Expression of δ-toxin by *Staphylococcus aureus* mediates escape from phago-endosomes of human epithelial and endothelial cells in the presence of β-toxin

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**Summary**

*Staphylococcus aureus* is able to invade non-professional phagocytes by interaction of staphylococcal adhesins with extracellular proteins of mammalian cells and eventually resides in acidified phago-endosomes. Some staphylococcal strains have been shown to subsequently escape from this compartment. A functional *agr* quorum-sensing system is needed for phagosomal escape. However, the nature of this *agr* dependency as well as the toxins involved in disruption of the phagosomal membrane are unknown. Using a novel technique to detect vesicular escape of *S. aureus*, we identified staphylococcal virulence factors involved in phagosomal escape. Here we show that a synergistic activity of the cytolytic peptide, staphylococcal δ-toxin and the sphingomyelinase β-toxin enable the phagosomal escape of staphylococci in human epithelial as well as in endothelial cells. The *agr* dependency of this process can be directly explained by the location of the structural gene for δ-toxin within the *agr* effector RNAIII.

**Introduction**

Whereas most *S. aureus* infections are caused by extracellular bacteria, the pathogen can invade host cells using pathogen adhesins that interact with extracellular matrix molecules and host cell surface receptors. For example, fibronectin-binding proteins bind to α5β1 integrin via fibronectin. This leads to rearrangement of the host cell cytoskeleton and subsequently to endocytotic uptake of the pathogen (reviewed in Sinha and Herrmann, 2005; Sinha and Fraunholz, 2010). *S. aureus*-containing phagoendosomes eventually fuse with lysosomes. The resulting phagolysosomes are decorated by lysosome-associated membrane proteins 1 and 2 (LAMP1 and LAMP2, respectively) (Jarry et al., 2008; Giese et al., 2009; Sinha and Fraunholz, 2010) and acidify to a pH of 5 (Giese et al., 2009). Within the hydrolytically active organelles most *S. aureus* are degraded, although a fraction of bacteria can persist as metabolically quiescent small-colony variants (Schröder et al., 2006). Additionally, bacteria can induce host cell death (Baran et al., 2001; Krut et al., 2003; Haslinger-Löffler et al., 2005; Kubica et al., 2008). It has been hypothesized that escape from the phagosome precedes host cell death (Menzies and Kourteva, 1998). A functional *agr* quorum-sensing system is required for phagosomal escape (Shompole et al., 2003; Jarry and Cheung, 2006; Kubica et al., 2008) and *agr* activation precedes phagosomal escape (Qazi et al., 2001). Among the *agr*-regulated proteins the pore-forming staphylococcal α-toxin has been viewed a prime candidate for mediating this escape. Whereas α-toxin is able to mediate translocation of phagocytosed *S. aureus* to the host cell cytoplasm in a cystic fibrosis airway epithelial cells (Jarry et al., 2008), we and others recently showed that α-toxin is not sufficient for phagosomal membrane disruption or permeabilization in a cystic fibrosis airway epithelial cell line complemented with wild-type cystic fibrosis transmembrane conductance regulator (CFTR) (Jarry and Cheung, 2006; Giese et al., 2009).

Phagosomal escape of *Staphylococcus aureus* usually is identified by ‘needle in the haystack’ approaches visualizing, for instance, the absence of a lysosomal membrane enclosure around internalized pathogens (Bayles et al., 1998; Shompole et al., 2003; Jarry and Cheung, 2006; Jarry et al., 2008; Kubica et al., 2008) or measuring alkalinization of infected phagosomes (Bayles et al., 1998; Shompole et al., 2003; Jarry and Cheung, 2006; Jarry et al., 2008; Kubica et al., 2008; Giese et al., 2009;
Lâm et al., 2010). We therefore developed a novel assay to quantify escape rates of recombinant *S. aureus* strains. Using this assay we here describe the activity of virulence factors mediating phagosomal escape in infected epithelial and endothelial cell lines and identify a direct link between *agr* activation and phagosomal escape.

**Results**

To facilitate the identification of phagosomal escape of *S. aureus* we exploit the immunoglobulin-binding staphylococcal protein A, a cell wall-anchored 50 kDa protein that is present in most *S. aureus* strains. We generated recombinant HeLa cells, cytoplasmically expressing YFP-Fc, a fusion protein of yellow fluorescent protein (YFP) and the *Fc* proportion of human immunoglobulin G1 (IgG1). In the cytoplasm of these cells, phagosomal escape of the pathogen should lead to an exposure of the staphylococcal *Fc* receptor, protein A, to YFP-Fc and thereby to the accumulation of YFP fluorescence at the bacterial cell wall (Fig. 1, schematic drawing). To induce phagosomal escape of *S. aureus* RN4220 (Kreiswirth et al., 1983), we infected HeLa YFP-Fc with recombinant *S. aureus* pLLO-Cerulean which expresses *Listeria monocytogenes* Listeriolysin O (LLO) and escapes from the phagosome (Giese et al., 2009) and compared it with a strain solely expressing a cyan-fluorescent marker, *S. aureus* pCerulean. Upon infection of HeLa YFP-Fc with *S. aureus* pLLO-Cerulean, fluorescent rings were visible around the staphylococci (Fig. 1A). By contrast, YFP-Fc was evenly distributed across the cytoplasm of HeLa infected with *S. aureus* merely expressing fluorescent marker (Fig. 1B). Both strains intracellularly possessed similar levels of protein A as we established by treating fixed infected HeLa with non-specific fluorophore-labelled immunoglobulin (Fig. S1A). We also infected a HeLa cell line that produced cytoplasmic YFP without *Fc*. This cell line did not show accumulation of YFP in the periphery of *S. aureus* pLLO-Cerulean or an alternative strain, RN6930B, which has been previously reported to escape from phagosomes (Shompole et al., 2003; Jarry and Cheung, 2006) (Fig. S1B). This indicated that the signal in Fig. 1A was due to interaction of protein A with the immunoglobulin proportion of YFP-Fc. We established that protein A is necessary for the observed YFP-Fc recruitment by using a LLO-expressing protein A-deficient mutant of strain RN4220 (Gründling and Schneewind, 2009).
2007), which did not recruit YFP-Fc to the staphylococcal cell wall (Fig. S1C). This further illustrated that in our assays the contribution of a second immunoglobulin-binding protein of *S. aureus*, Sbi, was negligible. The recruitment of YFP-Fc further correlates with the leakage of fluid-phase cargo as we demonstrate by adding a red fluorescent dye, Alexa Fluor 633 hydrazide, to the culture medium upon infection. The dye was internalized in endocytic vesicles along with staphylococci resulting in fluorescent seams of dye around internalized *S. aureus* (Fig. 1C, arrow). However, the fluid-phase cargo is not present when YFP-Fc is recruited (Fig. 1C, arrowhead). This strongly indicated that YFP-Fc recruitment was due to permeabilization of phagoendosomal membranes.

