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1 **Polysaccharide capsule and suilysin contribute to extracellular survival of *Streptococcus suis* co-**
2 **cultivated with primary porcine phagocytes**

3

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1 **Abstract**

2 *Streptococcus suis* is a major cause of meningitis, sepsis and arthritis in piglets and a zoonotic agent.
3 Survival in the blood circulation system represents a major step in pathogenesis of *S. suis* infections.
4 To get further insides into the mechanisms of *S. suis* survival in the host, we compared a highly
5 virulent *S. suis* serotype 2 strain with its non-encapsulated and suilysin-deficient mutants in their
6 abilities to resist phagocytosis and killing by polymorphonuclear neutrophils (PMNs) and
7 mononuclear cells. PMNs displayed a higher capacity to take up encapsulated bacteria than
8 mononuclear cells, whereas both cell types internalised efficiently non-encapsulated *S. suis*.
9 Differentiation of extracellular and intracellular survival of the WT strain revealed that in PMNs the
10 majority of the cell-associated streptococci were intracellular, whereas in mononuclear cells the
11 majority remained attached to the cell surface. *S. suis* survived mainly extracellularly, since both cells
12 killed intracellular bacteria to a similar extent. As a consequence of different resistance to
13 phagocytosis, only the encapsulated *S. suis* strains survived co-cultivation with PMNs. Comparison of
14 the WT strain with its encapsulated suilysin-deficient mutant revealed reduced survival of the mutant
15 after co-cultivation with PMNs. Involvement of suilysin in inhibition of phagocytosis was further
16 confirmed by use of anti-suilysin antibodies and recombinant suilysin. Kinetic experiments with
17 PMNs suggested that reduced survival of the mutant strain was mainly associated with an increased
18 uptake, whilst both strains adhered similarly. Concluding, our results indicate that the capsule and the
19 suilysin play important roles in *S. suis* survival in the host by interfering with phagocytic uptake.

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21 **Keywords:** *Streptococcus suis*; capsule; suilysin; phagocytosis.

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1 **1. Introduction**

2 *S. suis* is a major cause of meningitis, sepsis and arthritis in piglets and a zoonotic agent. Bacteria are
3 transmitted via the respiratory route and commonly colonize the palatine tonsils. Under certain, yet
4 unknown, circumstances *S. suis* crosses the respiratory epithelial barrier and disseminates in the blood
5 circulation system. A possible way how *S. suis* spreads into host tissues might be survival and
6 “travelling” inside (Williams, 1990; Williams and Blakemore, 1990), or closely associated to
7 monocytes and macrophages (Gottschalk and Segura, 2000). Alternatively, *S. suis* might migrate in
8 blood as free bacteria protected by a thick capsule layer, since a high level of bacteremia usually
9 precedes the onset of bacterial meningitis (Williams, 1990; Tunkel and Scheld, 1993). Nevertheless,
10 experimental evidence supporting either of these theories is limited.

11 Pathogenicity of many bacteria seems to rely on the protective effect of the capsule against
12 phagocytosis. For *S. suis* serotype 2 strains, the capsule represents a critical virulence factor, since it is
13 involved in protection against phagocytosis by alveolar macrophages (Charland et al., 1998; Smith et
14 al., 1999). On the other hand, interactions of *S. suis* with PMNs, is poorly described. These phagocytes
15 participate in the first defense line against *S. suis* infection, however, it is not clear whether they
16 efficiently take up and killed the bacteria (Wibawan and Laemler, 1994; Busque et al., 1998).

17 Another streptococcal factor probably associated with resistance to phagocytosis is suilysin, a
18 cholesterol dependent cytolysin related to streptolysin O of *S. pyogenes* and pneumolysin of *S.*
19 *pneumoniae* (Jacobs et al., 1994). These toxins seem to play a multifactorial role in pathogenesis by
20 their cytolytic activities and their inhibition of bactericidal activity of phagocytes (Sierig et al., 2003;
21 Orihuela et al., 2004). However, whether or not suilysin plays a similar role has not been reported yet.

