al., 1991). An alternative way is the direct linking of microbial RGD-exhibiting proteins to integrins (Hamzaoui et al., 2004; Tran et al., 1999). We hypothesized that the latter is
plausible for suilysin-integrin interaction. Therefore, we analyzed by flow cytometry, if \( \alpha_5 \beta_1 \) integrins were expressed on the cellular surface of unstimulated HEp-2 cells. The results clearly showed that \( \alpha_5 \beta_1 \) integrins were present on HEp-2 cells (Figure 4-4H), thus available as putative interaction partners for suilysin. Further studies will have to prove whether they in fact function as receptors.

In principle, CDC bind preferentially to cholesterol-rich microdomains (lipid rafts) (Gekara and Weiss, 2004; Shimada et al., 2002; Waheed et al., 2001), but membrane components required for CDC binding have not been characterized in detail. The impact of cholesterol on membrane binding of CDC and CDC function is still controversially discussed (Hotze and Tweten, 2011). First assumed as the cellular receptor, recognized by the fourth domain of the CDC monomer (Sekino-Suzuki et al., 1996), a recent study pointed out that cholesterol plays a role downstream of membrane binding by triggering the conversion of the prepore complex to a functional transmembrane pore (Giddings et al., 2003). Nevertheless, lipid rafts are known to function as a 'platform' for bacteria-host cell interaction, enhancing bacterial adherence and invasion (Lafont and van der Goot, 2005). It is proposed that this 'triggering effect' is mediated by clustering of cell surface receptors within cholesterol enriched microdomains. Receptors associated with lipid rafts are among other integrins. We have previously demonstrated that cholesterol is essential for cytolytic activity. Future studies will have to show how cholesterol contributes to cell-activation by suilysin.

Integrins can also transmit extracellular signals into the cell. The small family of Rho-GTPases, namely Rac, Rho and Cdc42, play a key role in integrin-mediated outside-in signalling. Integrin-ligand interaction triggers either a direct activation of GTPases or regulates GTPase effector proteins. Furthermore, activation of G-proteins in turn induces integrin-clustering and a higher ligand-affinity. This bidirectional interaction is known to regulate cytoskeletal rearrangements (Schwartz and Shattil, 2000) putatively involved in bacteria-cell interaction.

As described in our previous study suilysin promotes invasion of S. suis by a Rac-dependent activation of the actin cytoskeleton (Seitz et al., submitted; cf. chapter 3). We showed that rSLY specifically activated Rac1, a member of small
Rho-GTPases, in HEp-2 cells at subcytolytic concentration (100 ng/ml), thus suggesting a pore-independent mechanism. Moreover, the mutated suilysin rW461F, deficient in functional pore-formation, still retained its ability to activate Rac1. To elucidate, if the RGD-motif is required for activation of Rho signalling G-proteins, we performed pull down based GLISA analysis. HEp-2 cells were treated with rSVD in comparison to rSLY and rW461F used at subcytolytic concentration (100 ng/ml) for 15 min. Results revealed an activation of Rac1 after stimulation of HEp-2 cells with either rSLY or W461F (Figure 4-5A), confirming previous results (Seitz et al., submitted; cf. chapter 3). As anticipated, neither RhoA nor Cdc42 were inducible. Interestingly, in contrast to rSLY and W461F, rSVD failed to activate any Rho-GTPase (Figure 4-5A), indicating the requirement of the RGD-motif for suilysin-induced host cell signalling.

Since macropore-formation is the final step in cytolysis, we speculated that the point-mutations of suilysin might abolish macropore-formation. Therefore, we used the fluorescent marker calcein to test cell membrane integrity. HEp-2 cells were incubated with recombinant proteins (rSLY, rW461F or rSVD) at subcytolytic (100 ng/ml, Figure 4-5B) or lytic concentrations (400 ng/ml, Figure 4-5C) to induce large channel formation in lipid bilayers. As intact cell membranes are impermeable for calcein due to its size (diameter of 1.3 nm) macropore-formation (20 - 30 nm in size) is required for incorporation of calcein by cells. As anticipated, rSLY was capable to induce macropores in epithelial cells at lytic concentrations (Figure 4-5C), resulting in a respective shift of the curve (cell population) on the x-axis. Both rW461F and rSVD failed to form large pores. In accordance to these results, a homologous tryptophan-mutant of pneumolysin formed large pores to significant lower degree and less frequently compared to wild type pneumolysin analyzed by conductance measurement of patch clamps (El Rachkidy et al., 2008; Korchev et al., 1998). Furthermore, it has been found that rapid activation of RhoA and Rac1 in neuronal cells by pneumolysin preceded the formation of lytic macropores (Iliev et al., 2007). Likewise, the tryptophan-mutant of suilysin still retained Rac1-activation, but failed to induce large channels. This suggests that cell-activation occurs before
macropore-formation, as previously reported for the mechanism underlying suilysin-promoted invasion of *S. suis* (Seitz *et al.*, submitted; cf. chapter 3).

![Figure 4-4: Substitution of the RGD-motif of recombinant suilysin leads to a loss in membrane binding.](image)

Membrane binding was analyzed by immunofluorescent microscopy. HEp-2 cells were treated with rSLY (B + E), rW461F (C, F) or rSVD (D, G) at subcytolytic (100 ng/ml; B-D) or lytic concentrations (400 ng/ml; E-G). Untreated cells are shown in (A). The actin cytoskeleton was stained in red and suilysin in green. Bar represent 10 µm. (H) Detection of surface associated α₅β₁ integrins on HEp-2 cells via flow cytometry analysis. Shift of the curve on the x-axis represents fluorescent cells, indicating expression of respective integrin subunit.
Figure 4-5  Substitution of the RGD-motif leads to loss in Rac1-activation and macropore-formation. (A) Activation of Rac1, RhoA, and Cdc42 after a 15 min stimulation of HEp-2 cells with rSLY and its mutated derivates rW461F and rSVD at subcytolytic concentrations (100 ng/ml), expressed as percentage of control. (B, C) Flow cytometry analysis of HEp-2 cells treated with the fluorescent marker calcein and recombinant proteins (rSLY, rW461F or rSVD) at subcytolytic (B) or lytic (C) concentrations, respectively. Cells treated with calcein alone served as background control. Right shift of the curve on the x-axis represents fluorescent cells, indicting macropore-formation (cell permeable for calcein).

Concluding, our results showed that the pore-forming region as well as the RGD-motif are both needed for the cytolytic activity of suilysin. Interestingly, formation of a functional channel is not required for additional effects of suilysin, such as activation of morphological host cell response. However, the RGD-motif identified here seems to be essential for such activities since substitution of the RGD sequence led to loss of membrane binding and Rac1-activation. It is plausible to speculate that the RGD-motif of suilysin interacts with α5β1 integrins on the host cell surface to induce a Rac1-dependent remodelling of the actin cytoskeleton to allow invasion of S. suis. Further studies will have to be performed to elucidate the precise role of the RGD-motif in S. suis-host cell interaction and pathogenesis.
Experimental procedures

If not stated otherwise all materials were purchased from Sigma (Munich, Germany).

**Bacterial strain and growth conditions.** *Escherichia (E.) coli* strains BL21, BL21 (DE3) and DH5α were used for molecular cloning and protein expression experiments. *E. coli* were grown in Luria Bertani (LB) medium overnight under aerobic conditions at 37°C with vigorous shaking. For heterologously expression of suilysin *Lactococcus lactis* subsp. *cremoris* MG1363 (*L. lactis*) was used as described recently (Baums *et al.*, 2006).

**DNA techniques.** Routine molecular biology techniques including restriction endonuclease digestion, DNA ligations, agarose gel electrophoresis, transformation of *E. coli* and plasmid isolation were performed according to standard procedures (Sambrook *et al.*, 1989). Restriction enzymes were purchased from New England Biolabs (Frankfurt am Main, Germany). Plasmid preparations were performed with kits from Machery-Nagel (Dueren, Germany).

**Construction and expression of recombinant wild type suilysin and its point-mutated derivates W461F and RGD-SVD.** W461F and RGD-SVD substitutions were constructed using site-directed-mutagenesis according to the instruction manual of QuikChange® Site-Directed Mutagenesis Kit (Stratagene, Ja Jolla, CA) as described in a previous study (Seitz *et al.*, submitted; cf. chapter 3). As template DNA pET45bslynew was used (Kock *et al.*, 2009). For RGD replacement the oligonucleotide primers encoding R124-G125-D126 to S124-V125-D126 slyRGDMutfor (CAGTATTGCGTCGGTAGATCT GACGCTTAG) and slyRGDMutrev (CTAAGCGTCAGATCTACCGACGCAATACTG) were used. Mutation sites of each suilysin derivate were sequenced using the primers slyseqfor (GGATCATTCAGGTGCTTATG) and pET45seqrev (TGCTGGCGTTCAAATTTCGC). Recombinant His-tagged suilysin (rSLY) was expressed in *E. coli* BL21
pET45bslynew (Kock et al., 2009). In accordance, recombinant point-mutated suilysin W461F (rW461F) was expressed in *E. coli* BL21 pET45bslynewW461F as described in a previous study (Seitz et al., submitted; cf. chapter 3) and recombinant RGD-SVD (rSVD) in *E. coli* BL21 pET45bslynewRGD-SVD. Purification of all recombinant proteins was verified by immunoblot analysis with anti-Penta His antibodies (Qiagen, Hilden, Germany) as recommended and silver staining was used to control the purity grade. The proteins were stored at -20°C. The wild type suilysin was completely sequenced with standard oligonucleotide primers T7 (TAATACGACTCACTATAGGG) and T7 term (CTAGTTATTGCTCAGCGGT; Eurofins MWG operon, Ebersberg, Germany).