To further exclude an involvement of autophagy in YFP accumulation we infected HeLa YFP-Fc coexpressing the autophagic marker, mRFP-LC3 (Kimura et al., 2007). Accumulation of YFP-Fc around staphylococci was independent of LC3-mRFP signals (Fig. S2). Together our results demonstrate that recruitment of YFP-Fc to the periphery of *S. aureus* pLLO-Cerulean was due to the binding of YFP-Fc by staphylococcal protein A in absence of a phagosomal membrane barrier and thus constitutes a phagosomal escape assay with a positive read-out.

Phagosomal escape of *S. aureus* is preceded by activation of a functional *agr* quorum-sensing system (Qazi et al., 2001). We therefore assayed the *agr*-proficient laboratory strain *S. aureus* RN6390B (Peng et al., 1988) and its isogenic mutant RN6911, which is deficient in the complete *agr* operon (Novick et al., 1993). Infections of HeLa YFP-Fc with *S. aureus* RN6390B led to accumulation of YFP-Fc, whereas such fluorescence was not observed when using strain RN6911 (Fig. 2). Again, the absence of YFP-Fc in the periphery of RN6911 cells must be caused by a membrane barrier rather than absence of protein A, as protein A can be stained by non-specific fluorophore-labelled immunoglobulin after fixation and permeabilization of infected HeLa (Fig. S3).

Staphylococcal phagosomal escape has been reported to be *agr*-dependent. The *agr* effector RNAIII also encodes the 26 aa cytolysin δ-toxin. We therefore investigated the influence of this peptide on phagosomal membrane integrity. We constructed a plasmid, pHld-Cerulean, which synchronously produces the cytolysin as well as the cyan-fluorescent marker Cerulean. Upon infection of HeLa YFP-Fc with *S. aureus* RN4220 pHld-Cerulean, we observed a significantly higher percentage (*P* < 0.05) of staphylococci displaying YFP-‘rings’ (55.36 ± 16.45%; Fig. 3A and B) when compared with the control merely expressing fluorescent marker (0.08 ± 0.03%; Fig. 3B). Thus escape of *S. aureus* RN4220 pHld-Cerulean even exceeded those of the positive control, *S. aureus* pLLO-Cerulean (48.17 ± 9.14%). By contrast, only a minute number of *S. aureus* expressing α-toxin showed YFP accumulation (2.10 ± 0.59%). It has to be noted, that the few observed escape signals that we recorded with fixed.
preparations of the α-toxin-producing strain might have occurred during the fixation of the samples. Measurements with live cell infections never demonstrated YFP-Fc signal around staphylococci for control as well as α-toxin-expressing strains, whereas for strains expressing either δ-toxin or LLO YFP-Fc recruitment was prominent and frequent (not shown). Again, leakage of the fluid-phase endosomal cargo, Alexa 633, and YFP-Fc recruitment were mutually exclusive in infections with RN4220 expressing δ-toxin (Fig. 3C), thereby indicating disruption of the phagosomal membrane (compare with LLO-expressing RN4220 in Fig. 1C).
To ensure that the observed ability to escape conferred by δ-toxin is independent of the host cell type, we assayed two additional cell lines that were genetically engineered to express YFP-Fc. In the CFTR-complemented cystic fibrosis upper airway epithelial cell line S9 (Fig. S4A) as well as in the endothelial cell line EA.hy926 (Fig. S4B), S. aureus RN4220 expressing δ-toxin destroyed phagosomal membrane integrity as was evidenced by YFP-Fc recruitment.

It had been shown earlier that the RNAIII region harbouring the ORF for δ-toxin does not regulate virulence factors (Novick et al., 1993). In order to exclude that the mRNA for δ-toxin was responsible for the escape phenotype we nevertheless introduced mutations into the ORF; the start codon was mutated from AUG to AAG (designated M1K) thus abolishing translation initiation. In a second mutant we introduced a stop codon in the third codon position (Q3X). Both constructs did not lead to phagosomal escape (not shown) indicating that the δ-toxin peptide was necessary for the observed activity. Additionally, we co-infected HeLa YFP-Fc with a mixture of cyan-fluorescent, δ-toxin-expressing S. aureus RN4220 pHld-Cerulean, and a red-fluorescent control without toxin, S. aureus RN4220 pmRFPmars (Paprotka et al., 2010), in order to measure the effect of δ-toxin in direct comparison with a control within the same host cell. YFP-Fc recruitment was only observed for δ-toxin-expressing cyan-fluorescent S. aureus (Fig. 4, arrow). Isolated red-fluorescent S. aureus pmRFPmars were not labelled by YFP-Fc.

