Streptococcus suis DNase SsnA contributes to degradation of neutrophil extracellular traps (NETs) and evasion of NET-mediated antimicrobial activity

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Streptococcus suis is an important cause of different pathologies in pigs and humans, most importantly fibrinosuppurative meningitis. Tissue infected with this pathogen is substantially infiltrated with neutrophils, but the function of neutrophil extracellular traps (NETs) - a more recently discovered antimicrobial strategy of neutrophils - in host defence against Strep. suis has not been investigated. The objective of this work was to investigate the interaction of Strep. suis with NETs in vitro. Strep. suis induced NET formation in porcine neutrophils and was entrapped but not killed by those NETs. As the amount of NETs decreased over time, we hypothesized that a known extracellular DNase of Strep. suis degrades NETs. Though this nuclease was originally designated Strep. suis-secreted nuclease A (SsnA), this work demonstrated surface association in accordance with an LPXTG cell wall anchor motif and partial release into the supernatant. Confirming our hypothesis, an isogenic ssnA mutant was significantly attenuated in NET degradation and in protection against the antimicrobial activity of NETs as determined in assays with phorbol myristate acetate (PMA)-stimulated human neutrophils. Though assays with PMA-stimulated porcine neutrophils suggested that SsnA also degrades porcine NETs, phenotypic differences between wt and the isogenic ssnA mutant were less distinct. As SsnA expression was crucial for neither growth in vitro nor for survival in porcine or human blood, the results indicated that SsnA is the first specific NET evasion factor to be identified in Strep. suis.

INTRODUCTION

Streptococcus suis ranks among the five most important porcine pathogens worldwide causing mainly meningitis, but also other pathologies such as septicaemia, arthritis, serositis and endocarditis (Gottschalk, 2011). The pathogen is characterized by a great diversity of serotypes. Serotype 2 is worldwide the most prevalent among invasive isolates (Wei et al., 2009; Wisselink et al., 2000). Strep. suis meningitis can cause high rates of mortality in affected herds.

Furthermore, Strep. suis serotype 2 is also an important zoonotic pathogen causing meningitis and septicaemia in humans working with pigs or pork (Arends & Zanen, 1988; Gottschalk et al., 2010). Two Strep. suis outbreaks in China attracted substantial scientific interest as they were associated with streptococcal toxic shock-like syndrome and a high rate of mortality in humans (Tang et al., 2006). Further epidemiological studies in south-east Asia revealed that Strep. suis is one of the three most important causative agents of bacterial meningitis in adults in countries such as Vietnam and Thailand (Maï et al., 2008; Wangkaew et al., 2006).

Meningitis lesions in pigs (or humans) infected with Strep. suis are typically characterized by infiltrations with large numbers of neutrophils (Beineke et al., 2008; Lun et al., 2007; Vasconcelos et al., 1994; Williams & Blakemore, 1990). Neutrophils mediate the first line of innate immune defence against invading bacterial pathogens. The antimicrobial activity of these cells includes a combination of intracellular and extracellular killing mechanisms, i.e.
phagocytosis and degranulation of antimicrobial peptides, respectively. More recently, neutrophil extracellular traps (NETs) were characterized as a novel important defence mechanism of neutrophils against various bacterial pathogens (Brinkmann et al., 2004). These NETs consist of a DNA backbone associated with antimicrobial peptides, histones and proteases and can be released by activated neutrophils in response to infection and inflammation (reviewed by von Köckritz-Blickwede & Nizet, 2009). Two main functions of NETs can be differentiated: entrapment of bacteria in NETs to avoid subsequent spread into the surrounding tissue, and killing of bacteria within the NETs. It is still unclear whether NETs contribute to entrapment and/or killing of Strep. suis by activated neutrophils.

Importantly, in the absence of opsonizing antibodies, virulent Strep. suis serotype 2 strains are not efficiently killed by porcine neutrophils (Baums et al., 2009; Chabot-Roy et al., 2006). However, Scapinello et al. (2011) investigated the bactericidal activity of porcine neutrophil secretions against different bacteria including Strep. suis. The bactericidal effect of secretions of phorbol-myristate-acetate (PMA)-stimulated porcine neutrophils was significantly lower against Strep. suis than against Escherichia coli, indicating that Strep. suis has evolved efficient strategies to evade both intra- and extracellular antimicrobial activity of neutrophils. Several virulence-associated factors of Strep. suis have already been identified (Baums & Valentin-Weigand, 2009). One of the major phagocytosis evasion factors is the capsule (Charland et al., 1998; Smith et al., 1999). Isogenic unencapsulated mutants are highly attenuated in neutrophil killing assays. Furthermore, Strep. suis exhibits D-alanlyation of lipoteichoic acid, which is known to protect streptococci against cationic antimicrobial peptides released by neutrophils (Fittipaldi et al., 2008).