**Heterologous expression of SLY, W461F and SVD in *L. lactis***. Heterologous expression in *L. lactis* was performed as described previously (Baums et al., 2006). The *sly*-gene was amplified from chromosomal DNA of *Streptococcus suis* by PCR with oligonucleotide primers slyPstI (TAGTCTGCAGCTCCTAGCCTCTGGCTAA) and slyBamHIoptRBS (CAGAGGATCCAGGAGAAAACTTATGAGAAAAAG). After digestion of the PCR product with BamHI and PstI the fragment containing the whole *sly*-gene was cloned into the BamHI/PstI-digested shuttle vector pORI23 (Que et al., 2000). The resulting plasmid was named pORI23-SLY and transferred in *L. lactis* as described previously (Holo and Nes, 1989b). Transformants were screen by plating on GM17 agar plates containing 5 µg/ml erythromycin. For heterologous expression of W461F and SVD site-directed mutagenesis was performed as described above using purified plasmid DNA pORI23sly and the respective oligonucleotid primers slyTrp-Phefornew and slyTrp-Pherewnew as well as slyRGDMutfor and slyRGDMutrev. Resulting constructs pORI23-W461F and pORI23-SVD were electroporated in *L. lactis*.

**Immunoblot analysis**. Recombinant proteins were separated by SDS-polyacrylamid gel electrophoresis with a 4% stacking and a 10% separating gel under denaturing conditions and transferred to a PVDF-membrane (Serva, Heidelberg, Germany). For Immunoblot analysis, membrane-blocking was performed overnight with 3% milk powder in TBS with 0.5% Tween. Polyclonal antiserum raised against rSLY (Benga
et al., 2008) diluted 1:1,300 in 1% milk powder was used to detect either rSLY, rW461F, rSVD or suilysin and its mutated derivates in culture supernatants of the respective *L. lactis* strains (*L. lactis* pORI-SLY, *L. lactis* pORI-W461F and *L. lactis* pORI-SVD). For detection of His-tagged recombinant proteins membranes were blocked with 3% BSA in TBS with 0.5% Tween overnight and incubated with a monoclonal anti-Penta His antibody (Qiagen) diluted 1:2,000 in 3% BSA. Membranes were developed with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antiserum diluted 1:10000 in 1% milk powder (Amersham, Freiburg, Germany) followed by addition of SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, USA) according to the manufactures protocol.

**HEp-2 epithelial cell culture.** The human laryngeal epithelial cell line HEp-2 (ATCC CCL 23) was used. Cells were maintained in Dulbecco’s modified Eagle's medium (DMEM, Gibco-Invitrogen, Groningen, The Netherlands) supplemented with 10% fetal calf serum (FCS) and 5 mM glutamine at 37°C and 8% CO₂. The cells were subcultured every 2-3 days after detachment with 0.25% trypsin and 1 mM Na-EDTA (trypsin-EDTA, Gibco-Invitrogen). For cellular cytotoxicity assay 0.2 x 10⁵ cells were seeded on a 96 well plate. For immunofluorescence 0.8 x 10⁵ cells per well were seeded on a 12 mm diameter glass cover slips placed in 24 well plates. The cells were grown over-night and then used for experiments.

**Determination of haemolytic activity and cytotoxicity.** The haemolytic activity of rSLY, rW461F and rSVD was determined by a haemolysis assay as described in a previously (Seitz *et al.*., submitted; cf. chapter 3; Takamatsu *et al.*, 2001). Cytotoxicity of recombinant proteins was detected by an LDH-release assay as described previously (Seitz *et al.*; submitted; cf. chapter 3; Benga *et al.*, 2004). Briefly, *L. lactis* expressing either SLY, W461F or SVD (*L. lactis* pORI-SLY, -W461F and –SVD), respectively, were grown in GM17 medium overnight, adjusted to an optical density (OD₆₀₀) of 0.02 in pre-warmed media and then grown to late exponential growth phase (OD₆₀₀ 0.8). Subsequently bacteria were removed by centrifugation and culture supernatants were concentrated and purified using Amicon centrifugal filter devices 30 kDa (Millipore, Schwalbach/Ts., Germany) to remove
remaining medium components. Concentrated culture supernatants diluted 1:5 in 0.9% NaCl were used as test samples.

**Immunofluorescent microscopy.** Membrane binding of rSLY, rW461F and rSVD was determined by immunofluorescent microscopy. HEp-2 cells were incubated with the recombinant suilysin derivates at subcytolytic (100 ng/ml) or lytic (400 ng/ml) concentrations for 30 min at 37°C. Cells were washed three times to remove unbound suilysin, fixed with 2% paraformaldehyde and permeabilised with 0.1% Triton X-100 in PBS (Bio-Rad, Muenchen, Germany). Blocking was performed for 30 min at room temperature with PBS containing 10% FCS. Suilysin was stained using a polyclonal antiserum raised against rSLY (Benga *et al.*, 2008) diluted 1:1,300 in 1% milk powder for 1 h at room temperature followed by an incubation with FITC-conjugated goat anti-rabbit antibody (1:1,000 in 1% FCS in PBS, Dianova, Hamburg, Germany). F-actin was labelled with TRITC-conjugated Phalloidin (20 µg/ml, Invitrogen) for 40 min at room temperature. After final washing DAPI (Invitrogen) was used for staining the nuclei. Antifading reagent DABCO (Sigma) was used for sealing of the samples. Mounted samples were examined using an inverted immunofluorescence microscope Nikon Eclipse Ti-S equipped with a 40×, 0.6 S Plan Fluor objective (Nikon, Duesseldorf, Germany) driven by NIS Elements software BR 3.2..

**Detection of α5β1 integrin expression on HEp-2 cells.** HEp-2 cell-suspension was incubated with either a monoclonal anti-human CD29 antibody or a monoclonal anti-human CD49e (Bio Legend) both used at a concentration of 500 ng per 1.5 x 10^6 cells (Bio Legend, Fell, Germany). All antibodies were diluted in blocking buffer (PBS [pH 7.3] containing 3% BSA) and incubation was performed for 30 min at room temperature with gently agitation. After three washing steps with blocking buffer, cells were incubated with a TRITC-conjugated goat anti-mouse antibody (1:1000, Dianova, Hamburg, Germany). Stained cells were washed three times, fixed with 0.37% formaldehyde and resuspended in blocking buffer for subsequent measurement. Flow cytometry measurement was performed as described above.
Fluorescent cells were counted at channel FL-1 and unstained cells severed as background control.

**GLISA.** HEp-2 cells were treated with rSLY, rW461F or rSVD at subcytolytic concentration (100 ng/ml) for 15 min and rinsed twice with ice cold PBS to stop incubation. Lysis of cells was achieved by addition of 1 ml of ice cold lysis buffer (50 mM NaCl, 20 mM Tris-HCl, pH 7.4, 3 mM MgCl₂, 1% Nonidet-P 40, 0.25% Triton X-100, 5 mM dithiothreitol, 100 µM PMSF). Cells were scraped off, and the lysates were centrifuged at 14,000 rpm. The supernatant was used for a pull down-based G-LISA (Cytoskeleton, CO, USA) to determine activation of Rac1, RhoA, and Cdc42 according to the protocol by the supplier.

**Cell-permeability and macropore-formation assay.** Experiments were performed in PBS containing 0.9 mM CaCl₂/0.5 mM MgCl₂·6H₂O [pH 7.3] (Gibco-Invitrogen). Cell permeability was determined after co-incubation of HEp-2 cells (4 x 10⁵ cells/ml) with recombinant proteins (rSLY, rW461F, and rSVD) at subcytolytic (100 ng/ml) or lytic (400 ng/ml) concentrations (cf. cytotoxicity test) and the fluorescent marker calcein (2 µg/ml, Sigma, Taufkirchen, Germany). Incubation was performed for 2 h at 37°C on an end-over-end rotator. After two washing steps with PBS to remove extracellular calcein, cells were fixed with 0.37% formaldehyde (for 10 min at room temperature and then resuspended in 500 µl PBS for measurement of fluorescent cells using FACSkan® (Becton Dickinson, 488 nm Argon laser). Further analysis was performed with the software WinMDI (version 2.9.). For each determination at least 10,000 events were measured. Initial analysis of fluorescent cells was carried out by dot plot analysis (forward scatter [fsc] versus sideward scatter [ssc]) to define the cell population of interest (data not shown). Subsequently, fluorescent cells were detected at channel FL-1. Cells treated only with calcein served as background control.

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References


Chapter 5

Results, part III:

Establishment of an intranasal CD1 mouse infection model for colonization and invasion of *Streptococcus suis* serotype 2

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Running title: Intranasal mouse infection model for *S. suis*

(Manuscript in preparation)
Abstract

Streptococcus (S.) suis causes meningitis and various other diseases in pigs and humans. Healthy piglets carrying virulent S. suis strains on their mucosal surfaces are epidemiologically very important. The objective of this study was to establish an intranasal mouse model for colonisation and invasion of the respiratory tract. CD1 mice were intranasally infected with a highly virulent S. suis serotype 2 strain under different conditions of predisposition. Clinical, histological and bacteriological examination revealed that invasion of host tissues occurred only in mice predisposed intranasally with 1% acetic acid. This model is characterized by efficient colonization as all mice carried S. suis on their respiratory mucosa 7 days post infection. Furthermore, severe fibrinosuppurative or purulent necrotizing pneumonia associated with S. suis was a common manifestation in this model. The intranasal S. suis model was applied to investigate the relevance of suilysin in colonisation and invasion by comparison of the wild type with its isogenic sly-mutant. Results suggested attenuation in virulence but not in colonization of the suilysin mutant. In conclusion, this study revealed the first intranasal mouse model to study colonization and invasion of the respiratory tract by a highly virulent S. suis pathotype.
Introduction

*Streptococcus* (*S.*) *suis* is a major swine pathogen worldwide, causing severe diseases such as meningitis, septicaemia and bronchopneumonia (Higgins and Gottschalk, 2005). It is also an important zoonotic agent. Humans might be infected following contact with pigs or pork. Meningitis, septicaemia and the life-threatening *streptococcal toxic shock-like syndrome* (STSS) are important manifestations of *S. suis* infections in humans (Gottschalk *et al.*, 2007; Tang *et al.*, 2006).