To verify our results in a different strain we transferred our constructs into the agr deletion mutant strain RN6911. In the subsequent experiments we observed the same experimental outcomes as with strain RN4220: δ-toxin as well as LLO expression resulted in phagosomal escape of recombinant RN6911, whereas the vector control did not affect organellar integrity (data not shown). However, when we repeated the assays in S. aureus strain SA113 (Iordanescu and Surdeanu, 1976) only expression of LLO resulted in phagosomal escape; no escape was observed for S. aureus SA113 pHld-Cerulean (Fig. 5A). This suggested that in strains RN4220 and RN6911 a second factor must act synergistically with δ-toxin and that this factor was absent in strain SA113. SA113 does not express the sphingomyelinase β-toxin (gene designation: hib) due to the insertion of the lysogenic phage φ13 in its open reading frame (Coleman et al., 1986). Because it was established that δ-toxin engages in synergistic haemolysis with β-toxin (Kreger et al., 1971), we engineered a vector collinearly expressing δ-toxin, β-toxin and Cerulean (pHld-Hib-Cerulean) in order to investigate if both toxins also synergistically mediate phagosomal escape. In contrast to S. aureus SA113 pHld-Cerulean (0.40 ± 0.22%; Fig. 5A and C) solely expressing δ-toxin and S. aureus SA113 pHlb-Cerulean (0.86 ± 0.21%; Fig. 5C) merely expressing β-toxin, S. aureus SA113 pHld-Hib-Cerulean expressing both δ- and β-toxin, was capable of escaping from the phagosome (26.15 ± 5.42%; Fig. 5B and C) thereby even exceeding the LLO-expressing strain (20.84 ± 6.26%; Fig. 5C). As the strain recombinantly expressing β-toxin, S. aureus SA113 pHlb-Cerulean, and natural β-toxin producers, RN6911 and RN4220, do not escape from the HeLa phagosome, we thus demonstrate that the 26 aa peptide staphylococcal δ-toxin and the sphingomyelinase β-toxin can act synergistically in phagosomal escape of S. aureus.

It has been reported that phagosomal escape by S. aureus strains 8325-4 and DK2076 leads to induction of apoptotic cell death in HUVEC (Menzies and Kourteva, 1998). To test whether S. aureus escape from the phagosome is associated with reduced cell viability in our system, we determined the host cell numbers during infection with different recombinant S. aureus RN4220 strains. During 24 h of infection all infected wells showed increased host cell numbers with exception of the staphylococcal strain expressing LLO (Fig. 6). Host cell numbers in infections with δ-toxin-expressing RN4220 are comparable with those obtained with the strain merely expressing fluorescent reporter, suggesting that the additional presence of δ-toxin does not result in increased host cell death – despite the efficient phagosomal escape...
mediated by δ-toxin. HeLa within infected wells do not grow as fast as in uninfected control wells (Fig. 6). As previous studies have identified the common laboratory strain RN4220 as non-cytotoxic strain (Schnaith et al., 2007; Giese et al., 2009), the relative small numbers of host cells during the infections when compared with uninfected HeLa could result from growth inhibition of HeLa in the presence of bacteria. Expression of LLO results in lower host cell numbers than in any of the controls. When compared with upper airway epithelial cells (Giese et al., 2009) our data suggest that HeLa is less sensitive towards LLO-expressing S. aureus.

Staphylococcal δ-toxin is a phenol-soluble modulin (PSM). PSMs present a class of S. aureus cytolytic peptides structurally related to δ-toxin and regulated by agr. Their importance as virulence determinants was recently described (Wang et al., 2007). To find out if PSMs other than δ-toxin also promote vesicular escape of S. aureus we infected HeLa YFP-Fc with S. aureus SA113 pHld-Hlb-Cerulean (cross-hatched) results in 26.15 ± 5.42% escape efficiency, which is similar to that observed for S. aureus SA113 pLLO-Cerulean (20.84 ± 6.26%; dark gray). By contrast, SA113 producing either δ-toxin (white) or β-toxin (light gray) do not escape from HeLa phagosomes (0.40 ± 0.22% and 0.86 ± 0.21%, respectively). Values are given as escape events per cent of internalized bacteria ± standard deviation.
Fig. 6. Despite the capacity to mediate phagosomal escape, the effect of \( \delta \)-toxin-expressing RN4220 on host cell growth is comparable with the influence of RN4220 expressing only the fluorescent reporter Cerulean. Numbers of HeLa cells over a 24 h infection period indicate that HeLa infected with RN4220 pHld-Cerulean (3 h: \( 0.73 \pm 0.14 \times 10^5 \); 24 h: \( 1.12 \pm 0.12 \times 10^5 \) HeLa cells) and the control strain RN4220 pCerulean (3 h: \( 0.54 \pm 0.16 \times 10^5 \); 24 h: \( 0.95 \pm 0.18 \times 10^5 \) HeLa cells) continue to replicate, albeit less than an uninfected control (3 h: \( 0.65 \pm 0.20 \times 10^5 \); 24 h: \( 1.81 \pm 0.62 \times 10^5 \) HeLa cells). However, an infection with LLO-expressing RN4220 induces stagnating host cell numbers (3 h: \( 0.54 \pm 0.11 \times 10^5 \); 24 h: \( 0.44 \pm 0.23 \times 10^5 \) HeLa cells). Results are presented as means of data from three independent experiments performed in duplicates (\( \pm \) standard deviations).