In this study we characterized the interaction of Strep. suis with NETs to unravel the strategies of Strep. suis to avoid extracellular NET-mediated entrapment and killing. We finally demonstrate that Strep. suis serotype 2 exhibits induction of NET formation, entrapment in NETs, resistance against antimicrobial activity of NETs and finally degradation of NETs by expression of the previously described DNase SsNA.

**METHODS**

**Bacterial strains and growth conditions.** Strep. suis strain 10 is a virulent serotype 2 strain that has been used by different groups for mutagenesis and experimental infections of pigs (Baums et al., 2006, 2009; Smith et al., 1999). The capsule-deficient isogenic mutant 10cpsAEF was kindly provided by Hilde Smith (DLO-Lelystad) (Smith et al., 1999). Streptococci were grown on Columbia agar plates with 6% sheep blood or in Todd–Hewitt broth (THB; Bacto). In appropriate cases, antibiotics were added at the following concentrations for Strep. suis: 3.5 μg chloramphenicol ml \(^{-1}\), 2 μg erythromycin ml \(^{-1}\) and 100 μg spectinomycin ml \(^{-1}\).

Staphylococcus aureus nuc mutant (Berends et al., 2010) was grown in Brain Heart Infusion (BHI) medium at 37 °C with shaking. Fresh overnight cultures were diluted 1 : 100 in BHI and then grown to mid-exponential growth phase (OD\(_{600}\) of 0.7) until usage.

**Purification of porcine neutrophils.** Collection of heparinized blood from healthy pigs in our institute is registered at the Lower Saxonian State Office for Consumer Protection and Food Safety (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit) under no. 33.9-42502-05-11A137, and was conducted in line with the recommendations of the German Society for Laboratory Animal Science (Gesellschaft für Versuchstierkunde) and the German Veterinary Association for the Protection of Animals (Tierarztliche Vereinigung für Tierschutz e. V.) (http://www.gv-solas.de). Porcine neutrophils were purified using Ficoll Hypaque 1077 (Biochrom) and hypotonic lysis of erythrocytes as previously described (Benga et al., 2008).

**Purification of human neutrophils.** Neutrophils were isolated from fresh blood of healthy donors with the PolymorphPrep system (Axis Shield) as previously described (von Köckritz-Blickwede et al., 2010) and resuspended in RPMI 1640 (without phenol red; PAA).

**NET induction and entrapment assays.** Freshly isolated porcine neutrophils were seeded on poly-l-lysine-coated (0.001 % solution, Sigma Aldrich) coverslides (8 mm; Thermo Scientific) at a concentration of 2 × 10⁶ cells ml \(^{-1}\) (5 × 10⁶ cells per well in a 24-well plate) after centrifugation at 370 g for 5 min. Stacks of frozen bacterial suspensions including 15 % glycerol were thawed, diluted in RPMI to infect neutrophils at an m.o.i. of 1 : 2 at 37 °C and 5 % CO₂. At indicated time points, samples were fixed with 4 % paraformaldehyde and NET formation or entrapment was visualized as described below.

**NET degradation assays.** Porcine or human neutrophils were stimulated with 25 nM phorbol 12-myristate 13-acetate (PMA), seeded for 20 min on poly-l-lysine-coated (0.001 % solution, Sigma Aldrich) coverslides (8 mm; Thermo Scientific) at a concentration of 2 × 10⁶ cells ml \(^{-1}\), centrifuged (370 g, 5 min) and incubated for 4 h at 37 °C and 5 % CO₂ to induce NET formation. Stocks of frozen bacterial suspensions including 15 % glycerol were thawed, then diluted in RPMI to infect neutrophils at an m.o.i. of 1 : 2 for an additional 1 h at 37 °C and 5 % CO₂. Cells were fixed with 4 % paraformaldehyde. Alternatively, NETs were treated with filtered bacterial supernatants (Rotilabo 0.45 μm; Roth) of overnight cultures as indicated (leading to a final dilution of supernatants of 1 : 2). Micrococcoc nuclease (0.01 U ml \(^{-1}\); Worthington) was used as a positive control to verify efficient NET degradation.