Pigs and wild boars are considered the natural reservoir of *S. suis* (Baums *et al.*, 2007; Clifton-Hadley and Alexander, 1980; Higgins and Gottschalk, 2005). Different mucosal surfaces might be colonized by *S. suis*. In weaning piglets, *S. suis* is among other bacteria the most abundant colonizers of the upper respiratory and alimentary tract (Baele *et al.*, 2001; Lowe *et al.*, 2011; O’Sullivan *et al.*, 2011a; Su *et al.*, 2008). Healthy carriers of virulent *S. suis* strains play an important role in the epidemiology of *S. suis* diseases in pig and humans (Arends *et al.*, 1984; Ngo *et al.*, 2011).

*S. suis* is characterized by a high diversity as reflected by the presence of at least 33 serotypes. Serotype 2 is worldwide the most prevalent among invasive isolates of pigs and humans (Wei *et al.*, 2009; Wisselink *et al.*, 2000). The serotype is determined by the polysaccharide capsule. The capsule protects the bacteria against opsonophagocytosis and functions as an important virulence factor (Charland *et al.*, 1998; Smith *et al.*, 1999). A number of other surface-associated factors have also been demonstrated to contribute to pathogenicity of *S. suis* (Baums and Valentin-Weigand, 2009). The pathogenesis of *S. suis* meningitis is not well understood, and even less is known about the mechanisms employed by *S. suis* to colonize mucosa. As a matter of fact, not a single factor of *S. suis* has been demonstrated to be crucial for colonization.

Recently, a *S. suis* meningitis model was described in CD1 mouse, in which typical histopathological lesions and inflammatory responses were recorded. Sudden death of 20% of infected animals associated with high levels of systemic proinflammatory cytokines was observed. Infected mice that survived developed clinical signs of meningitis characterized by infiltrates of neutrophils and bacterial emboli.
(Dominguez-Punaro et al., 2007). Similar to many other experimental infections of mice, S. suis was applied intraperitoneally. As the upper respiratory tract and, in particular, the tonsils are considered to be the port of entry for S. suis (Williams and Blakemore, 1990), early steps in the pathogenesis of S. suis diseases cannot be studied in the CD1 mouse model described by Dominguez-Punaro et al. (2007). Thus, investigation of colonization of the respiratory mucosa requires a new murine intranasal S. suis model. Here, we describe for the first time that S. suis serotype 2 colonizes and invades mice tissue efficiently after intranasal application following predisposition. This work includes a description of an intranasal colonization and invasion model for S. suis using CD1 mice. Furthermore, the model was applied to identify suilysin-dependent phenotypes in vivo using encapsulated as well as unencapsulated strains.
Results and Discussion

To our knowledge, a mucosal infection model for *S. suis* in mice has not been described. In this study we investigated whether a virulent *S. suis* serotype 2 strain can infect CD1 mice after intranasal application. As healthy carrier animals play a crucial role in the epidemiology of *S. suis* diseases, there is also a urgent need to study colonization of mucosal surfaces. A major problem in swine is the restricted availability of *S. suis* free piglets for such investigations. Therefore, the second objective of this study was to introduce a mice model for *S. suis* colonization of mucosal surfaces. For this, we compared different conditions of experimental infection without and after predisposition with 1% acetic acid prior intranasal infection.

**Intranasal infection without predisposition.** A dose of $5 \times 10^9$ CFU of *S. suis* strain 10 was applied intranasally to Crl:CD1 (ICR) mice, as this mouse strain has been demonstrated to develop typical lesions of meningitis after intraperitoneal application of *S. suis* (Dominguez-Punaro *et al.*, 2007). Nine of 10 Crl:CD1 (ICR) mice did not show any clinical signs of infection over an observation period of 5 or 12 days (5 mice each, Table 5-2). The low morbidity in mice infected intranasally with a high dose of *S. suis* serotype 2 is in accordance with the findings of Williams *et al.* (1988). Apathy, continuing anorexia and a weight loss of more than 20% was registered in one mouse starting on the 4th dpi (Table 5-2; Table 5-S1). Histopathological screening revealed severe purulent meningitis and encephalitis associated with a bacterial load of 4,000 CFU per mg brain tissue (Table 5-3 and Table 5-4). Furthermore, the challenge strain was also reisolated in 3 of 10 mice in the TNL and in 2 of 10 mice in the lungs on the 5th day post intranasal infection. The bacterial load of the respiratory tract of these mice was below 100 CFU per μl TNL or mg tissue, respectively. These results suggest that *S. suis* serotype 2 colonized the respiratory tract and even caused meningitis, though only with low morbidity.
Intranasal infection model after predisposition with acetic acid. In piglets, *S. suis* mucosal infection models have been described which include experimental predisposition, such as infection with *Bordetella bronchiseptica* (Smith *et al.*, 1996; Smith *et al.*, 1999) or local application of 1% acetic acid (Baums *et al.*, 2006; Pallares *et al.*, 2003). Therefore we predisposed mice by nasal application of 12.5 µl 1% acetic acid per nostril 1 h pre intranasal infectionem with *S. suis*. In this experiment, morbidity was significantly higher in comparison to the previous experiment without predisposition (*P* = 0.003; Table 5-2). Sixty-seven percent of mice received a cumulative clinical index equal or above 3 and were, thus, classified as diseased (Table 5-1 and Table 5-2). Six (Table 5-S1) out of 24 infected mice developed severe clinical symptoms (anorexia, scruffy coat, rapid abdominal breathing, and apathy) and were euthanized one day post infection for reasons of animal welfare (mortality rate of 25%). Severe fibrinosuppurative or purulent necrotizing pneumonia as well as severe infiltration of the splenic red pulp with neutrophils was diagnosed in these six mice post mortem. *S. suis* was detected in the lungs of these six mice in high number (up to 23,000 CFU per mg lung tissue; Table 5-4). Purulent meningitis and/or encephalitis was found only in one of 24 infected mice. However, the challenge strain was reisolated from the brain in 46% of these animals. The bacterial load in the brain of these mice was below 150 CFU per mg brain tissue except in one mouse (34,000 CFU per mg brain tissue). Severe fibrinosuppurative and even purulent necrotizing rhinitis was a common finding in surviving mice. The challenge strain was frequently detected in the lungs but also in other inner organs (Table 5-4). Unexpectedly, in eight of 24 mice *St. aureus* was also detected in inner organs (see number in brackets in Table 5-4 and Table 5-S1). A likely explanation for this finding was that *St. aureus* colonizing the respiratory tract in mice might have invaded the host after local application of 1% acetic acid. However, infection with *St. aureus* was not a prerequisite for *S. suis* infection, since more than 50% of mice positive for *S. suis* in the brain, lung, heart or any other organ were free of *St. aureus* in these tissues. In this experiment, mice were sacrificed on the 3rd, 5th and 7th days post infection to monitor the time course of early colonization and invasion as shown in Table 5-4. The challenge strain was isolated from the upper respiratory tract (TNL...
was positive tested for the challenge strain in 92% of all infected mice) and inner organs from numerous mice on the 5th day post infection, but in mice sacrificed on the 7th day detection of \textit{S. suis} was restricted to the TNL (with the exception of one positive lung; Table 5-S1). In conclusion, intranasal application of 1% acetic acid 1 h pre infection significantly increased morbidity, mainly due to severe pneumonia associated with \textit{S. suis}. Invasion of inner organs by \textit{S. suis} was also enhanced in mice after acetic acid predisposition. Furthermore, \textit{S. suis} colonized the respiratory epithelium during the whole period of observation (up to seven days post infectionem).

To exclude coinfection with commensal \textit{St. aureus}, further experiments were conducted with the \textit{St. aureus}-free CD1 mice strain Hsd:ICR (CD-1\textsuperscript{®}). The challenge strain was detectable in almost all inner organs of six mice on the 3rd day post intranasal infection (Table 5-S1). Severe pneumonia was a common finding as in the 2nd experiment (Figure 5-1B). Accordingly, the pathoscore $\omega$ of experiments 2 and 3 were very similar (2.0 and 1.9, respectively). Whereas moderate to severe purulent necrotizing or fibrinosuppurative rhinitis was detectable in all infected animals (Figure 5-1A), meningitis and encephalitis were not observed in any of the infected mice. The challenge strain colonized the respiratory epithelium most efficiently as indicated by a reisolation rate of 100% of the challenge strain from TNL. As expected \textit{St. aureus} coinfection was not a problem in this experiment (Table 5-4).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image.png}
\caption{Histological findings of the nose and lung of mice infected intranasally with 5 x 10\textsuperscript{8} CFU of \textit{S. suis} after predisposition with 1% acetic acid \(\text{(A) Severe multifocal fibrinosuppurative rhinitis, HE 20x. (B) Severe multifocal fibrinous-necrotizing (arrow) pneumonia, HE 20x.}\)\
\end{figure}
The results indicated that application of acetic acid prior to experimental \(S. suis\) infection predisposed mice to rhinitis and pneumonia rather than to meningitis, the most common pathology of \(S. suis\) infection in swine and humans. This is in contrast to the effect of intranasal acetic acid predisposition in swine, leading mainly to \(S. suis\)-associated pleuritis, peritonitis and meningitis (Baums et al., 2006; Pallares et al., 2003) and the intraperitoneal model described by Dominguez-Punaro et al. (2007) associated with a prevalence of meningitis in 40% of mice. However, as demonstrated by bacteriology results, \(S. suis\) colonized the mucosal epithelium up to seven days post infectionem and invaded into tissue after intranasal application and acetic acid predisposition. Therefore, this model might be used to study colonization and invasion of \(S. suis\) in mice, using bacteriological screening of different inner organs as important read out parameter.