Discussion

In order to identify staphylococcal virulence factors involved in phagosomal escape we here present an assay that exploits staphylococcal protein A, a cell wall-anchored bacterial \( F_c \) receptor. Protein A recruits YFP-Fc in the host cell cytoplasm upon phagosomal membrane disintegration, e.g. by LLO-expressing staphylococci (Fig. 1). We established that both the Fc proportion of YFP-Fc as well as protein A of \( S. aureus \) are involved in YFP-Fc recruitment to the bacterial cell wall upon expression of LLO (Fig. S1). Because autophagy has been reported to play an important role in intracellular staphylococcal virulence within HeLa (Schnaith et al., 2007), we excluded involvement of autophagy in YFP-Fc accumulation (Fig. S2). Also, YFP-Fc recruitment is mutually exclusive with the presence of a fluid-phase fluorescent cargo within phago-endosomes (Figs 1C and 3C). Thus our YFP-Fc recruitment assay indeed reflects disintegration of a functional phagosomal membrane barrier and enables the assessment of escape efficiencies of bacterial strains. The assay only requires a wide-field fluorescence microscope, although quantitation of escape events is simplified using a motorized stage and confocal imaging. In contrast to a recently published method that measures the percentage of host cells in which \( S. aureus \)-like escape from their vacuole (Ray et al., 2010), quantification of the number of pathogens that actually escaped from the endomembrane vesicles is the hallmark of our assay, which will help to understand the contribution of phagosomal escape to virulence of \( S. aureus \). Also it enables a detailed assessment of transcriptome, proteome and metabolome signatures of pathogens recovered from host cells. The data now can be attributed to originate either from mainly membrane-enclosed or escaped staphylococci. Our assay can be performed using live as well as fixed samples and thus provides maximum flexibility for a variety of downstream analyses. Also, it should be applicable to other intracellular pathogens either naturally or heterologously expressing surface-anchored \( F_c \) receptors.

Here, we investigate \( S. aureus \) virulence factors directly involved in the process of \( S. aureus \) phagosomal escape. \( agr \) dependency of phagosomal escape has been shown for \( S. aureus \) (Qazi et al., 2001; Shompole et al., 2003; Jarry and Cheung, 2006) whereby often strains RN6390B (Peng et al., 1988) and its isogenic \( agr \) deletion mutant RN6911 (Novick et al., 1993) were used. We thus employed our assay to both strains (Fig. 2). Indeed, only for RN6390B YFP-Fc recruitment was observed, whereas RN6911 did not escape from the phagosomes thus supporting the outcomes of abovementioned studies. Interestingly, RN6390B yielded only punctuate YFP-Fc signals (Fig. 2, upper panels). This peculiar staining is reminiscent of the localization of protein A obtained for \( in vitro \) cultures of strains Newman and N315 (DeDent et al., 2007). Whereas virulence factors such as \( \alpha \)-toxin are upregulated when \( agr \) is switched on, protein A expression is negatively controlled by \( agr \) (Novick et al., 1993). Strains Newman and RN6390B display a strong \( agr \) expression (J. Panné-Farré, pers. comm.) and thus show reduced levels of the \( F_c \) receptor on their cell wall. The punctate staining in Fig. 2 thus reflects reduced levels of protein A in the \( agr \)-proficient bacteria (Janzon and Arvidson, 1990; Novick et al., 1993; Benito et al., 2000).

To date it is not known, which of the many virulence factors are responsible for phagosomal escape.

One prominent \( agr \) dependently expressed virulence factor of \( S. aureus \) is the cytolytic pore-forming protein, \( \alpha \)-toxin. Although \( \alpha \)-toxin is cytotoxic when applied to plasma membranes of mammalian cells (Bhakdi and Tranum-Jensen, 1991), it seems to promote phagosomal escape only in cells with a defect in CFTR (Jarry et al., 2008). In CFTR-proficient upper airway epithelial cells \( \alpha \)-toxin is not sufficient to disrupt or permeabilize phagosomes (Giese et al., 2009). We further demonstrated that several \( \alpha \)-toxin-expressing \( S. aureus \) strains are not able to escape from the phagosome (Lâm et al., 2010). Thus another virulence factor must be required in this process.

Upon \( agr \) activation, RNAIII is transcribed and regulates various proteins on transcriptional and translational level.
Fig. 7. Phenol-soluble modulins (PSMs) β but not α enable phagosomal escape of recombinant *S. aureus*. *S. aureus* RN4220 expressing pPSMβ1-2 and Cerulean (magenta) induce concentration of YFP-Fc (green) at the bacterial periphery in HeLa cells indicative for phagosomal escape (A). Blue: DNA; yellow: actin. Scale bars: lower panel: 5 μm; zoomed panels: 2 μm.

B. In a quantitative assay (25 fields of view per experiment; three independent experiments) approximately one-fifth of intracellular RN4220 pPSMβ-Cerulean escape (19.77 ± 0.72%), whereas the escape efficiency for RN4220 pPSMα-Cerulean is negligible (1.45 ± 0.72%).

C. Contrasting our findings from the phagosomal escape assays, haemolysis of RN4220 pPSMα-Cerulean is more prominent than that of RN4220 pPSMβ-Cerulean. Thus, phagosomal escape proficiency cannot directly be derived from the haemolytic phenotype.
Novick et al., 1993; Morfeldt et al., 1995; Geisinger et al., 2006). RNAIII further encodes the 26 amino acid residue amphipathic peptide, δ-toxin. δ-toxin has been shown to possess cytolytic properties and lyses bacterial protoplasts, lysosomes, lipid spherules, mitochondria and erythrocytes (Bernheimer and Schwartz, 1964; Kreger and Bernheimer, 1971; Kreger et al., 1971; Verdon et al., 2009). We therefore chose to investigate, if staphylococcal δ-toxin is involved in phagosomal escape. Because clinical isolates of S. aureus often are highly cytotoxic and express a plethora of virulence factors, we decided on the use of the non-cytotoxic laboratory S. aureus strain RN4220 as toxin-delivery vehicle in a gain-of-function approach. For toxin expression we modified a plasmid vector collinearly expressing toxin and the cyan-fluorescent marker, Cerulean, under control of an anhydrotetracycline-inducible promoter (Geissendörfer and Hillen, 1990; Bateman et al., 2001; Giese et al., 2009). When we expressed δ-toxin in strain RN4220, we observed phagosomal escape in rates similar to those of LLO (Fig. 3). By contrast, the controls with either only fluorescent marker or point mutants of hld did not escape (data not shown). Also, expression of α-toxin did not lead to phagosomal escape (Fig. 3), which is in line with our previous findings (Giese et al., 2009; Läm et al., 2010).

