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In vitro Endothelial Cell Damage is Positively Correlated with Enhanced Virulence and Poor Vancomycin Responsiveness in Experimental Endocarditis due to Methicillin Resistant *Staphylococcus aureus*

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Summary

The pathogenesis of *Staphylococcus aureus* infective endocarditis (IE) is postulated to involve invasion and damage of endothelial cells (ECs). However, the precise relationships between *S. aureus* – EC interactions *in vitro* and IE virulence and treatment outcomes *in vivo* are poorly defined. Ten methicillin-resistant *S. aureus* (MRSA) clinical isolates previously tested for their virulence and vancomycin responsiveness in an experimental IE model were assessed *in vitro* for their hemolytic activity, protease production, and capacity to invade and damage ECs. There was a significant positive correlation between the *in vitro* EC damage caused by these MRSA strains and their virulence during experimental IE (in terms of bacterial densities in target tissues; $P < 0.02$). Importantly, higher EC damage was also significantly correlated with poor microbiologic response to vancomycin in the IE model ($P < 0.001$). Interestingly, the extent of EC damage was unrelated to a strain's ability to invade ECs, hemolytic activity and protease production, or β-toxin gene transcription. Inactivation of the *agr* locus in two MRSA strains caused ~20% less damage as compared to the corresponding parental strains, indicating that a functional *agr* is required for maximal EC damage induction. Thus, MRSA-induced EC damage *in vitro* is a unique virulence phenotype that is independent of many other prototypical MRSA virulence factors, and may be a key biomarker for predicting MRSA virulence potential and antibiotic outcomes during endovascular infections.

Introduction

Throughout the industrialized world, *Staphylococcus aureus* is the leading cause of endovascular infections including vascular catheter sepsis and infective endocarditis (IE) (Fowler *et al.*, 2005, Miro *et al.*, 2005). The increased incidence of infections due to methicillin-resistant *S. aureus* (MRSA) and the high rates of vancomycin clinical failures have further complicated the management of patients who are infected with these strains (Chang *et al.*, 2003, Fowler *et al.*, 2004, Moise *et al.*, 2007, Moise *et al.*, 2000).

S. aureus expresses a large number of virulence factors that contribute to the establishment, course and ultimate outcome of endovascular infections such as IE (reviewed in (Moreillon

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et al., 2004, Moreillon *et al.*, 2002)). Upon entering the blood stream, *S. aureus* must avoid being cleared by host defense mechanisms in order to cause disease. In addition to persisting in the bloodstream, the organism must adhere to damaged cardiac valve endothelium to initiate IE. In order to persistently infect such sites, *S. aureus* must subsequently invade the cardiac endothelial cells. The ability of the organism to persist, invade, and proliferate at these initial infection sites is critical to the development of IE. Importantly, intracellular elaboration of exoenzymes by the organism may cause host cell lysis, and likely facilitates hematogenous spread to other target organs (e.g., kidneys and spleen). Recent studies suggest that EC damage plays a crucial role in the pathogenesis of valvular heart disease (reviewed in (Chorianopoulos *et al.*, 2009)]). Lysis of the endothelium harboring *S. aureus* would expose procoagulant subendothelial collagen, resulting in platelet adherence and promotion of vegetation formation. Moreover, induction of EC damage might play an important role in IE pathogenesis by providing a route for dissemination of *S. aureus* to distant sites.

In order to injure ECs, *S. aureus* must adhere to and invade these cells. The major adhesins involved in *S. aureus* invasion by ECs are thought to be fibronectin-binding proteins (FnBPs) (Peacock *et al.*, 1999, Piroth *et al.*, 2008, Que *et al.*, 2005, Sinha *et al.*, 2000). These proteins bind to fibronectin, which acts as a bridging molecule between *S. aureus* FnBPs and $\alpha_5\beta_1$ integrin on the EC surface (Jaffe *et al.*, 1978, Sinha *et al.*, 1999). The binding of FnBPs to $\alpha_5\beta_1$ integrin induces the rearrangement of the EC cytoskeleton, and subsequent endocytotis of the pathogen (reviewed in (Sinha *et al.*, 2010, Sinha *et al.*, 2005)). However, adherence to and subsequent invasion of ECs are required, but not sufficient for *S. aureus* to induce EC damage (Grundmeier *et al.*, 2010, Haslinger-Löffler *et al.*, 2005). Many factors have been suggested to mediate EC damage including bacterial toxins and immune response cytokines (reviewed in (Chorianopoulos *et al.*, 2009)).