Table 5-1: Clinical score sheet for health monitoring of mice after infection with \(S. suis\).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>0</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight</td>
<td>Constant or gain</td>
<td>&gt; 5% weight los</td>
<td>&gt; 20% weight loss</td>
</tr>
<tr>
<td>Coat</td>
<td>Flat, glossy</td>
<td>Rough, reduced grooming</td>
<td>Scrubby, failure of grooming</td>
</tr>
<tr>
<td>Breathing</td>
<td>Adequate, rhythmic</td>
<td>Rapid, shallow</td>
<td>Rapid, abdominal</td>
</tr>
<tr>
<td>Dehydration(a)</td>
<td>Normal skin elasticity</td>
<td>Moderately reduced skin elasticity</td>
<td>Persisting skin fold, sunken eyes</td>
</tr>
<tr>
<td>Bearing</td>
<td>Normal</td>
<td>Moderately curved back</td>
<td>Cowering, highly curved back</td>
</tr>
<tr>
<td>Eyes</td>
<td>Normal</td>
<td>Moderately protruding</td>
<td>Highly swollen</td>
</tr>
<tr>
<td>Activity</td>
<td>Normal active</td>
<td>Depression</td>
<td>Apathy, social isolation</td>
</tr>
<tr>
<td>Locomotion</td>
<td>No anomaly</td>
<td>Moderate incoordination</td>
<td>Apraxia, stagger</td>
</tr>
</tbody>
</table>

\(a\) Elapsing of a drawn up dorsal skin fold
### Table 5-2: Clinical course of CD1 mice intranasally infected with $5 \times 10^8$ CFU of *S. suis* strain 10 (*mrp*+, *epf*+, *sly*+, *cps2*).

<table>
<thead>
<tr>
<th>Expt</th>
<th>No. of mice</th>
<th>Mouse strain</th>
<th>Acetic acid&lt;sup&gt;a&lt;/sup&gt;</th>
<th>dpi&lt;sup&gt;b&lt;/sup&gt;</th>
<th>No. of mice/total no. of mice</th>
<th>Morbidity&lt;sup&gt;f&lt;/sup&gt;</th>
<th>Mortality</th>
<th>Severe clinical symptoms&lt;sup&gt;g&lt;/sup&gt;</th>
<th>Maximum. weight loss (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>10</td>
<td>Crl:CD1 (ICR)</td>
<td>-</td>
<td>$5^c$, $12^c$</td>
<td>1/10</td>
<td>0/10</td>
<td>0/10</td>
<td>2/10</td>
<td>1/10</td>
</tr>
<tr>
<td>2nd</td>
<td>24&lt;sup&gt;h&lt;/sup&gt;</td>
<td>Crl:CD1 (ICR)</td>
<td>12.5 µl</td>
<td>1, $3^d$, $5^d$, $7^d$</td>
<td>16/24</td>
<td>6/24</td>
<td>7/24</td>
<td>22/24</td>
<td>0/24</td>
</tr>
<tr>
<td>3rd</td>
<td>10</td>
<td>Hsd:ICR (CD-1&lt;sup&gt;g&lt;/sup&gt;)</td>
<td>12.5 µl</td>
<td>$2^e$, 3</td>
<td>4/10</td>
<td>2/10</td>
<td>2/10</td>
<td>7/10</td>
<td>1/10</td>
</tr>
</tbody>
</table>

<sup>a</sup> Volume of 1% acetic acid applied to each nostril 1 h pre infectionem

<sup>b</sup> Days post infection, on which mice were sacrificed or killed for reasons of animal welfare

<sup>c</sup> Five mice were sacrificed on each of these dpi

<sup>d</sup> Six mice were sacrificed on each of these dpi

<sup>e</sup> Two mice were killed 2 dpi for reasons of animal welfare

<sup>f</sup> Mice with a cumulative clinical score $\geq 3$ were regarded as diseased

<sup>g</sup> In particular persistent anorexia, apathy, and/or neural disorder leading to a cumulative clinical score $>6$

<sup>h</sup> Coinfection with *St. aureus* (isolation of *S. suis* and *St. aureus* from different affected tissues, see Table 5-4)
Table 5-3: Scoring of fibrinosuppurative and purulent necrotizing lesions of mice intranasally infected with 5 x 10^6 CFU of *S. suis* (mrrp* epf+ sly+ cps2).

<table>
<thead>
<tr>
<th>No. of mice/total no. of mice</th>
<th>Nose</th>
<th>Spleen</th>
<th>Lung</th>
<th>Brain and Spinal cord</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt</td>
<td>No. of mice</td>
<td>Mouse strain</td>
<td>Acetic acid</td>
<td>dpi</td>
</tr>
<tr>
<td>------</td>
<td>--------------</td>
<td>---------------</td>
<td>--------------</td>
<td>-----</td>
</tr>
<tr>
<td>1st</td>
<td>10</td>
<td>Ctrl:CD1 (ICR)</td>
<td>-</td>
<td>5(^{5}), 12(^{6})</td>
</tr>
<tr>
<td>2nd</td>
<td>24(^{k})</td>
<td>Ctrl:CD1 (ICR)</td>
<td>12.5 µl</td>
<td>1(^{1}), 3(^{d}), 5(^{d}), 7(^{d})</td>
</tr>
<tr>
<td>3rd</td>
<td>10</td>
<td>Hsd:ICR (CD-1(^{6}))</td>
<td>12.5 µl</td>
<td>2(^{e}), 3</td>
</tr>
</tbody>
</table>

\(^{a}\) Volume of 1% acetic acid applied to each nostril 1 h pre infectionem

\(^{b}\) Days post infection, on which mice were sacrificed or killed for reasons of animal welfare

\(^{c}\) Five mice were sacrificed on each of these dpi

\(^{d}\) Six mice were sacrificed on each of these dpi

\(^{e}\) Two mice were killed 2 dpi for reasons of animal welfare

\(^{f}\) Infiltration of splenic red pulp with neutrophilic granulocytes

\(^{g}\) Scoring of 4 and 5 indicates moderate to severe diffuse or multifocal fibrinosuppurative or purulent necrotizing inflammations of the indicated tissue

\(^{h}\) Scoring of 2 and 3 indicates mild focal fibrinosuppurative or purulent necrotizing inflammation of the respective organs

\(^{i}\) Individual single perivascular immune cells received a score of 1

\(^{j}\) \(\omega = \Sigma \) score\text{max/animals}\ (Baums *et al.*, 2006); rhinitis is not included in the score \(\omega\); lesions in the heart, kidney or liver were not recorded

\(^{k}\) Coinfection with *St. aureus* (isolation of *S. suis* and *St. aureus* from different affected tissues, see Table 5-4)
### Table 5-4: Reisolation of the challenge strain *S. suis* strain 10 (*mrp*+, *epf*+, *sly*+ *cps2*) in intranasally infected mice.

<table>
<thead>
<tr>
<th>Expt</th>
<th>No. of mice</th>
<th>Mouse strain</th>
<th>Acetic acid$^a$</th>
<th>dpi$^b$</th>
<th>No. of mice in which the <em>S. suis</em> challenge strain$^c$ was isolated/total no. of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TNL$^g,h$ Spleen, Liver, Kidney, Heart$^h$ Lung$^h$ Brain$^h$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+++ ++ + ++ +++ ++ + +++ ++ + +++ ++ +</td>
</tr>
<tr>
<td>1st</td>
<td>10</td>
<td>Crl:CD1 (ICR)</td>
<td>-</td>
<td>4$^c$</td>
<td>5$^c$ 0/5 0/5 3/5 0/5 0/5 0/5 2/5 1/5 0/5 0/5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>12$^c$</td>
<td>0/5 0/5 1/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>0/6 0/6 6/6 0/6 1/6 5(4)$^d$ 6 5(3)$^d$ 6 1/6 0/6 0/6 1/6 5(4)$^d$ 6</td>
</tr>
<tr>
<td>2nd</td>
<td>24</td>
<td>Crl:CD1 (ICR)</td>
<td>12.5 $\mu$l</td>
<td>3$^d$</td>
<td>0/6 0/6 6/6 0/6 0/6 4/6 0/6 0/6 3/6 0/6 0/6 2/6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5$^d$</td>
<td>0/6 3/6 2/6 1/6 0/6 2/6 0/6 1/6 2(1)$^d$ 6 1/6 0/6 2/6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7$^d$</td>
<td>0/6 2/6 3/6 0/6 0/6 0/6 0/6 0/6 1/6 0/6 0/6 0/6</td>
</tr>
<tr>
<td>3rd</td>
<td>10</td>
<td>Hsd:ICR (CD-1$^e$)</td>
<td>12.5 $\mu$l</td>
<td>2$^e$</td>
<td>1/2 0/2 1/2 0/2 1/2 1/2 0/2 0/2 2/2 0/2 0/2 2/2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>1/8 2/8 5/8 0/8 1/8 4/8 0/8 1/8 3/8 0/8 0/8 2/8</td>
</tr>
</tbody>
</table>

