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Subcytolytic effects of suilysin on interaction of *Streptococcus suis* with epithelial cells

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ABSTRACT

Suilysin is a pore-forming cholesterol-dependent cytolysin secreted by *Streptococcus suis* (*S. suis*), an important swine and zoonotic pathogen. The role of suilysin in *S. suis* host–cell interaction is still unclear. We found a higher adherence and invasion rate of an unencapsulated *sly*-positive strain in comparison to its isogenic *sly*-negative mutant. Electron microscopy revealed that formation of membrane ruffles accompanying invasion of the *sly*-positive strain was abolished in the *sly*-negative mutant. Inhibition experiments showed that the actin cytoskeleton was involved in suilysin-mediated effects. Point-mutation of the domain putatively responsible for macropore-formation resulted in abolished hemolytic and cytolysin activity, but had no effect on *S. suis* host cell association. Concluding, our results indicate that subcytolytic suilysin promotes *S. suis* association with epithelial cells.

1. Introduction

Streptococcus suis (*S. suis*) is one of the most important swine pathogens worldwide causing meningitis, arthritis, septicaemia, bronchopneumonia, and other pathologies. Furthermore, *S. suis* is an important zoonotic agent (Gottschalk et al., 2007). 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). Despite its emerging role as a (zoonotic) pathogen in swine the molecular mechanisms of *S. suis* virulence are only poorly known. Suilysin was identified as hemolysin of *S. suis* nearly two decades ago (Jacobs et al., 1994). It is a member of the pore-forming cholesterol-dependent cytolysin (CDC) family and its crystal structure was recently determined (Xu et al., 2010). 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 capsular serotypes. Though these isolates were mainly obtained from porcine cases of meningitis and septicaemia (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; Segura and Gottschalk, 2002; 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 subcytolytic concentrations, *e.g.* phosphorylation of p38

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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 *Streptococcus intermedius* (intermedilysin) it was reported that they may 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.* the extracellular adherent and/or intracellular bacteria. Using respective mutant strains and HEp-2 epithelial cells we identified a suilysin-dependent invasive phenotype of an unencapsulated *S. suis* serotype 2 strain. We showed that these effects did not require formation of a functional (cytolytic) pore.

2. Materials and methods

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

2.1. Bacterial strains and growth conditions

The unencapsulated isogenic mutant strain 10cpsΔEF of the virulent *S. suis* serotype 2 strain 10 was kindly provided by H. Smith (Lelystad, NL). It was originally generated though insertion of a spectinomycin resistance cassette in the genes *cps*2E and *cps*2F (Smith et al., 1999). The corresponding suilysin deficient mutant, strain 10cpsΔEFΔsly, was constructed by insertion of an erythromycin cassette in the *sly* gene of strain 10cpsΔEF using the plasmid pBlue/sly/erm as previously described (Benga et al., 2008). Mutants were controlled by PCR and Southern Blot analysis. *Escherichia coli* (*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_{600}) of 0.02 in pre-warmed media the next day and grown to late exponential growth phase (OD_{600} 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 2 μg/ml for *S. suis*; ampicillin 100 μg/ml for *E. coli*.

2.2. 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).

2.3. Expression of recombinant 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, Ja Jolla, CA). Purified plasmid DNA carrying the *sly* gene (pET45bslynew) was amplified using oligonucleotide primers encoding the W461 to F461 substitution (slyTrp-Phefornew [TACAGGATTAG-CATTTGAGTGGTGGAGAAC], slyTrp-Pherevnew [GTTCTCC-ACCACTCAAATGCTAATCCTGTAC]). The resulting plasmid pET45bslynewW461F was electroporated into *E. coli* BL21 (DE3), purified and controlled by sequencing. Expression and purification under native conditions of rSLY and rW461F were performed as described previously (Benga et al., 2008; Willenborg et al., 2011). Purified proteins were controlled by separation in SDS-polyacrylamide gels and immunoblot analysis using with anti-His antibodies (Qiagen, Hilden, Germany).

2.4. 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. Polyclonal antiserum raised against rSLY (Benga et al., 2008) diluted 1:1300 in 1% milk powder was used to detect either rSLY, rW461F or secreted suilysin in supernatants of infected cells. For detection of His-tagged recombinant proteins membranes were blocked with 3% BSA and incubated with a monoclonal anti-Penta His antibody (Qiagen) diluted 1:2000 in 3% BSA. Membranes were developed with horseradish peroxides-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.

2.5. 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 1.8×10^5 cells per well were seeded on 24 well tissue culture plates. For immunofluorescence and electron microscopy 0.5×10^5 and 1.5×10^5 cells per well were