At present, little is known about the specific role of *S. aureus* – EC interactions, especially EC damage, in the pathogenesis and antibiotic responsiveness of endovascular infectious syndromes. In order to address this fundamental void in knowledge, we studied the relationship between EC invasion and damage, and their correlations with *in vivo* virulence and antibiotic responsiveness in an experimental IE model, using ten recent MRSA clinical strains. Our results indicate that *S. aureus* - induced EC damage, rather than invasion, is crucial for innate virulence and vancomycin responsiveness in experimental IE model. EC damage might thus serve as a ‘biomarker’ for certain aspects of host - pathogen interactions *in vivo* related to *S. aureus*.

Results

Effects of inoculum and incubation time on EC damage

EC damage was assessed by a ^{51}Cr release assay in which ECs are loaded with ^{51}Cr. In this assay, ^{51}Cr is taken up by the ECs and binds to proteins in the cytosol. When the cells are damaged (due to necrosis and/or apoptosis), there is membrane leakage and the ^{51}Cr is released into the medium where it can be detected. Because the ^{51}Cr release assay has not been previously used to study *S. aureus* - induced EC damage, it was essential to identify an optimal multiplicity-of-infection (MOI; organism:EC ratio). We, therefore, determined the effect of MOIs ranging from 5 to 500 on the extent of EC damage caused by *S. aureus* strain 6850. This strain caused relatively low levels of damage at the 3h invasion time point at all three MOIs tested (Figure 1). However, strain 6850 caused significantly more EC damage after 24 h, inducing a specific chromium release of ~50% at a MOI of 50. As expected (Tuchscherr *et al.*, 2010), the small colony variant JB-1 induced significantly less EC damage than did its parental strain 6850 at 24h (Figure 2 A). Based on these data, this MOI was employed for all further EC damage assays, as a specific chromium release of ~50%

enabled us to reliably discriminate among the capacity of the different strains to induce EC damage (see below).

After 3h, all MRSA study strains caused little EC damage, and the amount of damage that was induced was similar to that induced by strain 6850 (data not shown). After 24 h there was a significant difference in the capacity of the various strains to induce EC damage (Figure 2 A). At this time point, isolates from the CC45 genetic background induced significantly more damage as compared to isolates from the CC5 background (Table 1).

The capacity of MRSA strains to invade ECs is independent of their capacity to induce EC damage

S. aureus must adhere to and invade EC in order to damage these cells (Haslinger-Löffler *et al.*, 2005). The adherence of the ten clinical MRSA strains to ECs has been previously reported (Seidl *et al.*, 2011). We found no correlation between adherence to ECs and virulence or vancomycin responses in terms of MRSA colony counts in target tissues in the IE model ($P > 0.1$ for all comparisons). We, thus, focused here on the subsequent step in *S. aureus* - EC interactions, i.e., the capacity of the various strains to invade ECs. The invasiveness of these strains was analyzed under the same *in vitro* conditions as in the damage assay, but at a MOI of 1. This latter MOI caused no detectable EC damage which would have prevented accurate quantification of invasiveness. As expected (Tuchscherr *et al.*, 2010), the small colony variant, JB-1, was significantly more invasive than its parental strain 6850 (Figure 2 B). Most of the MRSA clinical isolates invaded ECs similarly to control strain 6850. However, two CC45 and one CC5 strains invaded ECs significantly less than strain 6850 (Figure 2 B). There were no overall differences in the invasiveness of isolates from the two different genetic backgrounds (Table 1). Of interest, there was no relationship between the capacity of the different strains to invade ECs and their ability to induce EC damage ($P = 0.58$). These results are in accordance with previous findings (Grundmeier *et al.*, 2010, Haslinger-Löffler *et al.*, 2005), and indicate that *S. aureus* invasion of ECs and induction of EC damage are distinct processes.