---

$^a$ Volume of 1% acetic acid applied to each nostril 1 h pre infectionem

$^b$ Days post infection, on which mice were sacrificed or killed for reasons of animal welfare

$^c$ Five mice were sacrificed on each of these dpi

$^d$ Six mice were sacrificed on each of these dpi

$^e$ Two mice were killed 2 dpi for reasons of animal welfare

$^f$ Isolation of the challenge strain was confirmed in a multiplex PCR for detection of *mrp, sly, epf, arcA, cps1, cps2, cps7*, and *cps9* (Silva et al., 2006)

$^g$ Tracheo-nasal lavage

$^h$ Based on CFU per mg organ or per $\mu$l TNL, bacterial loads were classified as mild (+ <100), moderate (100 ≤ ++ <1000) or severe (+++ >1000)

$^i$ No. of mice in which *St. aureus* was detected in addition to *S. suis* in the respective tissues
Evaluation of colonization and invasiveness of isogenic sly-mutants. The intranasal mouse model with acetic acid predisposition was used to investigate the contribution of suilysin to the invasiveness of S. suis. Six mice each were infected either with the wild type strain 10 or its isogenic suilysin-deficient mutant (10Δsly). None of the mice infected with 10Δsly developed clinical symptoms except for a weight loss ≥5% one day post infectionem. In comparison, all mice infected with the wild type strain 10 showed mild clinical symptoms of general sickness including rough coat, moderate depression and persistent weight loss ≥5% (morbidity 100%). No case of severe sickness with obvious neural dysfunction was seen (Table 5-5). However, histopathological screening revealed one case of severe purulent encephalitis located in the olfactory bulb in a mouse infected with the wild type strain 10 (Table 5-S1). In accordance with differences in morbidity between wild type and 10Δsly infected mice, the general pathoscore ω was substantially lower in mice infected with the isogenic sly-mutant in comparison to the score of the wild type infected group (0.5 versus 1.7, Table 5-6). Attenuation in virulence of the isogenic sly-mutant is in accordance with results of experimental mouse infections reported by Allen et al. (2001), who observed substantial suilysin-dependent differences in mortality at high intraperitoneal doses. However, experimental infections of pigs with suilysin-negative and wild type strains did not reveal an attenuated phenotype of the sly-mutant (Allen et al., 2001; Lun et al., 2003).

In contrast to the two previous experiments with acetic acid predisposition cases of pneumonia were not observed in wild type infected mice (Table 5-6). Furthermore, the wild type strain was detectable only in the TNL but not in inner organs (Table 5-7). These differences might be related to differences in ages: Mice of experiments 2 and 3 were 4 weeks old at the time of infection, whereas mice of the 4th experiment were 6 to 7 weeks old. Northworthy, Williams et al. (1988) also observed that young mice were more susceptible to S. suis infection than old mice.
Table 5-5: Clinical course of Hsd:ICR (CD-1®) mice intranasally infected with $5 \times 10^8$ CFU of *S. suis* strain 10 (*sly*+, *cps2*), 10Δsly (*sly*-, *cps2*), 10cpsΔEF (*sly*+, *cps2*), and 10cpsΔEFAΔsly (*sly*-, *cps2*-) after predisposition with 1% acetic acid.

<table>
<thead>
<tr>
<th>No. of mice</th>
<th>Challenge strain</th>
<th>dpi(^a)</th>
<th>No. of mice/total no. of mice</th>
<th>Morbidity(^b)</th>
<th>Mortality</th>
<th>Severe clinical symptoms(^c)</th>
<th>Maximum weight loss (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td><em>S. suis</em> strain 10</td>
<td>5</td>
<td>6/6 0/6 0/6</td>
<td>6/6</td>
<td>0/6</td>
<td>6/6</td>
<td>0/6</td>
</tr>
<tr>
<td>6</td>
<td><em>S. suis</em> 10Δsly</td>
<td>5</td>
<td>0/6 0/6 0/6</td>
<td>6/6</td>
<td>0/6</td>
<td>6/6</td>
<td>0/6</td>
</tr>
<tr>
<td>6</td>
<td><em>S. suis</em> 10cpsΔEF</td>
<td>3</td>
<td>3/6 0/6 1/6</td>
<td>2/6</td>
<td>1/6</td>
<td>2/6</td>
<td>1/6</td>
</tr>
<tr>
<td>6</td>
<td><em>S. suis</em> 10cpsΔEFAΔsly</td>
<td>3</td>
<td>1/6 0/6 0/6</td>
<td>4/6</td>
<td>0/6</td>
<td>4/6</td>
<td>0/6</td>
</tr>
</tbody>
</table>

\(^a\) Days post infection, on which mice were sacrificed

\(^b\) Mice with a cumulative clinical score greater \(\geq 3\) were regarded as diseased

\(^c\) In particular persistent anorexia, apathy, and/or neural disorder leading to a cumulative clinical score \(\geq 6\)
Table 5-6: Scoring of fibrinosuppurative and purulent necrotizing lesions of Hsd:ICR (CD-1®) mice intranasally infected with 5 x 10^9 CFU of *S. suis* strain 10 (*sly*, *cps2*), 10Δsly (*sly*, *cps2*), 10cpsΔEF (*sly*, *cps2*), and 10cpsΔEFΔsly (*sly*, *cps2*) after predisposition with 1% acetic acid.

<table>
<thead>
<tr>
<th>No. of mice</th>
<th>challenge strain</th>
<th>dpi^a</th>
<th>Nose</th>
<th>Spleen</th>
<th>Lung</th>
<th>Brain and Spinal cord</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rhinitis</td>
<td>Splenitis^b</td>
<td>Pneumonia</td>
<td>Meningitis, Encephalitis, Ependymitis, Ventriculitis</td>
</tr>
<tr>
<td>6</td>
<td><em>S. suis</em> strain 10</td>
<td>5</td>
<td>4^c</td>
<td>2^d</td>
<td>1^e</td>
<td>4^c</td>
</tr>
<tr>
<td>6</td>
<td><em>S. suis</em> 10Δsly</td>
<td>5</td>
<td>3^c</td>
<td>1^d</td>
<td>1^e</td>
<td>0^c</td>
</tr>
<tr>
<td>6</td>
<td><em>S. suis</em> 10cpsΔEF</td>
<td>3</td>
<td>3^c</td>
<td>1^d</td>
<td>1^e</td>
<td>1^d</td>
</tr>
<tr>
<td>6</td>
<td><em>S. suis</em> 10cpsΔEFΔsly</td>
<td>3</td>
<td>3^c</td>
<td>2^d</td>
<td>0^e</td>
<td>0^c</td>
</tr>
</tbody>
</table>

^a Days post infection, on which mice were sacrificed  
^b Infiltration of splenic red pulp with neutrophilic granulocytes  
^c Scoring of 4 and 5 indicates moderate to severe diffuse or multifocal fibrinosuppurative or purulent necrotizing inflammations of the indicated tissue  
^d Scoring of 2 and 3 indicates mild focal fibrinosuppurative or purulent necrotizing inflammation of the respective organs  
^e Individual single perivascular immune cells received a score of 1  
^f ω = Σ score\_max/animals (Baums et al, 2006); rhinitis is not included in the score ω. Noteworthy, lesions in the heart, kidney or liver were not recorded  
^g Mild histiocytic interstitial pneumonia associated with the challenge strain was observed in one mouse
Table 5-7: Reisolation of the challenge strains *S. suis* strain 10 (*sly*, *cps2*), 10Δsly (*sly*, *cps2*), 10cpsΔEF (*sly*, *cps2*), and 10cpsΔEFΔsly (*sly*, *cps2*) in intranasally infected Hsd:ICR (CD-1®) after predisposition with 1% acetic acid.

<table>
<thead>
<tr>
<th>No. of mice</th>
<th>Challenge strain</th>
<th>dpi(^a)</th>
<th>No. of mice in which the <em>S. suis</em> challenge strain(^b) was isolated/total no. of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>TNL(^{cd})</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+++</td>
</tr>
<tr>
<td>6</td>
<td><em>S. suis</em> strain 10</td>
<td>5</td>
<td>0/6</td>
</tr>
<tr>
<td>6</td>
<td><em>S. suis</em> 10Δsly</td>
<td>5</td>
<td>1/6</td>
</tr>
<tr>
<td>6</td>
<td><em>S. suis</em> 10cpsΔEF</td>
<td>3</td>
<td>0/6</td>
</tr>
<tr>
<td>6</td>
<td><em>S. suis</em> 10cpsΔEFΔsly</td>
<td>3</td>
<td>0/6</td>
</tr>
</tbody>
</table>

\(^a\) Days post infection, on which mice were sacrificed

\(^b\) Isolation of the challenge strain was confirmed in a multiplex PCR for detection of *mrp, sly, epf, arcA, cps1, cps2, cps7,* and *cps 9* (*Silva et al., 2006*), a *cps2E* specific PCR, and a *sly* specific PCR

\(^c\) Tracheo-nasal lavage

\(^d\) Based on CFU per mg organ or per μl TNL, bacterial loads was classified as mild (+; <100), moderate (++; ≥100 but <1000) or severe (++; ≥1000); the challenge strain was not detected in kidney, heart, brain and liver
In conclusion, comparative evaluation of virulence of the wild type and the isogenic sly-mutant in the intranasal infection model suggested attenuation of the sly-mutant. Further experiments are necessary to clear why the wild type challenge strain was not detectable in inner organs and why pathohistological lesions of inner organs were relatively low, except for one case of severe encephalitis.