22 To get new insights into *S. suis* survival in the host we compared a highly virulent *S. suis* serotype 2
23 strain with its non-encapsulated and suilysin-deficient mutants in their abilities to resist phagocytosis
24 and killing by PMNs and mononuclear cells. We found evidence that CPS and suilysin prevent
25 phagocytic uptake, thereby allowing the bacteria to survive extracellularly.

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2. Materials and Methods

2.1 Bacterial strains and growth conditions. *S. suis* capsular serotype 2 strain 10 and its non-capsulated isogenic mutant strain 10 Δ *cps*EF (designated strain Δ *cps*) were kindly provided by H. Smith (Lelystad, NL) and have been described in an earlier study (Smith et al., 1999). In addition, a suilysin deficient mutant, strain Δ *sly*, was constructed from strain 10 as described below. The hosts for molecular cloning and protein expression experiments were *Escherichia (E.) coli* strains DH5 α and BL21 (DE 3), respectively. For co-cultivation experiments, *S. suis* was grown in Todd-Hewitt broth (THB, DIFCO Lab, Detroit, USA) over-night at 37°C. The next day, an equal volume of fresh THB was then added to the cultures, which were further incubated for 1.5 hours at 37°C. Then the bacteria were harvested, suspended in PBS (pH 7.3) and adjusted photometrically (600 nm) to a concentration of 2x10⁹ bacteria ml⁻¹, before use in further experiments. For some experiments, bacteria were opsonised using either fresh or complement-inactivated (30 min at 56 °C) porcine serum at 20% in RPMI (final concentration). Serum was obtained from an 8 weeks old piglet which had survived an experimental infection against *S. suis* (Beineke et al., 2008) and was tested positive for the presence of anti-*S. suis* antibodies by immunoblot as described earlier (Benga et al., 2004a).

2.2 Construction of the suilysin mutant strain 10 Δ *sly*. Techniques were performed according to standard procedures (Sambrook and Russel, 2001). Restriction enzymes were purchased from NewEnglandBiolabs (Frankfurt/Main, Germany). To inactivate the *sly* gene in the strain 10, the gene was interrupted by insertion of an erythromycin cassette. For this, a 1510 bp fragment of the *sly* gene was amplified by PCR using the primer pair SL4-L (AATCCATATGAGAAAAGTTCGCACTTGATTTT) and SL4-R (CGGGATCCTTACTCTATCACCTCATCCGCATAC). Subsequently, the product was cloned into the plasmid pCRII-TOPO (Invitrogen), then the fragment was digested with *SacI*-*PstI* and ligated into the plasmid pBluescript II SK(+) (Stratagene, Amsterdam, The Netherlands) digested with the same enzymes. The erythromycin cassette derived from pICerm (kindly provided by C. Baums) was digested with *PvuII* and ligated into the *HpaI* site of the *sly* gene to create pBlue/*sly*/erm. The last plasmid was electroporated (2.5 kV, 200 Ω , 25 μ F) into *S. suis* strain 10. Transformants which had undergone double-crossover events were genotypically confirmed using PCR and Southern Blot

1 analysis. The phenotype of the suilysin mutant was confirmed by a hemolysis assay, as described by
2 Takamatsu et al. (2001).

3 **2.3. Cloning, expression, and purification of recombinant suilysin and production of polyclonal**