EC damage, but not invasion, is positively correlated with virulence and reduced vancomycin responsiveness in an experimental IE model

The 10 clinical MRSA isolates were susceptible to vancomycin, with MICs of 0.5 or 1.0 μg/ml; none exhibited vancomycin tolerance based on *in vitro* kill-curve kinetics (Seidl *et al., submitted; Antimicrob Agents Chemother*). Population analyses revealed no evidence of vancomycin-heteroresistant subpopulations (Seidl *et al.*, submitted). These 10 isolates were originally selected for the study because they belonged to two distinct genetic backgrounds (CC45 and CC5) that have been associated with enhanced virulence in humans (Fusco *et al.*, 2009, McCalla *et al.*, 2008, Sakoulas *et al.*, 2002, van Leeuwen *et al.*, 2000). We recently analyzed these 10 strains in an experimental rabbit IE model for their comparative fitness and therapeutic response profiles to vancomyin (Table 2, Seidl *et al., submitted; Antimicrob Agents Chemother*). In this model, vancomycin treatment of IE caused by five of the ten strains (four CC45 strains and one CC5 strain; 'non-responders') was relatively ineffective, resulting in < 1.5 $\log_{10}$cfu/gram reductions in the bacterial load in the three target tissues (vegetations; kidneys, spleen). IE caused by the other five MRSA strains (one CC45 and four CC5 strains) responded well to vancomycin treatment ('responders'), with ≥ 5 $\log_{10}$cfu/g reductions in the number of CFUs in the vegetations, and ≥ 3 $\log_{10}$cfu/g reduction in bacterial counts in the kidney and spleen (Table 2, Seidl *et al., submitted; Antimicrob Agents Chemother*).

The extent of EC damage induced by the 10 MRSA isolates was directly correlated with their bacterial counts in target tissues in the IE model (Figure 3). Among the three target

tissues, the relationship between EC damage and tissue bacterial burden was the strongest for vegetations, and weakest for spleen. Furthermore, as a group, MRSA strains with a good response to vancomycin therapy in the experimental IE model induced significantly less EC damage than did MRSA strains that did not respond to vancomycin treatment (Table 3). In addition, CC45 strains inducing the greatest EC damage *in vitro* responded the least to vancomycin *in vivo* (Figure 2 A). Therefore, the capacity of CC45 isolates to damage ECs may in some manner enable it to avoid being killed by vancomycin *in vivo*.

Of interest, there was no significant association between the capacity of the study strains to invade ECs *in vitro* and their virulence during IE, as determined by respective bacterial densities in the infected target tissues ($P > 0.1$ for all comparisons). Moreover, the EC invasiveness of the strains that responded well to vancomycin in the IE model was similar to that of the strains that responded poorly to this antibiotic (Table 3). Thus, almost all of the study strains invaded ECs well, suggesting that this phenotype is required for subsequent pathogenetic steps in endovascular infections.

The presence of a functional agr locus is dispensable for EC invasion, but is necessary for greater EC damage

The accessory gene regulator (*agr*) locus is a principal global regulator within the overall staphylococcal virulon, regulating many adhesins and secreted virulence factors, such as proteases and toxins (e.g., α-toxin). The *agr* effector, RNAIII, also encodes the cytolysin, δ-toxin (reviewed in (Verdon *et al.*, 2009)). Because several previous studies have suggested that the *agr* locus plays a key role in *S. aureus* - induced host cell death (Giese *et al.*, 2011, Haslinger-Löffler *et al.*, 2005), we investigated the EC interactions of two engineered *agr* mutant strains. The two *agr* null mutants were constructed only in the CC45 backgrounds as we were not able to introduce the mutation into the CC5 background. The mutants produced no δ-toxin and had reduced α-toxin gene (*hla*) transcription as compared to their wild-type parent strains (Table 2). The EC invasion of strain 300-169Δ*agr* was significantly higher than that of its parental strain (Figure 4 A). In contrast, the EC invasion of strain 324-136Δ*agr* was slightly lower than that of its parental strain, without reaching statistical significance. Although relatively modest in extent, each mutant strain induced significantly less EC damage than did its respective parental strain, at both 3h and 24h (Figure 4 B). Collectively, these results indicate that *agr* is dispensable for MRSA invasion of ECs, although necessary for maximal induction of EC damage.