To exclude influences of the different recombinant strains on the integrity of endosomal membranes, we chose to co-infect HeLa YFP-Fc with a mixture of two recombinant S. aureus strains expressing either a red-fluorescent protein but no toxin or δ-toxin and Cerulean. Within the same host cell YFP-Fc recruitment was only observed around toxin producers indicating that phagosomal integrity of infected HeLa is compromised by the toxin and not due to cell-wide effects (Fig. 4).

Staphylococcus aureus toxins often target specific cell types. For example, there is differential sensitivity of multiple cell lines against the pore-former α-toxin (Valeva et al., 1997). We thus repeated our analyses in S9 upper airway epithelial cells (Flotte et al., 1993) as well as the endothelial cell line Ea.hy926 (Edgell et al., 1983). In all cell lines tested expression of δ-toxin by strain RN4220 led to phagosomal escape. This indicated that our results are independent of the selected host cell type (Fig. S4).

When we infected HeLa with recombinant versions of the agr-deficient mutant RN6911 we reproduced the results obtained for genetically engineered variants of strain RN4220. It thus seemed likely, that solely the δ-toxin presence might account for the disintegration of the phagosomal membrane. However, in another S. aureus laboratory strain, strain SA113, we were astonished to find that δ-toxin expression did not lead to YFP-Fc recruitment in infected HeLa YFP-Fc. We hypothesized that an additional factor is present in RN4220 and RN6911 but absent in SA113 and that this factor might account for the deviating observations. SA113 is a derivative of NCTC8325, a strain lysogenic for three prophages, whereas RN4220 and RN6911 are derived from phage-cured strain NCTC8325-4. In NCTC8325 the lysogenic phage φ13 is inserted into the structural gene for the sphingomyelinasen β-toxin (gene hlb) (Coleman et al., 1986). As a synergistic haemolytic activity of δ-toxin with β-toxin was described (Hebert and Hancock, 1985) we investigated a possible synergism for both toxins in phagosomal escape. Aside from the control strain expressing LLO, only the strain coexpressing δ-toxin and β-toxin escaped from the phagosome indicating that both toxins act synergistically in phagosomal escape of S. aureus (Fig. 5). The toxin expression levels were comparable or less than that of natural producers of β-toxin and δ-toxin as was judged from the width of haemolytic zones on sheep blood agar (Fig. S5).

Staphylococcal β-toxin is a neutral sphingomyelinasen C cleaving sphingomyelin to phosphocholine and ceramide moieties (Doery et al., 1983) and the sphingomyelinasen activity is responsible for haemolysis and monocyte cytotoxicity (Huseby et al., 2007). Sheep erythrocytes have a large proportion of sphingomyelin in their membrane (Nelson, 1967) and thus are highly susceptible to staphylococcal β-toxin, whereas erythrocytes from other species with a low sphingomyelin content are virtually insensitive to the toxin. For complete haemolysis of erythrocytes in sheep blood agar plates either a shift from 37°C to 4°C or additional factors must be present. Staphylococcus lugdunensis synergistic haemolysins (SLUSH) and δ-toxin have been recognized to cooperatively act with β-toxin in haemolysis of erythrocytes (Hebert and Hancock, 1985; Donvito et al., 1997). Sphingomyelin is present mainly in the outer leaflet of mammalian plasma membranes and thus also in the luminal face of phag-endosomes. It has been suggested that lysosomal ceramide does not contribute significantly to signalling processes (Tohyama et al., 1999). Therefore, endosomal ceramide patches might rather constitute interaction interfaces for δ-toxin. It has been shown that δ-toxin hardly binds to negatively charged phospholipids, poorly binds to cholesterol and sphingomyelin liquid-ordered raft domains, but binds strongly to liquid-disordered domains (Pokorny et al., 2006). Additionally, the involvement of δ-toxin in phagosomal escape directly explains the requirement of a functional agr system for escape in the β-toxin producer RN6390B (Qazi et al., 2001; Shompole et al., 2003; Jarry and Cheung, 2006). It recently was also shown that the staphylococcal agr system is confinement-induced and thus rather a diffusion-sensing than a quorum-sensing system, agr thus can be activated even at a single cell level (Shompole et al., 2003; Carnes et al., 2009). Even low numbers of phagocytosed staphylococci therefore are able to mount © 2010 Blackwell Publishing Ltd, Cellular Microbiology, 13, 316–329
defences to avoid degradation within host cell phagosomes. Our results indicated that in strain RN6911 heterologous δ-toxin expression was sufficient to mediate phagosomal escape in HeLa. However, RN6911 shows drastically reduced β-toxin production (Novick et al., 1993). We thus currently assume either that the residual amount of β-toxin produced by RN6911 is capable of inducing phagosomal escape in presence of δ-toxin or that alternative virulence factors can functionally replace β-toxin. In L. monocytogenes infections, for instance, a broad range phospholipase C is involved in escape from the endomembrane system (Goldfine et al., 1995; Marquis et al., 1995; Sibelius et al., 1996). With plc S. aureus possesses a homologue enzyme. Also, the Listeria metalloprotease mlp is able to disrupt infected HeLa phagosomes (Marquis et al., 1995). Aureolysin is the homologous protease in S. aureus. We therefore hypothesize that enzymes other than β-toxin can be co-opted for phagosomal escape.