4 **anti-suilysin antibodies.** The suilysin gene coding region of *S. suis* strain 10 was amplified by PCR
5 using the primers slyAgefor (TGTACCGGTGATTCCAAACAAGATATTAA) and slyAgerev
6 (CCAACCGGTCTCTATCACCTCATCCGCAT). The PCR product was digested using the enzyme
7 *AgeI* and inserted into the vector pET45b (Novagen, Darmstadt, Germany), which was also digested
8 by *AgeI*. The resulting vector pET45bsly, was amplified in *E. coli* DH5- α cells, then purified and
9 sequenced using the primers pET45b5forward (AGGATCGAGATCGATCTCG), pET45b5reverse
10 (TTCTTCTTTTCTCTGCGA) and pET45b (CCTGCAGGCGCGCCGTGTAC). Recombinant suilysin
11 was expressed in *E. coli* BL21 (DE3) cells after induction with isopropyl- β -D-thiogalactoside and was
12 purified according to „purification of polyhistidine-tagged proteins from *E. coli* under denaturing
13 conditions” with Protino[®] Ni-TED 2000 packed columns (Macherey-Nagel, Dueren, Germany). The
14 eluted protein was renatured by dialysis against 25 mM Tris-HCl with decreasing urea
15 concentrations. The last dialysis step was performed against a buffer containing 25 mM Tris-HCl (pH
16 7.0), 50 mM NaCl, 1 mM EDTA, 5% (v/v) Glycerol. Protein concentration was measured according to
17 ‘microplate assay protocol’ (Bio-Rad), and the haemolytic activity was tested as described as
18 described by Takamatsu et al. (2001). The protein was stored at -20°C after adding 5 mM
19 dithiothreitol. Polyclonal rabbit anti-rSLY antibodies were raised by immunisations of a New Zealand
20 white rabbits using the purified protein.

21 **2.4. Isolation of porcine phagocytes.** Freshly collected heparinised blood from healthy piglets was
22 mixed with an equal volume of 0.87% NaCl, then layered on Ficoll-Hypaque 1077 (Biochrom, Berlin,
23 Germany) and subsequently centrifuged for 30 min at 400 g and 20°C. Centrifugation resulted in
24 formation of two fractions, an interface containing mononuclear cells, and a pellet containing PMNs
25 and erythrocytes. Following, the erythrocytes were removed by hypotonic lysis, and the purified
26 PMNs were suspended in RPMI medium. The mononuclear fraction was harvested from the interface,
27 washed twice with 0.87% NaCl and then suspended in RPMI. The viability of the cells was >95% as

1 determined by trypan blue exclusion assay. The purified cell fractions were immediately used for
2 phagocytosis and killing assays.

3 **2.5. PMNs killing assay.** This was performed as described by Wessels et al.(1991). Briefly, 400 μ l
4 leukocyte suspension (10^7 cells/ml) was mixed 1:1 with *S. suis* in PBS, either alone, or in the presence
5 of 100 μ l porcine serum (fresh or complement-inactivated, 30 min at 56°C) and incubated at 37°C
6 under continuous end-over-end rotation (6 rpm/min). In some experiments, anti-suilysin polyclonal
7 antiserum or pre-immune rabbit serum, or rSLY were included. Aliquots of 20 μ l were plated
8 immediately and at the end of experiments (1 h). Samples containing only cells or bacteria were used
9 as controls. Results were expressed as log fold change in CFU.

10 **2.6. Determination of adherence, phagocytosis and intracellular survival by antibiotic protection**
11 **assay.** This was performed as described by Smith et al. (1999) with some modifications. Briefly,
12 porcine phagocytes (PMNs, mononuclear cells) (10^7 /ml) were mixed with equal numbers of pre-
13 opsonised bacteria (MOI 1:1) in a final volume of 2 ml and incubated for 15 min at 37°C under
14 continuous rotation to allow phagocytosis. To remove bacteria not associated with the cells phagocytes
15 were washed twice with ice-cold RPMI and resuspended in the initial volume of medium. Then,
16 parallel aliquots of 400 μ l were harvested either immediately ($t = 0$) or after further incubation for 45
17 min at 37°C ($t = 45$). These were treated either with 100 μ g gentamicin and 5 μ g penicillin ml^{-1} to kill
18 extracellular adherent *S. suis* and quantify the intracellular bacteria, or they were incubated further in
19 RPMI without antibiotics to quantify the total number of intra- and extracellular *S. suis* for 45 min at
20 37°C. The number of extracellular bacteria was determined by subtracting the intracellular bacteria
21 from the total number of intra- and extracellular bacteria. In some experiments, to determine the
22 phagocytic and intracellular killing ability of PMNs, samples were taken immediately ($t = 0$), and
23 after 60 min ($t = 60$) and treated with antibiotics for 45 min at 4°C (instead of 37°C) in order to
24 exclude additional killing of intracellular bacteria by neutrophils during antibiotic killing of
25 extracellular bacteria. The efficiency of antibiotic killing was proven by platings of the supernatants.
26 To determine the adherence of *S. suis* to PMNs, parallel aliquots of phagocytes were treated with 1
27 μ g/ml cytochalasin D (Calbiochem, Darmstadt, Germany) 30 min prior to infection, as well as 15 min
28 after the infection to inhibit the phagocytic uptake. Subsequently, the samples were washed twice, and