In vitro experiments with the respiratory epithelial cell line HEp-2 revealed a suilysin-dependent invasive phenotype of the isogenic unencapsulated mutant 10cpsΔEF (Seitz et al., submitted; cf. chapter 3). It is, however, not clear what role suilysin-dependent invasion of host cells might play for virulence. Unencapsulated S. suis strains are generally regarded as avirulent because of enhanced opsonophagocytosis (Charland et al., 1998; Smith et al., 1999). However, Smith et al. (1999) isolated the isogenic unencapsulated mutants 10cpsΔEF and 10cpsΔB from CNS, serosae and joints after intranasal infections of gnotobiotic piglets, though these piglets did not develop clinical signs of disease. Furthermore, unencapsulated non-typeable stains have been isolated from diseased pigs (Silva et al., 2006). Therefore, we used the intranasal mouse model to further investigate whether the unencapsulated strain 10cpsΔEF also showed a suilysin-dependent invasive phenotype by comparison with the double mutant 10cpsΔEFΔsly. In contrast to the experiment with the encapsulated strains, mice were sacrificed on the 3rd and not on the 5th day post infection to increase the probability of reisolation. However, neither the unencapsulated mutant 10cpsΔEF nor the isogenic suilysin double mutant (10cpsΔEFΔsly) was detectable in inner organs except for one reisolation for 10cpsΔEF from lung tissue (Table 5-6). Surprisingly, 50% morbidity (mild clinical symptoms) were observed in mice infected with 10cpsΔEF, of which one mouse (Table 5-S1) developed severe clinical symptoms (rapid abdominal breathing, kyphosis, and showed a weight loss >20%; Table 5-5) associated with a mild histiocytic interstitial pneumonia. The type of the latter lesion is atypical for S. suis infections. In comparison, only one out of six mice infected with 10cpsΔEFΔsly developed mild clinical symptoms, like shallow breathing and rough coat (16.7% morbidity). Though the comparison of morbidity suggested a further suilysin-dependent attenuation of the 10cpsΔEFΔsly double mutant, results have to be
interpreted with caution as the challenge strains were undetectable in inner organs. As discussed for the wild type strain, further experiments with younger animals might reveal different results.

The intranasal infection of mice with all different *S. suis* strains after acetic acid predisposition resulted in severe rhinitis in at least 50% of each group (Table 5-6). To avoid possible non-specific histopathological findings of the nose, rhinitis was excluded from the general pathoscore \( \omega \) and, therefore, differences in \( \omega \) between different strains are not due to rhinitis lesions (Table 5-5 and Table 5-6).

In all 4 groups at least 50% of mice were positive in the TNL for the respective challenge strain. Quantification of bacteria obtained from TNL revealed no significant difference between mice infected either with *S. suis* wild type strain 10 or its isogenic sly-negative mutant strain 10\( \Delta \)sly (Table 5-7). Comparably, infection with either 10cps\( \Delta \)EF or 10cps\( \Delta \)EF\( \Delta \)sly showed no clear difference of the recovered bacterial load of the upper respiratory tract, suggesting no deficiency in colonization of the sly-negative strains 10\( \Delta \)sly and 10cps\( \Delta \)EF\( \Delta \)sly.

In conclusion, an intranasal murine model for *S. suis* colonization and invasion was established in this work. Severe purulent necrotizing pneumonia and rhinitis, rather than meningitis, were common findings among infected mice. Furthermore, preliminary results suggested attenuation in virulence of an isogenic sly-mutant. But a suilysin-dependent phenotype with regard to early colonization was not recorded in these experiments. However, this murine model is suitable to study invasion and colonization of mucosal surfaces in mice in the future.
Material and Methods

Bacterial strains and culture conditions. *Streptococcus (S.) suis* serotype 2 wild type strain 10 was kindly provided by H. Smith (Lelystad, NL). This strain expresses EF, MRP, SLY, FBPS and OFS. It has been used by different groups successfully for mutagenesis and experimental intranasal infections of pigs (Baums *et al.*, 2006; Smith *et al.*, 1999; Vecht *et al.*, 1997). Strain 10cpsΔEF has been generated by insertional mutagenesis of the genes *cps2E* and *cps2F* involved in biosynthesis of the capsule and has been demonstrated to be severely attenuated in virulence, most likely due to increased opsonophagocytosis (Smith *et al.*, 1999). The corresponding suilysin deficient mutants of wild type strain 10 (designed 10Δsly) and 10cpsΔEF (designed 10cpsΔEFΔsly) were constructed as described previously (Benga *et al.*, 2008; Seitz *et al.*, submitted; cf. chapter 3). Streptococci were grown on Columbia agar supplemented with 7% sheep blood (Oxoid, Wesel, Germany) overnight under aerobic conditions at 37°C.

Preparation of infection culture. For infection of mice, streptococci were grown in Todd-Hewitt broth (THB, Difco, Detroit, USA) overnight at 37°C under aerobic conditions, adjusted to an optical density (OD$_{600}$) of 0.02 in pre-warmed media the next day and then grown to late exponential growth phase (OD$_{600}$ 0.8). Streptococci were harvested by centrifugation, resuspended in sterile PBS (pH 7.4) and adjusted to the final concentration of $5 \times 10^{11}$ CFU/ml for intranasal infection. Inoculum concentrations were verified by platting 10-fold serial dilutions on Colombia agar plates with 7% sheep blood after infection.

Intranasal infection of mice. Four (expt. 1 to 4) or six (expt. 5) week old specific-pathogen-free female mice of the outbreed strain Crl:CD1 (ICR) were obtained from Charles River Laboratories (Sulzfeld, Germany). Mice of the *Staphylococcus (St.) aureus* free outbred strain Hsd:ICR (CD1®) were purchased from Harlan Laboratories (AN Venray, The Netherlands). Animals were randomly divided into groups consisting of five to six animals each. Mice were allowed to acclimate for one week.
and cared for in accordance with the principles outlined in the European Convention for the Protection of Vertebrate Animal Used for Experimental and Other Scientific Purposes [European Treaty Series, no. 123: http://conventions.coe.int/treaty/EN/Menuprincipal.htm; permit no.33.9-42502-04-08/1589]. Before infection mice were anesthetized via inhalation of isofluran (IsoFlo®, Albrecht, Germany). In experiment 2 to 5 mice were pre-treated with 12.5 µl 1% acetic acid [pH 4.0] placed in each nostril 1 h prior intranasal infection. After a controlled recovery phase and re-anaesthesia per isofluran inhalation, mice were infected with $5 \times 10^9$ CFU of either $S.\ suis$ wild type strain 10, $10\Delta\text{sly}$, $10\text{cps}\Delta\text{EF}$ or $10\text{cps}\Delta\text{EF}\Delta\text{sly}$. The dose was applied in two drops of 12.5 µl volume placed in front of the nostrils.

**Clinical score.** Animals were clinically scored every 8 h. The health status was rated using a clinical score sheet (Table 5-1), including weight development, clinical signs of general sickness (rough coat, rapid breathing, dehydration), clinical signs indicating meningitis (apathy, apraxia), and septicaemia (swollen eyes, depression), developed on the basis of mouse clinical monitoring score described by the Research office of the Australian National University [http://www.anu.edu.au/ro/ORI/Animal/ AEEC001_MouseMonitoringSOP.doc]. A cumulative score of 3 to 4 indicated mild clinical symptoms, a score of 5 to 6 moderate clinical symptoms and a score greater than 6 severe clinical symptoms, in particular persistent anorexia, apathy, and/or neural disorder. Mice with a cumulative score equal or greater than 3 were classified as diseased (calculation of morbidity). In the case of severe weight loss (> 20%) and/or enduring severe clinical signs, mice were euthanized for reasons of animal welfare by inhalation of CO$_2$ and cervical dislocation.

**Histological screening.** Immediately after euthanasia, necropsy was conducted and the organs were aseptically removed and split for histological and bacteriological screenings, including spleen, liver, kidney, heart, lung, brain, spinal cord, and nose. The histological screenings were carried out as blind experiments. Findings were
scored as described for piglets (Baums et al., 2006). In contrast to piglets, in addition to fibrinosuppurative lesions, purulent necrotizing lesions associated with the challenge strain were scored as well (see results). Rhinitis was not included in the general score ω designed to reflect lesions caused by invading streptococci.

Reisolation of *S. suis* strains from tissue and tracheo-nasal lavage (TNL). One half of each organ was suspended in 5 ml PBS [pH 7.4] and weighed. All organs were homogenized with an Ultra Turrax (IKA, Staufen, Germany). Ten-fold serial dilutions of samples were plated on blood agar plates. Colony forming units (CFU) were counted the next day after incubation at 37°C for 24 h and CFU per mg organ was determined.