**Visualization of NETs and entrapment of bacteria by NETs.** NETs were stained with a monoclonal antibody against a histone H2A-H2B-DNA complex as previously described (Berends et al., 2010). Briefly, after blocking and permeabilization, neutrophils were incubated with a mouse monoclonal antibody against H2A-H2B-DNA complex (PL2-6) (Losman et al., 1992; 2.65 mg ml \(^{-1}\), diluted 1 : 2000 in PBS containing 2 % BSA, 0.2 % Triton X-100) overnight at 4 °C. Finally, an Alexa 488-conjugated goat anti-mouse antibody (Dy Light 488 conjugated highly cross-absorbed, Thermo Scientific; diluted 1 : 1000 in PBS containing 2 % BSA, 0.2 % Triton X-100) was used as the secondary antibody. To label Strep. suis in red (entrapment assays), samples were additionally incubated with a polyclonal rabbit anti-Strep. suis antibody (diluted 1 : 500) (Beineke et al., 2008) followed by a goat anti-rabbit Alexa 633-conjugated secondary antibody (1 : 500; Invitrogen). After washing, the glass slides were embedded in ProLong Gold antifade reagent with DAPI (Invitrogen). Samples were recorded using a Leica TCS SP5 confocal inverted-base fluorescence microscope with a HCX PL APO 40 x 0.75–1.25 oil immersion objective. Settings were adjusted with control preparations using an isotype control antibody. For each sample, a
Minimum of four randomly selected images per independent experiment were acquired and used for quantification of NET-producing cells (NET induction) or area covered with NETs (NET degradation). A detailed explanation of calculation of area covered with NETs to analyse NET degradation is shown in Fig. S1, available in Microbiology Online.

**Determination of antimicrobial activity of porcine NETs through comparison to growth controls not exposed to neutrophils.** Porcine neutrophils were plated in non-treated tissue culture plates as previously described (Berends et al., 2010) and treated with 25 nM PMA for 4 h to induce NETs, and then with 10 μM cytochalasin D for 20 min to block residual phagocytosis. Then, after centrifugation at 370 g for 5 min and removing the supernatant, the neutrophils were infected with bacteria in RPMI at an m.o.i. of 2, centrifuged again and incubated for 90 min at 37 °C in 5% CO₂. Serial dilutions in sterile PBS were plated on agar plates for enumeration of surviving bacteria. The percentage of surviving bacteria was calculated in comparison to bacterial growth control grown under the same conditions in the absence of cells.

**Determination of antimicrobial activity of porcine and human NETs on *Strep. suis* wt in comparison to 10ΔssnA.** Neutrophils were plated in non-treated tissue culture plates (Nunc, Sigma) and the antimicrobial activity of NETs was determined as previously described (Berends et al., 2010) except that FCS was not included. The survival factor was calculated for the time points at 30 min and 90 min by dividing the specific bacterial content (cf.u. ml⁻¹) determined for these time points by the starting content (cf.u. ml⁻¹ at 0 min).

**DNA techniques and primer sequences.** Routine DNA manipulations were performed as described (Sambrook et al., 1989). Primers were designed based on the sequence of SSU1760 in the genome of *Strep. suis* P117 (www.sanger.ac.uk). Chromosomal DNA of strain 10 served as the template in all PCRs conducted for generation of inserts. DNA fragments were amplified with Phusion polymerase (Promega).

**Targeted mutagenesis of ssnA.** In-frame deletion mutagenesis of ssnA was conducted in *Strep. suis* strain 10 with the thermosensitive plasmid pSET5ΔssnA. To construct this vector, a 922 bp 5'-ssnA amplification product generated with the primer pair ssnA_new_for_BamHI (5'-CCAGATCTGCAAACTATCTATG-3') plus ssnA_new_rev_SalI (5'-GGTGGCACTATAATGTTAAGGATAAA-3') and a 917 bp 3'-ssnA amplification product amplified with the primer pair ssnA_SalI_3 (5'-CCAGATCTGCAAACTATCTATG-3') plus ssnA_SphI_3 (5'-CCAGCTGCGAAAGGGCGAATTCCTT-3') were cut with the restriction enzymes indicated in the names of the primers. Both amplimons were inserted between the BamHI and PstI site of pSET5s (Takamatsu et al., 2001) to generate pSET5ΔssnA. Restriction analysis and sequencing were carried out to verify this plasmid and in particular the in-frame deletion. The temperature-sensitive replication of this vector enabled allelic exchange of *ssnA* as previously described (Baums et al., 1989). After blocking 5% dry milk powder in Tris-buffered saline (Twen 20) in TBST], membranes were incubated with a polyclonal rabbit serum raised against SsnA (Gomez-Gascón et al., 2012) in TBST (1:2000) with 1% dry milk powder. Blots were washed thoroughly four times. After labelling with a horse-radish peroxidase-conjugated antibody (1:7500; Cell Signaling Technology) signals were developed using Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific) as recommended by the manufacturer.