The peptide δ-toxin is a PSM. PSMs have been originally identified in Staphylococcus epidermidis (Mehlin et al., 1999) and were recently discovered in S. aureus (Wang et al., 2007). PSMs are also regulated by agr and their importance as virulence determinants was described recently (Vuong et al., 2004; Wang et al., 2007; Kretschmer et al., 2010). PSMs of type β are homologues of SLUSH and about 40 AA in length, whereas PSMα are shorter (< 20 AA) and share structural similarities with δ-toxin (Wang et al., 2007). Haemolysis of PSMα was shown to be higher than that of PSMβ (Wang et al., 2007). Figure 7C corroborates these findings and illustrates production of functional PSM by our transgenic laboratory strains. However, in our analyses PSMα did not lead to phagosomal escape, whereas the recombinant strain expressing the PSMβ gene cluster efficiently escaped from the phagosome of HeLa (Fig. 7B). Our studies thus suggest that α-type PSMs might only play a subordinate role in phagosomal escape. In contrast, PSMβ can functionally substitute δ-toxin in phagosomal escape (Fig. 7). The differential behaviour between PSMα and PSMβ was unexpected, as δ-toxin is structurally more similar to PSMα than to PSMβ (Wang et al., 2007).

In summary we conclude that S. aureus can employ at least two peptide toxins to avoid degradation within the phagosome, δ-toxin and PSMβ. However, the detailed roles of the toxins identified in this work and possibly additional factors contributing to evasion of intracellular host cell defences as well as clinical significance of the toxins need further elucidation.

Among the recombinant strains used in our study δ-toxin expressing S. aureus led to phagosomal escape; however, host cell numbers were comparable with the vector control (Fig. 6). Phagosomal escape thus might not be directly linked to host cell death. Thus, non-cytotoxic strains are a valuable tool in gain-of-function approaches to observe specific host–pathogen interactions.

**Experimental procedures**

**Bacterial and host cell culture**

Staphylococcus aureus strains (Table S1) were grown in tryptone soy broth using appropriate antibiotics. All cell lines were grown Mycoplasma-free in T75 or T175 tissue culture flasks (Invitrogen, Karlsruhe, Germany) in media supplemented with 10% fetal calf serum (PAN-Biotech, Munich, Germany) and penicillin/streptomycin (100 U ml⁻¹ and 100 μg ml⁻¹, respectively). HeLa was grown in RPMI1640, EA.hy926 (Edgell et al., 1983) and S9 (Flotte et al., 1993) were grown in DMEM: F12 (1:1). All media were purchased from PAN-Biotech.

**Cloning procedures**

pCerulean and pmRFPmars were constructed as described (Paprotka et al., 2010). The toxin expression vectors were generated as follows: δ-toxin was amplified from S. aureus DNA using primers hld-f (5’-GGTTAAACATGGCACAAGATCTCA TTTCAACATCGGTGAC-3’) and hld-r (5’-CCTAGGTTATT TTTAATGAACTTTGATCTC-3’) in a polymerase chain reaction (PCR). For generation of hld-M1K, which lacks a suitable translation initiation codon, hldM1K-f (5’-GGTTGTTTTAAACA AAGCACAAGATCATTTC-3’) was substituted for hld-f. The PCR-products were cloned into pCR2.1-TOPO and transformed into E. coli TOP10 (Invitrogen) according to the manufacturer’s instructions. The Pmel-AvrII fragments containing the coding region for δ-toxin or its untranslated pendant, hld-M1K, were subsequently cloned in pCerulean, yielding pHld-Cerulean and pHld-M1K-Cerulean. Generation of pLO-Cerulean and pHLA- Cerulean has been described previously (Giese et al., 2009). β-toxin was amplified from S. aureus RN4220 DNA by PCR using oligonucleotides hb-f (5’-CCTAGGAGTGAATGGATGTTG-3’) and hb-r (5’-CCTAGGCTATTTACTAGGC-3’).

According to the above procedures the product was TOPO-cloned and sequenced. The AvrII fragment was ligated into AvrII-opened pHld-Cerulean, yielding pHld-Hib-Cerulean. The orientation of hb was verified by restriction analysis. The gene clusters for PSMα and PSMβ were amplified via PCR from genomic DNA of S. aureus 6850 (Proctor et al., 1994) using either primers psma1-f (5’-GGTTAAACATGGCACAAGATCATTTC-3’) and psma4-r (5’-CCTAGGTTATTTTGGCAAAATGT CG-3’) or psmb1-f (5’-GGTTAAACATGGCACAAGATCATTTC-3’) and psmb2-r (5’-CCTAGGTTATTTTGCAAAATGT CG-3’), respectively, were cloned into pCR2.1 TOPO and sequence analysis of the products was verified. Using endonucleases Pmel and AvrII the fragments were excised from the cloning vector and inserted in the according restriction sites of pHld-Cerulean, thereby replacing the ORF for δ-toxin. Correct insertion of the genes was confirmed via dye-terminator sequencing and activity of recombinant S. aureus strains was assessed by haemolysis on sheep blood agar.

YFP-Fc was generated by using 200 ng B-Cell cDNA as a PCR template essentially after Shibasaki et al., 2006). The 680 bp PCR product of Fc contained chain 2 and 3 as well as the hinge region of human IgG1. The product was amplified using oligo-
nucleotides Fc-fwd (5'-GACAAACTCACACATGCCCACCCG-3') and Fc-rev (5'-TCATTTCGGAGGACAGGGAGG-3') using Phusion DNA Polymerase (Finnzymes, Espoo, Finland) according to the manufacturer's instructions. The PCR product was gel-purified and cloned into pCDNA6.2-N-YFP-TOPO (Invitrogen) and transformed in E. coli TOP10 (Invitrogen) according to the recommended protocols. The resulting plasmid, pYPF-Fc, was confirmed by DNA sequencing. An expression vector of cytoplasmically localized YFP, pYPF-3A-Fc, was generated by removing the Fc-tag via EcoRI digestion of pYPF-Fc followed by religation. HeLa cells were transfected with pYPF-Fc using jetPEI-RGD (PolyPlus, Illkirch, France) and selected with 3 μg ml⁻¹ blasticidin (Invitrogen). Table S2 lists all constructs used in this study.