For sampling TNL the trachea was opened and a retrograde irrigation of the nasal cavity with 300 µl PBS was collected. Number of typical α-haemolytic streptococci per µl TNL was determined by serial plating on blood agar. Isolated α-haemolytic streptococci were investigated in a *S. suis* multiplex PCR for the detection of *mrp, epf, sly, arcA, gdh, cps1, cps2, cps7*, and *cps9* (Silva et al., 2006). Isolates received from mice challenged either with strain 10Δsly, 10cpsΔEF or 10cpsΔsly were additionally tested in a *cps2E*-specific PCR (with oligonucleotide primers *cps2Efor* (TTTCGCACTTTCAAGACGTG) and *cps2Erev* (GGACGGGTACCGACTAGACTC) and *sly*-specific PCR using primers *slyAgefor* (TGTACCGGTGATTCCAAACAAGATATTAA) and *slyAge3new* (TTAACCGGTACTCTATCACCTCATCCG). Based on CFU per mg organ or per µl TNL, bacterial loads were classified as mild (+; <100), moderate (++; ≥100 - <1000) or severe (+++; >1000).
Acknowledgements

We thank Hilde Smith (DLO-Institute for Animal Science and Health, The Netherlands) for providing strain 10 and the non-encapsulated isogenic mutant 10cpsΔEF. Oliver Goldmann (Helmholtz Centre for Infection Research, Braunschweig, Germany) kindly introduced us to intranasal application techniques and anaesthesia of mice.

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References


Table 5-S1: Register of all infected mice including the clinical course of infection, scoring of fibrinosuppurative and purulent necrotizing lesions and reisolation of the challenge strain (table includes intravenously infected mice).

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<th>CFUb</th>
<th>dpi</th>
<th>Morbiditye</th>
<th>Mortality</th>
<th>Severe clinical symptomsf</th>
<th>Maximum weight loss (%)</th>
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<sup>a</sup> Intranasal (i. n.) or intravenous (i. v.) application of bacterial inoculum  
<sup>b</sup> Volume of 1% acetic acid applied to each nostril 1 hr pre infection  
<sup>c</sup> Infection dose  
<sup>d</sup> Days post infection, on which mice were sacrificed or killed for reasons of animal welfare  
<sup>e</sup> Mice with a cumulative clinical score greater >3 were regarded as diseased  
<sup>f</sup> In particular persistent anorexia, apathy, and/or neural disorder leading to a cumulative clinical score >6
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Results, part III

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\(^g\) Coinfection with *St. aureus* (isolation of *S. suis* and *St. aureus* from different affected tissues)

\(^h\) Infiltration of splenic red pulp with neutrophilic granulocytes

\(^i\) Scoring of 4 and 5 indicates moderate to severe diffuse or multifocal fibrinosuppurative or purulent necrotizing inflammations of the indicated tissue

\(^j\) Scoring of 2 and 3 indicates mild focal fibrinosuppurative or purulent necrotizing inflammation of the respective organs

\(^k\) Individual single perivascular immune cells received a score of 1

\(^l\) Severe necrotizing hepatitis associated with the challenge strain

\(^m\) Mild histiocytic interstitial pneumonia associated with the challenge strain
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\(^n\) Isolation of the challenge strain was confirmed in a multiplex PCR for detection of \textit{mrp}, \textit{sly}, \textit{epf}, \textit{arcA}, \textit{cps1}, \textit{cps2}, \textit{cps7}, and \textit{cps 9} (Silva et al., 2006) a \textit{cps2E} specific PCR, and a \textit{sly} specific PCR; Based on CFU per mg organ or per µl TNL, bacterial loads were classified as mild (+ <100), moderate (100 ≤ ++ <1000) or severe (+++ >1000).

\(^a\) Tracheo-nasal lavage;

\(^p\) Departed; estimation of bacterial load (<8 h after death) or determination not applicable
Chapter 6

General discussion
General discussion

*S. suis* is considered as a major problem worldwide due to high economical losses in swine husbandry. To reduce infection threat preventive antimicrobial medication and early treatment with antibiotics in the case of clinical signs are the only treatment options. Moreover, most currently available vaccines only provide homologous protection. Therefore, understanding of pathogenesis and identification of bacterial virulence (-associated) factors involved in colonisation, persistence, invasion and immune evasion are urgently needed for better control and prevention measures. Suilysin, the haemolysin of *S. suis*, has been identified many years ago (Jacobs et al., 1994), but is still controversially discussed as a virulence factor of *S. suis*. In vivo experiments using suilysin knock-out mutants demonstrated attenuation in virulence in mice, but only slight attenuation was seen in piglets (Allen et al., 2001; Lun et al., 2003). Furthermore, immunisation studies with a vaccine containing purified suilysin completely protected mice against challenge with a highly virulent *S. suis* serotype 2 strain, but failed to fully protected piglets (Jacobs et al., 1994; Jacobs et al., 1996). Nevertheless, the *sly* gene is prevalent in almost all highly virulent *S. suis* strains isolated from diseased pigs and humans in Europe and Asia. Taken together suilysin is currently considered to contribute to virulence, though the toxin seems not to be essential for pathogenesis. At present, it remains unclear which biological activities are relevant for virulence of *S. suis*. Suilysin belongs to the large family of cholesterol-dependent pore-forming cytolysins (CDC), thus membrane damage followed by cell lysis (Figure 6-1A) is the obvious but possibly not the only biological effect which contributes to pathogenesis (Billigton et al., 2000).

As described in the general introduction, pathogenesis of *S. suis* includes three crucial steps. Briefly, colonisation and invasion, dissemination within the bloodstream and finally translocation into target tissues (Gottschalk and Segura, 2000). In chapter 3 biological activities of suilysin which play a role in early stages of *S. suis* pathogenesis were investigated. The first barrier of defence after the intranasal route of infection is the mucosal epithelium lining of the upper respiratory tract. Although *S. suis* is considered an extracellular pathogen, interaction with epithelial cells to
breach the mucosal barrier is a crucial step. It is largely unknown which bacterial factors and host cell components are involved in this process. The present study showed that suilysin promotes invasion of an unencapsulated \textit{S. suis} serotype 2 strain \textit{in vitro} in a trigger-like uptake mechanism associated with membrane ruffles (Figure 3-1). The polysaccharide capsule is assumed to interfere with the ability of \textit{S. suis} to interact with host tissue (Benga \textit{et al.}, 2004; Vanier \textit{et al.}, 2004). Since its main function is protection against phagocytosis after entering the bloodstream, it has been proposed that the capsule is down-regulated during colonisation of the mucosal epithelium to allow adherence and invasion (Gottschalk and Segura, 2000; Okamoto \textit{et al.}, 2004; Willenborg \textit{et al.}, 2011). In accordance, unencapsulated \textit{S. suis} strains showed a higher adherence and invasion rate in comparison to the well encapsulated wild type strains (Benga \textit{et al.}, 2004). Furthermore, we found evidence that the signal protein kinase Rac, belonging to the small family of Rho-GTPases, is a key regulator in suilysin promoted uptake of \textit{S. suis} (Figure 3-2). Recruitment of the Rho family guanosine triphosphatases, namely Rac1, Rho, Cdc42, for bacterial internalisation has been previously reported for several bacterial species (Agarwal and Hammerschmidt, 2009; Burnham \textit{et al.}, 2007; Criss and Casanova, 2003; Finlay, 2005). Rho-GTPases can act as targets of bacterial toxins (Aktories \textit{et al.}, 2000) like the cytotoxic necrotizing factors CNF1 and CNF2 from \textit{Escherichia coli} (Schmidt and Aktories, 2000) or the \textit{Salmonella} outer protein E (Hardt \textit{et al.}, 1998). An interaction of CDC with G-proteins has only been described for pneumolysin so far (Iliev \textit{et al.}, 2007).

It still remains open whether these \textit{in vitro} results reflect the \textit{in vivo} situation of \textit{S. suis} pathogenesis. Therefore, an intranasal mouse infection model for colonisation and invasion for \textit{S. suis} was established (\textbf{chapter 5}). As asymptptomatically colonized carrier pigs play a key role in epidemiology of \textit{S. suis}, a important step in pathogenesis is sufficient colonisation of (respiratory) mucosal surfaces. Using an intranasal instillation of a highly virulent \textit{S. suis} serotype 2 strain after nasal predisposition with acetic acid we were able to induce colonisation of the upper respiratory tract (Table 5-4). We observed that invasive disease associated with rhinitis, pneumonia and/or meningitis was only seen after pretreatment with acetic
acid. This corresponds well to the situation of natural infection, where coinfections with other pathogenic agents, such as PRRSV or \textit{Bordetella bronchiseptica}, mucosal irrigation or stress are predisposing factors for \textit{S. suis} infections (Pallares \textit{et al.}, 2003). In contrast to typical histopathological lesions for \textit{S. suis} infection in swine, which are characterized by fibrinopurpurative inflammation of the respective tissue (Beineke \textit{et al.}, 2008; Williams and Blakemore, 1990), we observed purulent necrotizing alteration of the respiratory apparatus (nose and lung) and the central nervous system (CNS) in mice. Similar pathologies of the CNS were described for mice after intraperitoneal challenge (Dominguez-Punaro \textit{et al.}, 2007; Vecht \textit{et al.}, 1997). Presumably, induction of necrosis is directly linked to cytotoxic effects of suilysin as observed for different cell types \textit{in vitro} (Charland \textit{et al.}, 2000; Gottschalk and Segura, 2000; Lalonde \textit{et al.}, 2000; Norton \textit{et al.}, 1999; Tenenbaum \textit{et al.}, 2005; Vanier \textit{et al.}, 2004). Tenenbaum \textit{et al.} (2006) found necrosis of porcine choroid plexus epithelial cells after incubation with a \textit{sly}-positive \textit{S. suis} strain. A similar distribution of \textit{S. suis} and suilysin in affected brain tissues of experimental infected pigs associated with massive infiltration of neutrophils and acute necrosis of neurons was described by Zheng \textit{et al.} (2009).