**Real-Time PCR from reverse transcribed RNA extracted from *Strep. suis*.** Bacterial RNA was extracted from exponential THB cultures of *Strep. suis* wt and the isogenic mutant 10ΔssnA as described previously (Willenberg et al., 2011). Real-time PCR of reverse transcribed RNA (qRT-PCR) was designed to analyse expression of four genes in close proximity to *ssnA* (purA and gshA upstream as well as hslO and SSU1762 downstream of *ssnA*), two randomly selected virulence-associated genes (sly and ofs) and the housekeeping gene gyrb. The respective primers are listed in Table S1. The qRT-PCR was conducted as described previously (Willenberg et al., 2011) with the following modified program: initial denaturation at 95 °C for 20 min and 40 cycles of denaturation at 95 °C for 25 s, annealing at 55 °C for 30 s, and amplification at 72 °C for 20 s. Products were verified by melting curve analysis and 1.5% agarose gel electrophoresis. Data were normalized to a non-regulated housekeeping gene (gyrb). The relative ΔC_T values were determined for expression of the six targeted genes in *Strep. suis* wt and the mutant 10ΔssnA. C_T is the cycle number at the chosen amplification threshold, ΔC_T = C_T gene - C_T Reference (gyrb), and ΔΔC_T = ΔC_T *ssnA - ΔC_T *ssnA Calderon (sly). The fold change in expression (2−ΔΔC_T) was calculated as the read-out parameter.

**Preparation of murein-associated proteins (MAPs).** The MAP fractions of *Strep. suis* wt (strain 10) and its isogenic mutant 10ΔssnA were essentially prepared as previously described (Baums et al., 2009). Briefly, bacteria grown to an OD_600 of 0.7 at 37 °C were centrifuged and resuspended in a buffer containing 30 mM Tris/HCl pH 7.5, 25% (v/v) sucrose, 0.01 M NaEDTA and 0.2 mg ml⁻¹ lysozyme. After incubation at 37 °C for 1 h, the resulting protoplasts were centrifuged. The supernatant was recovered, and MAPs were resuspended in PBS after precipitation.

**SDS-PAGE and Western blot analysis.** Separation of proteins was conducted in SDS-PAGE with 10% separating and 4% stacking gels. For Western blot analysis, proteins were transferred to a nitrocellulose membrane (Roth) by standard procedures (Sambrook et al., 1989). After blocking 5% dry milk powder in Tris-buffered saline (Twen 20) incubated for 4.5 h at 37 °C supplemented with DNase buffer, respectively. Samples were incubated with 1 : 2000) with 1 % dry milk powder. Blots were washed thoroughly four times. After labelling with a horse-radish peroxidase-conjugated antibody (1 : 7500; Cell Signaling Technology) signals were developed using Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific) as recommended by the manufacturer.

**Nuclease assay.** Nuclease activity of bacterial supernatants and pelleted bacteria was determined by testing different conditions. Bacteria were grown overnight and pelleted via centrifugation (2600 g, 5 min). The supernatants were transferred to new tubes after filtration (Rotilabo 0.45 μm; Roth) and mixed with either RPMI 1640 or THB supplemented with 50% DNaSE buffer (3 mM MgCl₂, 3 mM CaCl₂, 300 mM Tris; pH 7.4) in ratios of 1 : 1 and 1 : 2, respectively. Two μg calf thymus DNA (Sigma) was added to 70 μl and 105 μl supernatants diluted with RPMI 1640 and THB supplemented with DNaSE buffer, respectively. Samples were incubated for 4.5 h at 37 °C. Visual examination of DNaSE activity was conducted after 1% agarose gel electrophoresis and staining of DNA with RotiSafe (Gelstain ready-to-use, Roth).

Alternatively, bacterial pellets of overnight cultures were washed in PBS, adjusted to an OD_600 of 1.8, and 20 μl of these suspensions were incubated in 100 μl THB supplemented with 40 μl DNaSE buffer (3 mM MgCl₂, 3 mM CaCl₂, 300 mM Tris; pH 7.4) and 4 μg calf thymus DNA.