1 the phagocytic cells were lysed for 15 min at room temperature in 400 μ l of 1% saponin. The number
2 of CFU in the suspensions was determined by platings on THB agar.

3 **2.7. Determination of adherence and phagocytosis by double immunofluorescence microscopy.**

4 Approximately 10^7 pre-opsonised bacteria were added to $\sim 10^6$ phagocytes (PMNs, mononuclear cells)
5 in 500 μ l RPMI and incubated for 1.5 h at 37°C using end-over-end rotation. Then the phagocytes
6 were allowed to adhere for 1 h to glass coverslips placed in 24 well plates. Subsequently, the wells
7 were washed, fixed with 3.7% formaldehyde and examined by differential immuno-fluorescence (DIF)
8 for intra- and extracellular bacteria as previously described (Benga et al., 2004b).

9 **2.8 Cell cytotoxicity assays.** Trypan blue exclusion assay (0.2% solution) and lactate dehydrogenase
10 (LDH) release assays were used to assess a possible damage of porcine phagocytes during co-
11 cultivation with *S. suis* as described previously (Benga et al., 2004b)

12 **2.9 Statistical analysis.** Experiments were performed at least 3 times, and results were expressed as
13 means +/- standard deviation (SD). Data were analyzed by the Wilcoxon test. A *P* value < 0.05 was
14 considered significant.

15 16 **3. Results**

17 **3.1. Extra- and intracellular survival of *S. suis* after co-cultivation with PMNs and mononuclear**

18 **cells.** To determine the contribution of different phagocytic cell types to opsonophagocytic killing of
19 *S. suis*, we assessed the ability of the virulent encapsulated *S. suis* strain 10 to resist phagocytosis and
20 killing by porcine PMNs and mononuclear cells. PMNs displayed a higher capacity to phagocytose
21 encapsulated *S. suis* than mononuclear leukocytes (fig. 1). After 45 min, approximately 67% of the
22 PMN-associated bacteria were detected intracellularly, whereas approx. 75% of bacteria associated
23 with the mononuclear cells were found extracellularly. After further incubation for 45 min, approx.
24 80% of the internalized bacteria were killed, whereas the number of extracellular bacteria increased by
25 approx. 70%, independent of the phagocyte cell type (fig. 1). To confirm these findings we used DIF
26 to distinguish between intra- and extracellular bacteria. In correlation with the plating results, PMNs
27 phagocytosed efficiently encapsulated *S. suis*, i.e. numerous intracellular bacteria were detected and
28 only few adherent bacteria were seen. In contrast, mononuclear cells were significantly less effective

1 in phagocytosis, i.e. only few intracellular bacteria were detected (fig. 1). The non-encapsulated strain
2 Δcps was efficiently taken up by both phagocytes (data not shown).

3 **3.2 Role of suilysin and capsule in *S. suis* survival after co-cultivation with PMNs.** The relevance
4 of suilysin for survival after co-cultivation with PMNs was evaluated by comparing the strains 10 and
5 its suilysin-deficient mutant Δsly . The non-encapsulated mutant Δcps was included as a phagocytosis
6 sensitive control.