To further investigate the role of suilysin in early stages of \textit{S. suis} pathogenesis \textit{in vivo} we applied this model to perform a comparative study using a highly virulent serotype 2 strain, its isogenic \textit{sly}-negative strain (10Δsly), as well as the respective isogenic unencapsulated strains (10cpsΔEF and 10cpsΔEFΔsly). Preliminary results suggested attenuation of \textit{sly}-deficient strains indicated by reduced morbidity and less histopathological changes (Table 5-5 and Table 5-6). In contrast, colonisation of the respiratory epithelium by suilysin-negative strains was unaffected. Thus, suilysin seems to be not critical for colonisation, but contributes to invasion of \textit{S. suis} \textit{in vivo}. Nevertheless, the results are not conclusive because induction of invasive disease such as meningitis or pneumonia failed in this trial except for two cases of meningitis in the wild type infected group and rhinitis in all infected animals. For the closely related pneumococci proven intranasal pneumonia and bacteraemia models in mouse have been described previously (Berry \textit{et al.}, 1989; Medina, 2010; Saeland \textit{et al.}, 2000). Intranasal challenge studies showed that pneumolysin deficient mutant
strains were less virulent in mice indicated by a significant longer median survival time, reduced apathetical alterations in the lung and diminished onset of bacteraemia (Alexander et al., 1998; Berry et al., 1989; Jounblat et al., 2003). In contrast, pneumolysin production was not required for successful nasopharyngeal colonization (Rubins et al., 1998).

As described in chapter 3, we observed that suilysin effects did not require cytolytic activities. Therefore, to further dissect functional domains involved in biological activities of suilysin, we introduced point-mutations of the tryptophan-rich pore-forming region (W461F) and the RGD-motif (SVD) within the sly gene (chapter 4). Both mutations resulted in a loss of haemolytic and cytolytic activity (Figure 4-2). The tryptophan-mutant had retained its ability to bind to the host-cell membrane and to activate Rac. In contrast, an unmodified RGD-motif seems to be required for efficient membrane binding (Figure 4-6) and more strikingly for activation of Rac1 (Figure 4-7). Therefore we hypothesized that the RGD-motif of suilysin interacts with heterodimeric transmembrane cell surface receptors, namely α5β1 integrins, to mediate suilysin-promoted activation of Rac1 and subsequent invasion of S. suis in target cells. In addition, the proven cholesterol dependency of suilysin would be explainable since integrins are most frequently expressed on cholesterol enriched microdomains (lipid rafts) (Lafont and van der Goot, 2005). Nevertheless, it remains hypothetical and the role of the first domain of suilysin and integrins in S. suis-host cell interaction should be addressed in future studies.

Based on our results we propose the following model of suilysin-promoted invasion of the upper respiratory tract by S. suis after colonisation of the mucosal surface as illustrated by Figure 6-1B. Secreted suilysin binds to cholesterol enriched microdomains (lipid rafts?) of the host cell membrane either via domain 4 and/or via an interaction between α5β1 integrins and the RGD-motif of domain 1 (1). If applicable, the prepore complex is formed and a downstream suilysin-mediated activation of Rac1 (2) leads to induction of F-actin (3) and the formation of membrane ruffles. Finally, S. suis is taken up by the cell (4). Suilysin-promoted invasion into host tissue and intracellular persistence might be crucial for bacterial pathogenesis, since they allow pathogens to escape the immune system for further dissemination and invasive
disease development (Finlay and Falkow, 1997; Garzoni and Kelley, 2009). On the other hand suilysin might be beneficial for the host, since subcytolytic concentrations of suilysin may be sensed by epithelial cells to commence an early onset of innate immune response in the host as proposed by Ratner et al. (2006) for other bacterial toxins. This study contributed to the knowledge on virulence mechanisms of S. suis, but many open questions remain to be addressed in future studies to further dissect mechanisms underlying pathogenesis and to evaluate the role of suilysin in virulence of S. suis.

![Hypothetical model of suilysin-promoted interaction of S. suis with epithelial cells.](image)

Figure 6-1: Hypothetical model of suilysin-promoted interaction of S. suis with epithelial cells.

(A) Direct cytotoxic effects of suilysin: Pore-formation followed by cell lysis modified from Gottschalk et al. 2010; Willenborg (unpublished). (B) Subcytolytic effects of suilysin: Suiysin-induced invasion of S. suis by a Rac1-dependent remodelling of the actin cytoskeleton. Secreted suilysin binds to the membrane, forms the prepore complex (1) and activates Rac1 (2). Activation of Rac1 leads to induction of F-actin (3) and the formation of membrane ruffles which results in an uptake of S. suis by the cell (4).
References


Chapter 7

Summary
Summary

*Streptococcus* (*S.*) *suis* is a swine pathogen of the upper respiratory tract causing meningitis, arthritis and septicaemia. Due to high economical losses *S. suis* is considered as an important pathogen in pig husbandry worldwide. Asymptomatically colonized carrier-pigs are the most important source of infection. Moreover, *S. suis* is a zoonotic agent associated with meningitis and the *streptococcal toxic shock like syndrome* (STSS) in humans.

Suilysin is a secreted virulence-associated haemolysin of *S. suis*. It is present in many *S. suis* strains and belongs to the family of Cholesterol-dependent pore-forming cytolysins (CDC). At high concentrations these multifunctional toxins lyse host cells. Additional biological activities of CDC such as the activation of host cells or immune modulation, occur at subcytolytic concentrations. The role of suilysin in pathogenesis of *S. suis* is still unclear.

The aim of this study was to analyse the role of suilysin in pathogen-host cell interaction and to characterise the molecular mechanisms underling suilysin-mediated biological effects.

Using an isogenic suilysin-negative mutant strain and the human respiratory epithelial cell line HEp-2 a suilysin-dependent invasive phenotype of an unencapsulated *S. suis* serotype 2 strain was identified *in vitro*. Subcytolytic concentration of suilysin activated the small Rho-GTPase Rac1, which resulted in formation of membrane ruffles and finally led to the uptake of *S. suis* by the cell. Furthermore, point-mutation of the putative pore-forming region of the suilysin gene abolished cytolytic activity, but activation of the G-protein Rac1 was unaffected. A second putative functional region, a RGD-motif, was identified and also point-mutated, resulting in loss of lytic activity, membrane binding and the ability to activate Rac1. Thus, the RGD-motif is proposed to be involved in suilysin-mediated host cell-activation. Integrins as possible interaction partners were identified on the surface of HEp-2 cells, but further studies are needed to proof the precise role of integrins in suilysin-mediated host cell-interaction.
After characterization of suilysin-dependent effects \textit{in vitro}, an intranasal mouse infection model for \textit{S. suis} was established to investigate the role of suilysin in colonisation and invasion of \textit{S. suis} \textit{in vivo}. After predisposition with acetic acid followed by mucosal application of a \textit{S. suis} serotype 2 strain we were able to induce colonisation of the upper respiratory tract as well as invasive disease associated with rhinitis, pneumonia and/or meningitis in mice. The suilysin-negative mutant strain colonised the respiratory epithelium, but was slightly attenuated in virulence in comparison to the wild type strain.

Taken together, these results show that subcytolytic activity of suilysin plays a role in pathogen-host cell interaction. The established mouse model is suitable to study the relevance of subcytolytic activities of suilysin \textit{in vivo} in the future. The results of this work improved our understanding of \textit{S. suis} pathogenicity mechanisms.
Chapter 8

Zusammenfassung
Zusammenfassung


Ziel dieser Arbeit war es daher, die Bedeutung von Suilysin in der Erreger-Wirtszell-Interaktion und die molekularen Mechanismen Suilysin-vermittelter biologischer Effekte näher zu untersuchen.

Mit Hilfe einer isogenen Suilysin-negativen Mutante konnte in in vitro Untersuchungen an der humanen respiratorischen Epithelzelllinie HEp-2 ein Suilysinabhängiger invasiver Phänotyp eines unbekapselten S. suis-Serotyp-2-Stammes gezeigt werden. Die durch subzytolytische Suilysin-Konzentration induzierte Aktivierung der kleinen Rho-GTPase Rac1 vermittelte hierbei die Ausbildung von membrane ruffles, wodurch S. suis in die Zelle aufgenommen wurde. Des Weiteren konnte durch gezielte Punktmutagenese im Suilysin-Gen der putative Poren-bildende Bereich ausgeschaltet werden, was zu einem Verlust der zytolytischen Aktivität führte, nicht aber die G-Protein-aktivierende Eigenschaft des Suilysins beeinflusste. Neben dem für die Porenbildung notwendigen Bereich im Suilysin-Gen, wurde eine...

Nach der Charakterisierung Suilysin-abhängiger Effekte im Zellkultursystem wurde ein intranasales Mausinfektionsmodells für \textit{S. suis} etabliert, um die Bedeutung des Suilysins für die Kolonisation und Invasion von \textit{S. suis in vivo} zu untersuchen. Nach vorheriger Prädisposition mit Essigsäure war es möglich durch mukosale Applikation des \textit{S. suis}-Serotyp-2-Stammes eine Besiedlung des oberen Respirationstraktes sowie das Auftreten von Rhinitis, Pneumonie und/oder Meningitis in der Maus zu induzieren. Im Vergleich zum Wildtyp-Stamm erwies sich die Suilysin-negative Mutante als geringgradig virulenzattenuiert, wohingegen keine Unterschiede in der Kolonisation beobachtet wurden.

Chapter 9

Literature
Literature


Seitz, M., Baums, CG, Gerhard, R., Just, I, Neis, C., Benga, L., Fulde, M., Nerlich, A, Rohde, M., Goethe, R., and Valentin-Weigand, P. Subcytolytic activity of suilysin promotes invasion of *Streptococcus suis* in HEP-2 epithelial cells by Rac-dependent activation of the actin cytoskeleton. Ref Type: submitted to Cellular Microbiology


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