**Determination of survival in porcine and human blood.** Killing of *Strep. suis* in human or porcine blood was investigated using freshly drawn heparinized blood. Stocks of frozen bacterial suspensions

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Fig. 1. *Strep. suis* induces NETs in vitro and is entrapped but not killed in those NETs. (a) Entrapment of *Strep. suis* wt (strain 10) in NETs after infection of purified porcine neutrophils was visualized by immunofluorescence microscopy using DAPI staining of DNA (blue), Alexa 488-conjugated antibodies against histone H2A-H2B-DNA complexes to label NETs (green) and Alexa 653-conjugated antibodies against *Strep. suis* serotype 2 (red, arrows). (b) Formation of NETs after infection of porcine neutrophils with *Strep. suis* wt (strain 10) at an m.o.i. of 1:2 for 30 to 120 min. (c) NET-survival capacity of *Strep. suis* wt and its unencapsulated mutatant 10cpsΔEF (Δcps). Prior to infection, neutrophils were treated with 25 nM PMA for 4 h to induce NETs and with 10 μM cytochalasin D for 20 min to block phagocytosis. Survival of bacteria was compared to their respective growth control (determination of c.f.u.). A *Staphylococcus aureus* nuclease-deficient strain (Δ) was used as control to verify antimicrobial activity of porcine NETs, as it is a known NET-susceptible strain under these experimental conditions. All data are shown as mean ± SEM of three independent experiments; *P<0.05; **P<0.005; ***P<0.001; n.s., not significant by one-tailed Mann–Whitney test (b) or paired one-tailed t-test (c).
including 15% glycerol were thawed prior to the bactericidal assay and 1.5 × 10^5 c.f.u. were mixed with 500 μl of heparinized blood. The specific bacterial contents (c.f.u. ml⁻¹) were determined after 0 min, 60 min and 120 min of incubation on a rotator at 37 °C.

**Statistical analysis.** Data were analysed using Excel 2010 (Microsoft) and GraphPad Prism 5.0 (GraphPad Software). Normal distribution of data was verified by Kolmogorov–Smirnov normality test (GraphPad software) prior to statistical analysis. Differences between two groups were analysed using a one-tailed paired (when all individual samples were performed within one individual experiment) or unpaired Student’s t-test in the case of normal distributed data, or by using a one-tailed Mann–Whitney test (non-parametric data). Probabilities lower than or equal to 0.05 were considered significant, lower than or equal to 0.1 as statistically noticeable.

**RESULTS AND DISCUSSION**

**Strep. suis induces NETs and is entrapped in NETs**

As neutrophils are the dominating immune cells infiltrating lesions of acute *Strep. suis* infection, we investigated whether interaction of *Strep. suis* with neutrophils might result in NET formation and subsequent entrapment and/or killing of bacteria by NETs. As shown in Fig. 1(a, b), a significant (P=0.014) increase in NET formation (12.3 ± 6.0%) was observed in porcine blood-derived neutrophils after 30 min of infection with *Strep. suis* wt compared with uninfected neutrophils (4.7 ± 2.0%). Although it is well known that NET formation can be