Infection assays

Overnight cultures of S. aureus were diluted to an optical density at 540 nm (OD₅₄₀) of 0.4 in broth containing 100 ng ml⁻¹ anhydrotetracycline. After reaching OD₅₄₀ = 0.6, bacteria were harvested by centrifugation at 3000 g for 10 min, resuspended in infection medium and added to host cells. For HeLa a multiplicity of infection of 160 was used, whereas for EA.hy926 and S9 multiplicities of infections of 60 and 40 were applied respectively. One day before infection with the S. aureus strains, 0.7 × 10⁶ host cells were seeded in 12-well plates with 18 mm cover slips (for end-point assays). For live cell microscopy, LabTek II chambered coverslips (Nunc, Langenselbold, Germany) were inoculated with 0.2 × 10⁶ HeLa per well. One hour before infection the medium containing 100 U ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin and 3 μg ml⁻¹ blasticidin was changed to infection medium without antibiotics. One hour after infection, the infection medium was replaced by medium containing 20 μg ml⁻¹ lysostaphin and 100 μg ml⁻¹ gentamicin for 30 min. After removal of extracellular bacteria, the monolayers were rinsed twice with PBS (pH 7.4) and incubated in full medium supplemented with 100 μg ml⁻¹ gentamicin. When recombinant bacteria were used, 10 μg ml⁻¹ chloramphenicol and 100 ng ml⁻¹ anhydrotetracycline (Acros Organics, Geel, Belgium) were added. For investigation of host cell numbers in presence of pathogen, HeLa cells were seeded in 12-well plates without coverslips and were infected as described above. Three and 24 h after infection the cells were detached by Trypsin/EDTA (PAN Biotech) and enumerated in a Neubauer chamber. All cell culture media contained 10% FCS (PAN Biotech). For semi-automated image analysis (Fig. S6), the Hoechst, Cerulean or SNARF channels were thresholded and particle counting was applied to detect bacteria or nuclei. After detection, the regions of interest (ROI) were enlarged in diameter by 0.2 μm. Subsequently, the Cerulean and the YFP channels were measured in 12-bit depth and plotted against each other using ImageJ thereby generation scatterplots. A gate threshold was applied and YFP signals beyond this gate were counted as escape events whereas the localization of the remainder of the staphylococci was classified as phagosomal. Escape rates were calculated by dividing the number of ROIs with YFP signals above the given threshold by the number of detected ROIs. Upon assessment of escape proficiency, classified ROIs (‘escaped’, ‘not escaped’) were visually inspected to ensure accuracy of detection and classification. Significance levels of the escape phenotypes were determined by a two-tailed Student’s t-test.

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We are indebted to the following colleagues: Annett Weltrowski for early cloning work, Nicole Schubert and Franziska Preuß for USA300 was stained before infection by incubation in 0.08% (w/v) SNARF-1 succinimidyl ester (Invitrogen) in PBS at room temperature for 30 min. For non-specific immunofluorescence staining of immunoglobulin-binding staphylococcal proteins, the monolayers were blocked after the quenching step with PBS₂⁻T containing 2% (w/v) BSA for 1 h. Subsequently, the cells were treated with PBS₂⁻T containing 0.2% (w/v) BSA and an non-specific Alexa647-coupled polyclonal donkey anti-sheep antibody (Invitrogen), overnight at 4°C. Subsequent washings with PBS₂⁻T/0.2% (w/v) BSA, with PBS₅, and with deionized water completed the procedure. The slides were mounted with ProLong Gold. Live cell microscopy was performed in LabTek II chambered cover glasses after removal of extracellular bacteria by lysostaphin/gentamicin treatment (see above). Fluorescence microscopy was performed on a Zeiss LSM510 META confocal microscope (Carl Zeiss, Jena, Germany). Confocal images, unless noted otherwise, represent 0.6 μm thick confocal slices of the specimen. Hoechst 34580 was excited with the 405 nm diode laser and a 405 nm dichroic mirror. A 420–480 nm band pass (BP) was used to obtain the fluorescence signal. The 458 nm emission line of the argon-ion laser was used for excitation of cyan-fluorescent protein, Cerulean. Cerulean fluorescence was recorded using a 458 nm dichroic and 457–507 nm BP filter. YFP-Fc was excited with the 514 nm line of the argon-ion laser. Fluorescence was detected using a 514 nm dichroic and the META detector with a detection range from 518 nm to 539 nm. mRFP was excited by the 561 nm line of the yellow diode laser. Emission was recorded using the 561 nm dichroic mirror and a 575–615 BP. MFP647-conjugated phalloidin and Alexa Fluor633 hydrazide were imaged using the 633 nm line of the HeNe-Laser in combination with a 633 nm dichroic and a spectral window of 646–753 nm of the META detector. Transmitted light images were acquired with the longest excitation wavelength of any multi-track channel set-up.

For quantitative assays, 25 fields of view per sample each covering 225 × 225 μm (40 × oil immersion objective; NA 1.3) were analysed in two replicates and results were obtained from three independent experiments unless otherwise noted. Host cells and bacteria were enumerated either by visual inspection or using the image analysis software ImageJ (Abramoff et al., 2004). For semi-automated image analysis (Fig. S6), the Hoechst, Cerulean or SNARF channels were thresholded and particle counting was applied to detect bacteria or nuclei. After detection, the regions of interest (ROI) were enlarged in diameter by 0.2 μm. Subsequently, the Cerulean and the YFP channels were measured in 12-bit depth and plotted against each other using ImageJ thereby generation scatterplots. A gate threshold was applied and YFP signals beyond this gate were counted as escape events whereas the localization of the remainder of the staphylococci was classified as phagosomal. Escape rates were calculated by dividing the number of ROIs with YFP signals above the given threshold by the number of detected ROIs. Upon assessment of escape proficiency, classified ROIs (‘escaped’, ‘not escaped’) were visually inspected to ensure accuracy of detection and classification. Significance levels of the escape phenotypes were determined by a two-tailed Student’s t-test.