Hemolysin and protease production did not correlate with EC damage

There was no significant correlation between the hemolytic activity of the individual strains and their capacity to induce EC damage (Figure 5 A). For instance, strain 088-237 was non-hemolytic, but was nevertheless able to induce more EC damage than strain 010-016, which was strongly hemolytic (Figures 2 A and Figure 5 A). Similarly, there was no apparent relationship between extracellular protease production and EC damage (Figure 2 A and Figure 5 B). Thus, strain 301-103, which induced greater EC damage than did strain 6850, exhibited very low protease activity. In contrast, strain 010-016, which caused the least EC damage, demonstrated strong protease production (Figures 2 A and Figure 5 B).

β-toxin is not required for induction of EC damage

It was recently reported that the combined elaboration of β-toxin and δ-hemolysin is required for *S. aureus* to escape from phago-endosomes and induce EC damage (Giese *et al.*, 2011). β-toxin is variably produced by *S. aureus* strains, depending on the presence or absence of β-toxin converting phages whose insertion inactivates the β-toxin gene *hlb*. While strains COL and 6850 exhibited *hlb* transcripts, none of the ten clinical MRSA strains used in this study expressed the *hlb* gene *in vitro* (Figure 6 A). To determine if the lack of

hlb transcript in these latter strains was due to the presence of *hlb*-converting phages, we tested for their presence by multiplex PCR. The control strains, COL and 6850, both possessed an intact *hlb*-gene. Like control strain N315, all five CC5 strains had an integrated *hlb*-disrupting phage (Figure 6 B). No PCR product was obtained for any of the CC45 strains; the reason for their lack of *hlb* transcription remains unclear. However, these data demonstrate that EC damage in this strain cohort is unambiguously independent of β-toxin elaboration.

Discussion

Although *S. aureus* has been principally considered an extracellular pathogen, recent data have confirmed that this organism is internalized by a variety of non-professional phagocytes, such as ECs. While the interactions between vascular ECs and circulating bacteria, as noted above, are considered to be important in the initial phases of *S. aureus* IE pathogenesis (i.e., tissue infection), little is known about the specific impact of the *S. aureus*:EC interactions upon the virulence and response to antibiotic therapy in endovascular infections. It has been recognized that internalized *S. aureus* can avoid host immune defenses, as well as the bactericidal effects of many antimicrobial agents, thus fostering persistent and/or relapsing infections (Reviewed in (Garzoni *et al.*, 2009)]). Moreover, the intra-EC milieu appears to foster small colony variant morphotypes, an additional intracellular persistence strategy for *S. aureus* (Schröder *et al.*, 2006, Sendi *et al.*, 2009, Vesga *et al.*, 1996). On the other hand, *S. aureus* - infected ECs could initiate a brisk inflammatory response, prompting production of key cytokines, chemokines and adhesion molecules. Such responses might either assist in efficiently eliminating invading bacteria, or in contrast, promote further disease pathogenesis at the infection site (Golias *et al.*, 2007). Therefore, in the present study, we defined *S. aureus* - EC interactions, focusing on two key post-EC binding events: **i**) invasion (internalization); and **ii**) the ability to induce EC damage. Importantly, the effects of these two interaction metrics on virulence and antibiotic treatment outcome was defined in a relevant model of endovascular infection.

Our present study demonstrated several pivotal findings. *First*, the extent of EC damage by different MRSA strains predicted their virulence in terms of achievable bacterial densities in IE target tissues. Those MRSA strains which induced less EC damage were also significantly less virulent, as compared to MRSA strains that caused greater EC damage. *Second*, there was a significant inverse correlation between MRSA-induced EC damage and vancomycin therapeutic responses in the IE model. Thus, with one exception (strain 300-246), higher EC damage correlated with worse response to vancomycin therapy in the IE model. This differential vancomycin outcome among strains was not linked to several standard *in vitro* vancomycin susceptibility yardsticks, including MICs, killing kinetics, tolerance or population analyses profiles. The ability of individual MRSA strains to induce EC damage was significantly related to their genetic background in terms of CC types: i.e., CC45 isolates caused significantly more EC damage than did the CC5 isolates. Furthermore, these CC45 isolates were significantly more virulent than the CC5 strains in the IE model (Seidl *et al.*, *submitted; Antimicrob Agents Chemother*). It has been shown that CC45 MRSA strains can cause severe human infectious syndromes (Fusco *et al.*, 2009). Collectively, these data suggest that induction of EC damage is a key event during endovascular infections, and that this process is not only associated with the innate virulence of an individual strain, but also its capacity to persist despite vancomycin therapy.