7 Although PMNs phagocytosed and killed the wt strain to a certain extent, as described above, it
8 seemed that phagocytosis and killing rates were not efficient to eliminate all bacteria, since wild-type
9 (wt) *S. suis* survived co-cultivation with isolated PMNs (fig. 2). Moreover, it was able to multiply, in
10 contrast to strain Δcps , which was killed very efficiently. Interestingly, no difference in survival of the
11 wt strain was seen after opsonisation. On the contrary, opsonisation of the non-encapsulated mutant
12 strain resulted in a further increase of killing in comparison with non-opsonic conditions (fig. 2). After
13 opsonisation with heat-inactivated serum this strain was killed as efficiently as with fresh serum (data
14 not shown). Strain Δsly survived at intermediate levels in comparison with the wt and strain Δcps
15 when co-cultivated with isolated porcine PMNs (fig. 2). Similar to strain 10, strain Δsly was able to
16 multiply and no difference was seen after opsonisation, in contrast to strain Δcps . However, the
17 suilysin mutant multiplied to significantly lower extent than the wild type when co-cultivated with
18 PMNs.

19 To assess the possibility that the increased survival of the wt strain as compared to strain Δsly was due
20 to suilysin-mediated cytotoxicity, we evaluated the cell viability at the end of the experiments by
21 trypan blue staining and measurement of LDH release in the supernatant. Both tests revealed that all
22 strains were not cytotoxic under our experimental conditions (data not shown). Moreover, all three
23 strains grew equally well in the control assay without cells. To further confirm the role of suilysin in
24 the protection of *S. suis* against PMNs, we produced recombinant suilysin and raised rabbit polyclonal
25 antiserum against it. In the PMNs killing assay, we observed that treatment with anti-suilysin
26 antibodies lead a decreased survival of the wt strain 10 (fig. 3A). On the other hand, complementation
27 of strain Δsly with recombinant suilysin at sublytic concentrations resulted in an increased growth
28 capacity in the presence of porcine PMNs restoring the wt phenotype (fig. 3B).

1 **3.3. Suilysin mediates inhibition of the phagocytic uptake by PMNs.** In order to see whether the
2 differences in survival of the 3 tested strains were related to resistance either to phagocytosis or to
3 intracellular killing, we quantified the ability of porcine PMNs to take up and kill the wt strain, strain
4 Δsly and strain Δcps in a modified antibiotic protection assay. Phagocytic uptake was recorded after 15
5 min of co-cultivation ($t=0$), and bacterial resistance to intracellular killing was determined after further
6 incubation for 60 min. To avoid possible killing of ingested bacteria by PMNs during antibiotic
7 treatments, these were done at 4°C. As expected, we found a significantly higher extent of
8 internalization in strain Δcps in comparison with the encapsulated strains at $t = 0$ (fig. 4A). Moreover,
9 strain Δsly was significantly better internalized than the wt strain. After 60 min, the percentage of
10 intracellular bacteria decreased substantially in strain Δcps but to a lesser extent in encapsulated
11 strains (fig. 4A). To exclude the possibility that the increased internalization of the strain Δsly
12 compared to the wild-type was due to an increased ability to adhere to the PMNs, we assessed in
13 parallel samples the adherence capacity of the three tested *S. suis* strains after inhibition of phagocytic
14 uptake by cytochalasin D. Both encapsulated strains adhered equally well to the PMNs (fig. 4B).

15

16 **4. Discussion**

17 After invasion into deeper tissues, dissemination in the blood circulation is a crucial step in
18 establishment of *S. suis* infection. As postulated previously (Gottschalk and Segura, 2000), *S. suis*
19 might be found in blood either as free bacteria or in association with blood cells, i. e. monocytes. In
20 the latter case, the bacteria might “travel” either intracellularly (Williams, 1990; Williams and
21 Blakemore, 1990) or closely bound to the cell surface (Gottschalk and Segura, 2000). The published
22 reports supporting either of the two theories provided some evidence that *S. suis* survives co-
23 cultivation with porcine phagocytes (Smith et al., 1999; Chabot-Roy et al., 2006). At present,
24 however, it is not clear how *S. suis* survives killing by phagocytes, and to what extent different
25 phagocytes (PMNs, mononuclear cells) and bacterial factors contribute to this process.