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**Fig. 2.** The isogenic *Strep. suis* mutant 10ΔssnA (ΔssnA) is neither attenuated in growth *in vitro* nor in survival in porcine or human blood. (a) -SsnA Western blot analysis of culture supernatants and murein-associated proteins (MAPs) of *Strep. suis* wt (strain 10) and its isogenic mutant 10ΔssnA. SsnA has a theoretical molecular mass of 114 kDa. (b) Demonstration of DNase activity in *Strep. suis* wt (strain 10) but not in 10ΔssnA by 1% agarose gel electrophoresis of calf thymus DNA incubated with bacterial supernatants in the presence of RPMI (medium in NET assays) or THB containing DNase buffer with Ca²⁺ and Mg²⁺ for 270 min at 37 °C. Controls were incubated with PBS rather than bacterial supernatants. (c) Growth of *Strep. suis* wt (strain 10) and 10ΔssnA in THB at 37 °C. (d) Survival factors of *Strep. suis* wt and 10ΔssnA in porcine and human blood *ex vivo* (freshly drawn heparinized blood). C.f.u. were determined at 0 min, 60 min and 120 min. No significant differences between the survival factors of *Strep. suis* wt (strain 10) and 10ΔssnA were found. All data are shown as mean±SEM of three independent experiments; * P<0.05; ** P<0.005; *** P<0.001 by paired one-tailed t-test.
Fig. 3. The isogenic mutant 10ΔssnA (ΔssnA) is attenuated in NET degradation. (a) Comparative evaluation of NET degradation by Strept. suis wt (strain 10) and 10ΔssnA (OD$_{600}$ = 1.3). PMA-stimulated human neutrophils were infected for 1 h either with Strept. suis wt (strain 10) or 10ΔssnA (OD$_{600}$ = 1.3) at an m.o.i. of 1 : 2. (b) Comparative evaluation of NET degradation in PMA-stimulated human neutrophils incubated with supernatants of overnight cultures of Strept. suis wt (strain 10) and 10ΔssnA. (c) Degradation of NETs formed by PMA-stimulated human neutrophils was visualized by immunofluorescence microscopy using DAPI staining of the DNA (blue) and Alexa 488-conjugated antibodies against histone H2A-H2B-DNA complexes to label NETs (green). PMA-stimulated human neutrophils were infected for 1 h either with Strept. suis wt (strain 10) or 10ΔssnA (OD$_{600}$ of 1.3) at an m.o.i. of 1 : 2 or treated with micrococcal nuclease (MN) as positive controls.
triggered by different pathogens, substantial differences exist in NET induction between different bacterial species (Pilsczek et al., 2010). Further studies are anticipated to comparatively evaluate NET formation in porcine neutrophils after infection with different porcine pathogens and to characterize Strep. suis NET-induction factors.

Importantly, using immunofluorescence microscopy, we were able to detect efficient entrapment of Strep. suis in those porcine NETs (Fig. 1a and Fig. S2). However, assays determining the antimicrobial activity of NETs revealed that both Strep. suis wt (strain 10) and its isogenic phagocytosis-sensitive unencapsulated mutant (10cps∆EF) can are resist these antimicrobial effects (Fig. 1c). The nuc mutant of Staph. aureus was used as a positive control in this assay, since it was demonstrated to be susceptible to NET-mediated killing (Berends et al., 2010). Both Strep. suis strains, wt and its unencapsulated mutant (10cps∆EF), showed a significant survival capacity in the presence of NETs compared with the control Staph. aureus nuc mutant (Fig. 1c). Thus, entrapment of Strep. suis in NETs without killing is reminiscent of results obtained for Streptococcus pneumoniae (Beiter et al., 2006) or Neisseria meningitidis (Lappann et al., 2013). Pneumococci as well as meningococci are trapped but, unlike other pathogens (e.g. Streptococcus pyogenes (Buchanan et al., 2006) and Staph. aureus) not efficiently killed by NETs (Berends et al., 2010; Brinkmann et al., 2004; Chow et al., 2010).

Interestingly, when investigating the NET formation of porcine neutrophils in response to Strep. suis infection over time, the amount of NETs significantly decreased again at 90 min of co-incubation of Strep. suis wt with porcine neutrophils (Fig. 1b). These data lead to the suggestion that Strep. suis wt is able to degrade NETs in vitro, and thereby possibly joins other leading Gram-positive pathogens such as Staph. aureus (Berends et al., 2010), Strept. pneumoniae (Beiter et al., 2006), Strept. pyogenes (Buchanan et al., 2006) and Streptococcus agalactiae (Derré-Bobillot et al., 2013) by evading NET-entrapment through NET degradation by nuclease.

Expression of the extracellular nuclease SsnA by Strep. suis is not crucial for in vitro growth and survival in porcine and human blood