The relationship between the capacity of an organism to damage ECs *in vitro* and virulence in experimental animal models of infection has also been reported for *Candida albicans* and *Aspergillus fumigatus* (Chiang *et al.*, 2008, Sanchez *et al.*, 2004). However, a unique finding in the current study was that except for one strain (300-246), MRSA isolates with the

greatest capacity to induce EC damage also induced infections that had the poorest response to vancomycin therapy. Although the exact cause of this relationship remains to be determined, we speculate that the strains that cause the greatest host cell damage may produce the largest areas of necrosis within the host. Such necrosis may also stimulate cytokine-mediated and/or tissue factor-induced procoagulant activity at these sites (Lopes-Bezerra *et al.*, 2003, Mattsson *et al.*, 2008, Matussek *et al.*, 2005, Veltrop *et al.*, 1999), leading to exuberant platelet-fibrin vegetation formation. The net result of these events may well render the organism better able to survive within such areas of necrosis-vegetation formation (e.g., due to reduced vancomycin penetrations).

A number of different assays have been utilized to assess *S. aureus* - induced cytotoxicity (Park *et al.*, 2008, Strober, 2001). In the present study, we used a ^{51}Cr release assay, which has been used to detect damage in endothelial and epithelial cells induced by *Candida albicans* (Filler *et al.*, 1995, Park *et al.*, 2009, Phan *et al.*, 2000) and *Aspergillus fumigatus* (Bezerra *et al.*, 2004, Kamai *et al.*, 2006). To our knowledge, this assay has not been previously used to assess *S. aureus* - induced EC damage. We determined that the extent of EC damage induced by the control *S. aureus* strain 6850 was inoculum-dependent, similar to the finding of Haslinger-Loeffler *et al.* (Haslinger-Löffler *et al.*, 2005). In parallel, confirming other investigations, very little EC damage was induced by strain JB-1, a stable small colony variant of strain 6850 (Sendi *et al.*, 2009, Tuchscherr *et al.*, 2010). These results demonstrate that the ^{51}Cr release assay is a convenient, reliable, and reproducible method for assessing the capacity of *S. aureus* strains to induce EC damage. Our finding that the extent of EC damage measured using this assay directly correlated with virulence during IE and poor response to vancomycin argues that this assay is an excellent tool for elucidating key aspects of the host-pathogen interaction during MRSA IE.

Another interesting finding was that the capacity of the MRSA study isolates to invade ECs did not correlate with their ability to induce EC damage. This is in accordance with previous findings, which suggest that even though invasiveness is a prerequisite for *S. aureus*-induced cytotoxicity, it does not necessarily lead to EC activation and/or cytotoxicity (Grundmeier *et al.*, 2010, Haslinger-Löffler *et al.*, 2005, Tuchscherr *et al.*, 2010). These results strongly suggest that EC invasion and damage are distinct and separate processes, which are likely governed by different MRSA genetic networks and phenotypic effectors.

Other investigators have reported that many factors are associated with *S. aureus* - induced EC damage, including the elaboration of α-, β-, and δ-toxins. The RNAIII effector molecule of the *agr* locus, which encodes δ-toxin, has been reported to be a key factor in *S. aureus*-induced cytotoxicity (Shompole *et al.*, 2003). δ-toxin has been shown to possess cytolytic properties (reviewed in (Verdon *et al.*, 2009)), and has been suggested to mediate phagosomal escape by *S. aureus* (Giese *et al.*, 2011, Shompole *et al.*, 2003), a proposed prerequisite for induction of host cell death (Klein *et al.*, 2006). We found that *agr* null mutants caused approximately 20% less EC damage than did their respective parental strains. Thus, although *agr*-induced δ-toxin production plays a modest role in inducing EC damage, other factors must also contribute to this process.

A recent investigation demonstrated that β-toxin in combination with δ-toxin was required for phago-endosomal escape from ECs; the authors speculated that this process induced EC death (Giese *et al.*, 2011). β-toxin is variably produced by *S. aureus* strains, depending on the presence or absence of *hlb*-converting phages, whose insertion inactivates *hlb*. We found that none of our 10 MRSA clinical isolates expressed detectable *hlb* mRNA. In five of the ten isolates, the absence of *hlb* mRNA was due to the presence of a β-toxin converting phage. Because the majority of these isolates still induced significant EC damage, β-toxin production appears to be dispensable for this process. Therefore, phago-endosomal escape

may not necessarily cause cell death. Alternatively, the necessity of β-toxin might be host cell-dependent.