26 We designed experiments that allowed further evaluation of *S. suis*-phagocyte interactions. Our results
27 indicated that PMNs and mononuclear cells differed significantly in their capacity to internalize
28 encapsulated *S. suis*. PMNs phagocytosed encapsulated *S. suis*, whereas mononuclear cells

1 internalized the bacteria very poorly. On the other hand, both cell types phagocytosed efficiently non-
2 encapsulated strains. Once internalized, even encapsulated *S. suis* were easily killed by both
3 phagocytes. However, in the case of encapsulated *S. suis* the internalization rate seemed not to be
4 sufficient to prevent infection, since encapsulated *S. suis* were able to multiply extracellularly after co-
5 cultivation with phagocytes. Our results suggest that the capacity of the bacteria to avoid efficient
6 phagocytic uptake contributed to their survival, and that *S. suis* survived closely attached to the cell
7 surface.

8 We further showed that encapsulated wt strain and strain Δsly , but not Δcps survived phagocytic
9 clearance when co-cultivated with PMNs independent of opsonisation, further supporting the
10 important role of the capsule as an antiphagocytic resistance factor (Charland et al., 1998; Smith et al.,
11 1999). Opsonisation substantially increased killing by PMNs of the non-encapsulated mutant Δcps
12 (which was killed also at non-opsonic condition), whereas the encapsulated bacteria survived co-
13 cultivation by leukocytes even after opsonisation. There was no difference in killing of the capsule
14 mutant when complement deprived serum was used, suggesting that complement mediated
15 opsonisation did not play an essential role, similar to what has been described for group B streptococci
16 (Valentin-Weigand et al., 1996). Nevertheless, the precise role of the complement system in virulence
17 of *S. suis* remains to be elucidated.

18 In other pathogenic streptococci the cholesterol binding toxins have been shown to be involved in
19 bacteria-phagocyte interactions. For example, expression of streptolysin O impaired killing of *S.*
20 *pyogenes* by PMNs *in vitro* and enhanced their virulence *in vivo* in a mouse infection model (Sierig et
21 al., 2003). And in pneumococci, Orihuela et al. (2004) have elegantly demonstrated the multifactorial
22 role of the pneumolysin in pathogenesis. In our study we demonstrated that suilysin contributed to
23 bacterial survival in the presence of porcine phagocytes *in vitro*, in accordance to a recently published
24 study (Chabot-Roy et al., 2006). Moreover, we were able to show that anti-suilysin antibodies could
25 neutralize these effects and that complementation with recombinant suilysin restored the wt
26 phenotype. Since we performed our experiments at subcytolytic conditions, cytotoxic effects on
27 phagocytes could be excluded. Rather, an adherence-dependent action of the toxin seems to be the

1 mechanism involved in survival. Based on our findings we assumed that beside the capsule, suilysin
2 contributed to extracellular survival of *S. suis* after cocultivation with PMNs.
3 Recently, it has been shown that streptolysin O prevents internalisation of *S. pyogenes* by
4 oropharyngeal keratinocytes (Hakansson et al., 2005). Moreover, this toxin mediated evasion of
5 lysosomal uptake (Hakansson et al., 2005), thus facilitating the survival of bacteria localized mainly
6 extracellularly. Consistent with this, we demonstrated that the suilysin-deficient mutant Δsly was taken
7 up significantly better than the wild type strain, whereas the adherence rates of the two strains were
8 similar. This suggests that suilysin is involved in bacterial dissemination in the blood circulation.
9 Indeed, very recently it was shown that the expression of the *sly* gene was up-regulated in blood (Tan
10 et al., 2008). Earlier studies showed that a *sly* mutant was attenuated *in vivo* in a murine (Allen et al.,
11 2001) but not a porcine (Lun et al., 2003) infection model, which may be explained by the differences
12 between the mouse and porcine infection models for *S. suis* (Vecht et al., 1997).
13 Taken together, depending on the route and stage of infection, suilysin may well contribute to
14 virulence by interfering with phagocytosis, especially in low-encapsulated *S. suis* strains. This is
15 supported by the fact that suilysin expressing capsule-deficient *S. suis* strains could be isolated from
16 invasive locations in pigs (Allgaier et al., 2001; Benga et al., 2004b). Future studies will have to
17 address the molecular mechanisms of suilysin-mediated effects on host cells, and will help to dissect
18 the possible interplays of the different virulence-associated factors of *S. suis* in infection.