As our data lead to the hypothesis that Strep. suis wt is able to evade NET-mediated entrapment and antimicrobial activity by the production of a NET-degrading nuclease, we performed a mutagenesis study with a previously described extracellular nuclease, designated SsnA (Fontaine et al., 2004). We constructed a new in-frame ssnA deletion mutant to exclude polar effects through insertion of foreign DNA. qRT-PCR showed that ssnA was not expressed in the new isogenic ssnA mutant, designated 10ΔssnA. Numerous attempts failed to construct an ssnA complementation vector on the basis of pGA14. It was noted that expression of active nucleases in E. coli might be toxic (Midon et al., 2011). However, transcript levels of two randomly selected virulence-associated genes (sly and ofs) and the four neighbouring genes were not different between Strep. suis wt and 10ΔssnA, confirming that in-frame deletion mutagenesis did not result in polar effects (Fig. S3). Western blot analysis of culture supernatants of wt and 10ΔssnA revealed lack of four bands between 50 and 100 kDa recognized by a polyclonal rabbit serum raised against rSsnA (Gómez-Gascón et al., 2012). A likely explanation for multiple zSsnA-reactive bands is rapid degradation of SsnA in vitro. Accordingly, numerous bands between 50 and 100 kDa showed DNase activity in Strep. suis wt but not in the previously investigated ssnA mutant (Fontaine et al., 2004). Furthermore, SsnA was detected in the murein-associated protein fraction (Fig. 2a). This was expected, as the ORF encodes a typical C-terminal cell wall anchoring domain (Fontaine et al., 2004). Thus, detection of SsnA in the culture supernatant suggested partial release of SsnA and not secretion, as is known for other bacterial nucleases such as Nuc of Staph. aureus (Beiter et al., 2004). Attenuation in degradation of eukaryotic DNA was demonstrated for the new in-frame 10ΔssnA deletion mutant in the presence of Mg2+ and Ca2+ (Fig. 2b) in accordance with previously published results (Fontaine et al., 2004). It is noteworthy that DNA was also degraded within 4.5 h by Strep. suis wt (strain 10) in the presence of RPMI included in our previous NET assay, but distinctly less DNA degradation was recorded for the mutant 10ΔssnA under these conditions (Fig. 2b). Next, we investigated whether expression of SsnA is crucial for growth in vitro and for survival in blood. Importantly, the 10ΔssnA mutant is neither attenuated in growth in THB (Fig. 2c) nor in survival in freshly drawn human or porcine blood (Fig. 2d). This is in contrast to results obtained for the cell wall-attached nuclease SpnA of Strep. pyogenes that is crucial for survival in human blood ex vivo (Chang et al., 2011). In summary, SsnA was found to be dispensable for growth in THB and for survival in human and porcine blood ex vivo. The latter indicates that SsnA is not crucial for protection against opsonophagocytosis.

SsnA is involved in NET degradation by Strep. suis

An important objective of this study was to investigate putative SsnA-dependent NET degradation of Strep. suis. We conducted these studies with human and porcine neutrophils, because Strep. suis is an important pathogen in both species. However, our results indicated that 90–100 % NET formation after 4 h stimulation with 25 nM PMA,
Fig. 4. Determination of porcine NET degradation by *Strep. suis* wt and the isogenic mutant 10ΔssnA (ΔssnA). (a) Comparative evaluation of NET degradation of *Strep. suis* wt (strain 10) and 10ΔssnA (OD600 of 1.3). PMA-stimulated porcine neutrophils were infected for 1 h either with *Strep. suis* wt (strain 10) or 10ΔssnA (OD600 of 1.3) at an m.o.i. of 1:2. (b) Comparative...
which is best suited for these assays (Fuchs et al., 2007), is inducible in human but not in porcine neutrophils (compare controls in Figs 3 and 4). Reasons for these differences are unclear, but a lower sensitivity of porcine neutrophils to PMA has already been described (Bre´a et al., 2012). High levels of NETs were induced with PMA in human neutrophils and afterwards incubated with *Strep. suis* wt and 10ΔssnA. As depicted in Fig. 3(a, c), the area covered with NETs was significantly reduced to less than 60% after incubation with *Strep. suis* wt in comparison with the control (THB medium alone, *P* = 0.002). In contrast, incubation of NETs with the *Strep. suis* mutant 10ΔssnA did not result in a significant reduction of the area of NETs in comparison with the control (Fig. 3a, c). Differences between *Strep. suis* wt and 10ΔssnA in this NET degradation were significant (*P* = 0.0116; Fig. 3a). Furthermore, incubation of NETs with supernatants of *Strep. suis* wt but not of 10ΔssnA resulted in significant NET degradation (*P* = 0.037, Fig. 3b), in accordance with the partial release of SsnA in the supernatant (Fig. 2a). As mentioned above, NET induction by PMA in porcine neutrophils was restricted to less than 10% of neutrophils after 4h PMA stimulation (controls in Fig. 4a–c). However, incubation of these PMA-treated porcine neutrophils with *Strep. suis* wt led to a statistically noticeable reduction in the area of NETs in comparison with the control (*P* = 0.057), which was not observed to the same degree after incubation with 10ΔssnA bacteria (*P* = 0.24; Fig. 4a, c). Though differences between porcine NET degradation of wt and 10ΔssnA bacteria were not significant (*P* = 0.17), the tendency is in accordance with the significant attenuated phenotype of 10ΔssnA obtained with human NETs. Furthermore, substantial differences in porcine NET degradation were observed between supernatants of wt and 10ΔssnA bacteria (Fig. 4b, *P* = 0.1), suggesting that SsnA is also responsible for degradation of porcine NETs by *Strep. suis* wt, though involvement of an additional nuclease cannot be ruled out.