The role of *S. aureus* α-toxin in cytotoxicity is not clear, with results from previous investigations being rather inconsistent (Reviewed in (Sinha *et al.*, 2010)). We found no relationship between α-toxin expression and EC damage among the MRSA strains tested. For instance, strain 088-237 did not produce α-toxin, but still induced significant EC damage. Taken together, our findings suggest that individually, α-, β-, and/or δ-toxins play a modest role in the induction of EC damage by MRSA. On the other hand, it is also possible that the effects of deletion/absence of a single toxin gene are masked by the increased expression of other toxin genes. Additional experiments using strains that lack multiple toxin genes are necessary to evaluate this possibility.

In summary, *S. aureus* - induced EC damage significantly contributes to both virulence and responsiveness to antimicrobial (vancomycin) therapy in experimental IE. The ^{51}Cr release assay used to quantify *S. aureus* - induced EC damage provides an excellent technique to study biologically relevant host-pathogen interactions under controlled experimental conditions. We are currently using this assay to test additional MRSA strains with different genetic backgrounds and with distinct gene knockouts to better understand the MRSA - EC interactions in IE pathogenesis and antibiotic treatment outcomes.

Experimental procedures

Reagents

M199 medium, streptomycin, penicillin and HBSS were obtained from Gibco (Grand Island, NY, USA); tissue culture plastics were from Costar (Cambridge, MA, USA). Fetal bovine serum and bovine calf serum were purchased from Gemini Bioproducts (West Sacramento, CA, USA). Trypsin was purchased from Irvine Scientific (Santa Ana, CA, USA). Human albumin and fibronectin from human serum, collagenase, lysostaphin and casein sodium salt from bovine milk were from Sigma-Aldrich (St. Louis, MO, USA). Agar plates contained 5% Sheep in Tryptic Soy Agar Base (Hardy Diagnostics, Santa Maria, CA, USA).

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. The ten MRSA strains studied here originated from a multi-national *S. aureus* bacteremia clinical trial collection, conducted between 2002 and 2005 (Fowler *et al.*, 2006). All isolates had similar *in vitro* growth kinetics. When not otherwise specified, bacteria were grown overnight in Bacto BHI broth (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at 37°C. In addition to the above ten strains, *agr* deletion mutants of two isolates were used (Seidl *et al.*, *submitted; Antimicrob Agents Chemother*).

In the EC damage assays (described below), the well-characterized *S. aureus* strains 6850 and JB-1 (kindly provided by R. Proctor; University of Wisconsin) were included as controls. Strain 6850, a clinical isolate from a patient with invasive infection (osteomyelitis), was used as a high damage-inducing strain, because it was previously shown to induce apoptosis in human ECs (Haslinger-Löffler *et al.*, 2005, Tuchscherr *et al.*, 2010). Strain JB-1, a stable gentamicin-induced small colony variant (SCV) of 6850 was used as a negative control because it is known to invade ECs without causing substantial EC damage (Balwit *et al.*, 1994).