19

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1 **Figure legends**

2 **Figure 1**

3 Kinetics of adherence, phagocytosis and killing of *S. suis* strain 10 by porcine PMNs (upper graph) and
4 mononuclear cells (lower graph). The total numbers of extra- (black bars) and intracellular (white bars) bacteria
5 were determined by antibiotic protection assay as described in Material and Methods. Data are expressed as total
6 extra- or intracellular CFU and represent means and SD of three independent experiments. Adherence and
7 phagocytic uptake by porcine PMNs and mononuclear cells was also analyzed by double differential
8 immunofluorescence. Representative microscopic pictures are shown in the inset.

9
10 **Figure 2**

11 Contribution of capsule and suilysin to survival of *S. suis* to co-cultivation by porcine PMNs. *S. suis* strains 10,
12 Δsly and Δcps were incubated with PMNs, either alone (non-opsonised) or in presence of 20 % porcine serum.
13 CFU of surviving bacteria were quantified by platings at the beginning and at the end of the experiment. Data are
14 expressed as mean log fold change in CFU after 1 h. Means and SD of five independent experiments are shown.
15 *, *P* values <0.05.

16
17 **Figure 3**

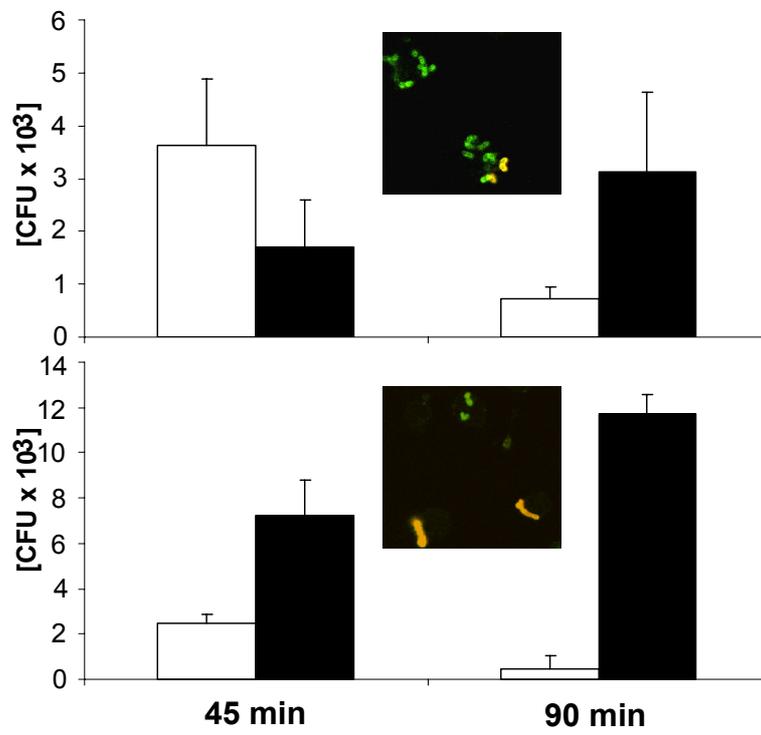
18 Neutralization (A) and complementation (B) of the suilysin in the *S. suis* WT and suilysin mutant respectively.
19 For this, *S. suis* strains 10 and Δsly were incubated with PMNs for 1h at 37°C, in the presence of 20 %
20 antisuilysin serum or preimmune serum (A) or in the presence of rSly (10 µg/ml) (B). CFU of surviving bacteria
21 were quantified by platings at the beginning and the end of the experiment. Data are expressed as % survival of
22 the fold change in CFU after 1 h achieved by the controls. Means and SD of five independent experiments are
23 shown. *, *P* values <0.05.