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Acknowledgements

We are indebted to the following colleagues: Annett Weltrowski for early cloning work, Nicole Schubert and Franziska Preuß for
assistance, Andreas Peschel (Tübingen, Germany) for providing strain SA113, Susanne Engelmann (Greifswald, Germany) for strains RN6390B and RN6911, Olaf Schneewind (Chicago, IL) for S. aureus SEJ1, Tamotsu Yoshimori (Osaka, Japan) for mRFP-LC3, Piotr Grabarczyk (Greifswald, Germany) for human B-cell cDNA, and Jan Panè-Farrè (Greifswald, Germany) for valuable comments. This work has been funded by the German Ministry for Science and Research (BMBF) within the program ‘Entrepreneurial Regions: Competence Centers’ under code 03ZIK011. B.S. was supported by a grant of the German Research Council (DFG) under code SFB/TR34, project C6. The authors declare no competing interests.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Interaction of protein A and YFP-Fc is responsible for accumulation of fluorescence. (A) Protein A is present on the surface of S. aureus RN4220 pLLO-Cerulean (upper panels) and toxin-deficient S. aureus RN4220 pCerulean (lower panels) within infected HeLa, since fluorophore-labelled immunoglobulin (green) is bound by both bacterial strains (magenta). Z-Projection of confocal slices. Bars: 5 μm. (B) Both staphylococcal strains (magenta) that had been shown to accumulate YFP-Fc, S. aureus RN4220 pLLO-Cerulean and RN6390 pCerulean, do not recruit YFP (green) where the Fc part has been deleted. (C) S. aureus RN4220 Δspa pLLO-Cerulean (magenta), a protein A deletion mutant of strain RN4220 is unable to recruit YFP-Fc (green). Blue: DNA. Bars: 5 μm.

Fig. S2. Ring-shaped accumulation of YFP-Fc is not due to autophagy. HeLa expressing YFP-Fc (green) and the autophagic marker mRFP-LC3 (red) were infected with recombinant S. aureus (magenta). Staphylococcal expression of LLO (upper panels) and δ-toxin (middle panels) demonstrates that YFP-Fc accumulation is independent of autophagy. Lower panels: In rare cases α-toxin expressing S. aureus pHla-Cerulean recruits YFP-Fc (compare with Fig. 3). However, the signal is not surrounded by autophagic membrane. Bars: 5 μm.

Fig. S3. S. aureus strains within infected HeLa are capable of non-specific immunoglobulin-binding. S. aureus RN6390 and its isogenic agr deletion mutant RN6911, each expressing fluorescent marker Cerulean (magenta), as well as S. aureus RN4220 pHLA-Cerulean (magenta) bind immunoglobulin (green) non-specifically. These results indicate that the failure of S. aureus RN6911 Cerulean and S. aureus RN4220 pHLA-Cerulean to recruit YFP-Fc from the cytoplasm of transgenic HeLa is due to the presence of a phagosomal barrier rather than the absence of protein A. Bars: 5 μm.

Fig. S4. The competence of δ-toxin for phagosomal escape is not restricted to the epithelial cell line HeLa. YFP-Fc rings (green) surrounding S. aureus RN4220 pHLD-Cerulean (magenta) are also observed in the CFTR-proficient upper airway epithelial cell line S9 (A) and in the endothelial cell line EA.hy 926 (B). Blue: DNA; yellow: actin. Scale bars: lower panels: 5 μm; zoomed panels: 2 μm.

Fig. S5. Haemolysin of recombinant S. aureus strains in comparison with mRFP-expressing clinical strain 6850. Toxin expression driven by the various plasmids was at levels at which no prominent synergistic haemolysis is observed and diameters of β-toxin lysis were comparable between recombinant (SA113 pHld-Hlb-Cerulean, SA113 pHlb-Cerulean) and wild-type β-toxin producers (RN4220 pHld-Cerulean, 6850 pmRFPm). All strains contained plasmids and thus were streaked on Columbia Sheep blood agar containing an equivalent of 10 mg ml⁻¹ chloramphenicol and 200 ng ml⁻¹ anhydrotetracycline. Plates were incubated at 37°C overnight.

Fig. S6. Semi-automated quantification of phagosomal escape. Host cells were infected with S. aureus containing various toxin-expression plasmids and fixed with paraformaldehyde. For semi-automated microscopic quantitation of phagosomal escape of the recombinant strains, 25 fields of view were collected per replicate each covering 225 × 225 μm (40 × oil immersion objective; NA 1.3). For each strain three infections of three independently grown colonies were analysed in two technical replicates. Bacteria were enumerated by the image analysis software ImageJ (Abramoff et al., 2004) by thresholding the Cerulean channel, followed by automated particle detection. For scatterplots, after thresholding for detection of bacteria, ROI were enlarged in diameter by 0.2 μm. Subsequently, the mean Cerulean and YFP fluorescence was measured in 12-bit depth and plotted against each other using ImageJ. A gate threshold was applied and YFP signals beyond this gate were counted as escape events, whereas the localization of the remainder of the staphylococci was classified as phagosomal. Upon assessment of escape proficiency, classified ROIs (‘escaped’, ‘not escaped’) were visually inspected to ensure accuracy of detection and classification.

Table S1. Staphylococcus aureus strains used in this study.

Table S2. Plasmids used in this study.

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