**SsnA is involved in protection of *Strep. suis***

**against antimicrobial activity mediated by human NETs**

As SsnA was found to be crucial for NET degradation exhibited by *Strep. suis*, we investigated whether SsnA mediates protection against NET-mediated antimicrobial activity. Thirty minutes of coinubcation with PMA-stimulated human neutrophils did not result in significant differences between the survival factors of *Strep. suis* wt (mean = 0.89, SD = 0.17) and 10ΔssnA (mean = 0.87, SD = 0.08). However, after 90 min the survival factor of *Strep. suis* wt (mean = 0.69, SD = 0.15) was significantly higher than the survival factor of 10ΔssnA (mean = 0.43, SD = 0.13, *P* = 0.05; Fig. 5a), indicating that SsnA is involved in protection against NET-mediated antimicrobial activity. Attenuation in survival after 90 min but not 30 min co-incubation is reminiscent of the phenotype shown by the nuc mutant of *Staph. aureus* (Berends et al., 2010).
In contrast to the results obtained for human neutrophils, incubation of *Strep. suis* with porcine neutrophils treated with PMA and cytochalasin D did not result in efficient reduction of surviving bacteria of *Strep. suis* wt or 10ΔssnA, but in bacterial proliferation. *Strep. suis* wt and 10ΔssnA showed mean survival factors of 5.61 (SD = 2.57) and 5.19 (SD = 2.42), respectively, after 90 min incubation and differences between both strains were not significant (P=0.35; Fig. 5b). One plausible explanation for the differences between the results of the assays with human and porcine neutrophils is variation in the degree of NETosis after PMA stimulation (see above). To the best of our knowledge, experiments leading to NET induction in porcine neutrophils comparable to assays with PMA-stimulated human neutrophils have not been described. Thus, it is currently very challenging to test the NET-mediated antimicrobial activity of porcine neutrophils.

In conclusion, we identified SsnA as the first factor of *Strep. suis* to be involved in interactions with NETs *in vitro*. SsnA mediates degradation of NETs and thereby resistance against NET-mediated antimicrobial activity of the bacteria, at least in interaction of NETs formed by human neutrophils. Similar functions have been shown for other nucleases expressed by other Gram-positive pathogens. However, it might be hypothesized that *Strep. suis* produces additional virulence factors that mediate a high resistance against NET-mediated antimicrobial activity of the wt strain. A further candidate as a factor involved in NET evasion is DltA, as it protects *Strep. suis* against antimicrobial peptides by D-alanylation of lipoteichoic acid (Fittipaldi et al., 2008). Resistance against antimicrobial peptides has been shown for other pathogens to be associated with a resistance against antimicrobial activity of NETs (Lauth et al., 2009; Wartha et al., 2007). Future studies are anticipated to reveal further insights into NET evasion of this important pathogen and to demonstrate its impact on pathogenesis.

**ACKNOWLEDGEMENTS**

We thank H. Smith (DLO-Lelystad, Netherlands) for *Strep. suis* strains 10 and 10cpsΔEF, Daisuke Takamatsu (National Institute of Animal Health, Japan) kindly provided the plasmid pSETs and Marc Monestier (Temple University School of Medicine, USA) the PL2-6 antibody. We also acknowledge Lydia Gómez-Gascón and Immaculada Luque (both Universidad de Córdoba, Spain) for providing the rabbit serum against SsnA and Tina Basler (University of Veterinary Medicine Hannover, Germany) for supporting qRT-PCR analysis. N. d. B. was funded by a fellowship of the Ministry of Science and Culture of Lower Saxony (Georg-Christoph-Lichtenberg Scholarship) within the framework of the PhD program ’EWI-Zoonosen’ of the Hannover Graduate School for Veterinary Pathobiology, Neuroinfectiology, and Translational Medicine (HGN). A. N. was funded by a grant provided by the Akademie für Tiergesundheit, Germany, within the framework of the PhD program ’EWI-Zoonosen’ of the HGNI.

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Edited by: P. Langford