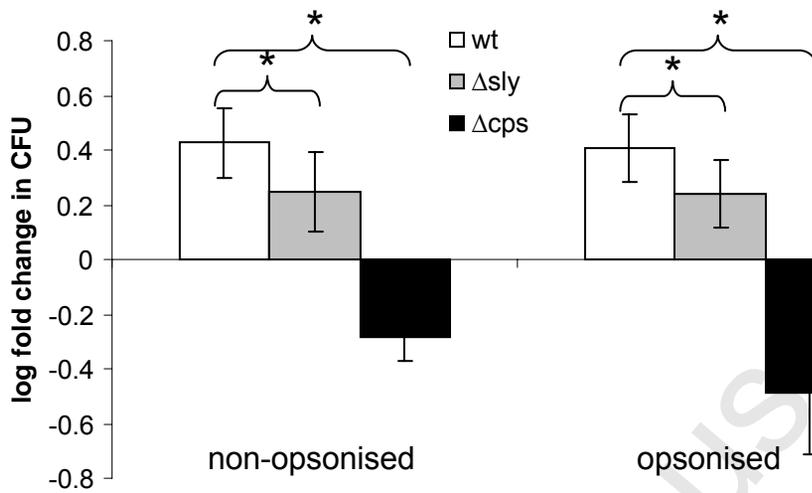
24
25
26 **Figure 4**

27 Contribution of suilysin and capsule to adherence (B), phagocytosis and killing (A) by porcine PMNs. *S. suis*
28 strains 10, Δsly and Δcps were opsonised with 20% porcine immune serum, then co-cultivated with PMNs for 15
29 min at 37°C to allow phagocytosis. Subsequently, the phagocytes were washed and the number of intracellular
30 bacteria was determined by platings after 0 and 60 min of further incubation. To determine the adherence
31 parallel aliquots of PMNs were treated with cytochalasin D to inhibit the cytoskeleton mediated uptake. The total
32 number of adherent and intra-cellular CFU in the suspensions was quantified by platings. Results were expressed

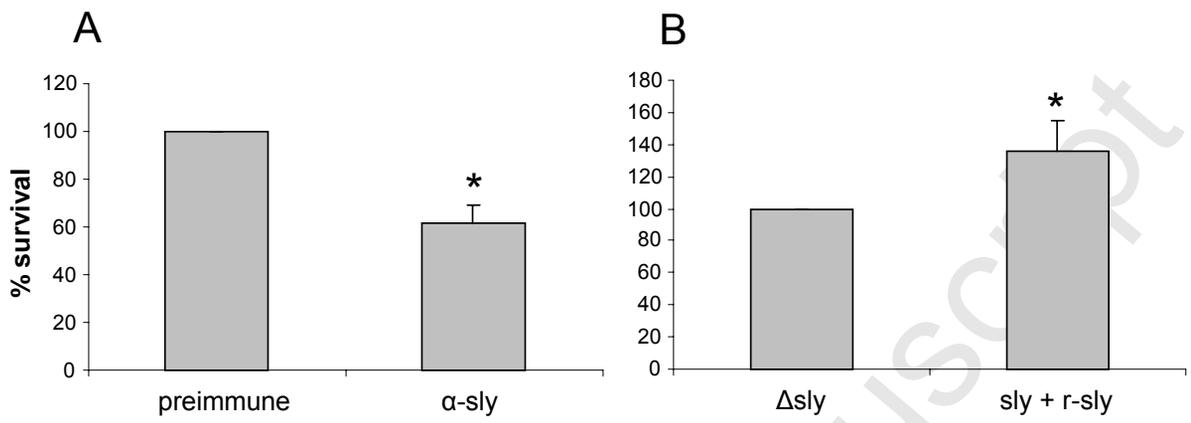
- 1 as % adherence or invasion (% recovered CFU of the inoculum). Means and SD of five independent experiments
- 2 are shown. *, P values <0.05 .

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