Endothelial cell (EC) damage

ECs were harvested from human umbilical cord veins by the method of Jaffe *et al.* (Jaffe *et al.*, 1973) and maintained as previously described (Filler *et al.*, 1995). For use in the damage assays, confluent ECs (second or third passage) in 24-well tissue culture plates were incubated overnight in complete M-199 medium (with 10% fetal bovine serum, 10% bovine calf serum plus penicillin, 100IU/ml; streptomycin, 100 μg/ml) containing $Na_2{}^{51}CrO_4$ (6 μCi per well; MP Biomedicals, Solon, Oh). Prior to inoculation, unincorporated ^{51}Cr was aspirated from the ECs and the wells were rinsed twice with warm HBSS. The various bacterial strains were grown overnight, washed twice and added to the ECs in invasion medium (1% human albumin and 25 mM HEPES, pH 7.3 in M199 without serum or antibiotics, (Haslinger-Löffler *et al.*, 2005, Tuchscherr *et al.*, 2010)) at the indicated multiplicities of infection (MOIs). After 3 h incubation at 37°C in 5% CO_2, the medium was aspirated and collected (3 h wash), wells were washed with HBSS and 500 μl of fresh complete M-199 medium containing 10 μg/ml lysostaphin was added (Cheung *et al.*, 2007, Vann *et al.*, 1987). After a total incubation time of 24 h at 37°C in 5% CO_2, 0.25 ml of medium was gently aspirated from each well, after which the endothelial cells were lysed by the addition of 0.5 ml of 6 N NaOH. The lysed cells were aspirated and collected, and wells were rinsed twice with Radiac Wash (Atomic Products, Inc. Shirley, NY). These rinses were added to the lysed cells, and the ^{51}Cr activity of the medium and the cell lysates was determined. Uninfected control wells which underwent the same washes were processed in parallel to measure the spontaneous ^{51}Cr release. After corrections were made for the differences in the incorporation of ^{51}Cr in each well, the specific release of ^{51}Cr at the 3 h and 24 h time points were calculated as previously described (Filler *et al.*, 1995).

EC invasion

The capacity of the various strains to invade ECs was determined by the lysostaphin protection assay (Cheung *et al.*, 2007, Suzuki *et al.*, 2011) using conditions similar to those used for damage assays (see above) at an MOI of 1 (10^5 bacteria per well). Briefly, invasion was allowed to proceed for 3h before extracellular bacteria were lysed with 10μg/ml lysostaphin in complete M-199 medium, after which the ECs were lysed and the number of internalized organisms was determined by quantitative culture as described (Cheung *et al.*, 2007). Previously published equations were used to determine relative invasiveness (Suzuki *et al.*): percent invasion = (number of bacteria internalized/number of bacteria initially added) × 100; Growth index = total number of bacteria at 3h/number of bacteria initially added; corrected invasion = percent invasion/growth index.

Assessment of hemolytic and protease activity

To investigate the hemolytic and proteolytic capacities of the study strains, the cells were grown overnight on sheep blood agar and resuspended in physiological NaCl solution to a density of 0.5 McFarland units (~10^8 cells/ml). Next, 2 μl of the suspension was spotted onto sheep blood agar (for hemolytic activity) and onto 1% caseinate agar (for proteolytic activity) (Ismail *et al.*, 2000) and incubated at 37°C for 24 h. Zone sizes (in mm) around each colony were measured. Each strain was tested at least twice.

Northern blot and multiplex PCR analyses for β-hemolysin

Both Northern blotting and multiplex PCR were used to detect *hlb* transcription and the presence of an intact β-toxin gene. For Northern blotting, RNA from bacterial cultures grown to late exponential (4h) growth phase was isolated using the RNAeasy kit (Qiagen, Valencia, CA). A total of 5 μg of total RNA from each sample were separated through a 1.5% agarose-20 mM guanidine thiocyanate gel in 1× Tris-borate-EDTA running buffer (McCallum *et al.*, 2006). Digoxigenin-labeled DNA probes, produced by using the PCR

DIG Probe synthesis kit (Roche, Basel, Switzerland), were used for the detection *hlb* transcripts by Northern hybridization, according to the instructions of the manufacturer using previously published primers (Said-Salim *et al.*, 2003). The Northern blot analyses were performed using two independent RNA samples.

Multiplex PCR to detect the presence of *hlb*-converting phages was carried out using primers hlb-2, hlb-527 and θN315int-for as previously published (Goerke *et al.*, 2006). Primers hlb-2 and hlb-527 span the attB-site, and therefore can be generated only in the absence of the phage, an *hlb*-specific amplicon. The integrase gene of the phage is detected by primers θN315int-for and *hlb*-527, thus only the *hlb*-integrated phage is detected. Strain COL, that has an intact *hlb* gene and N315 that has an integrated phage were used as control strains.

Statistical analysis

Means, medians, and standard deviations of continuous variables were calculated for all strains. For the analysis of the relationships of continuous variables to genotype, EC damage, and vancomycin responses, univariate analyses were performed using the student's t-test or the Wilcoxon rank sum test. For the analysis of the relationship between continuous variables and virulence in terms of bacterial density in target tissues, univariate analyses were performed with simple linear regression or Spearman rank-correlation, as appropriate.

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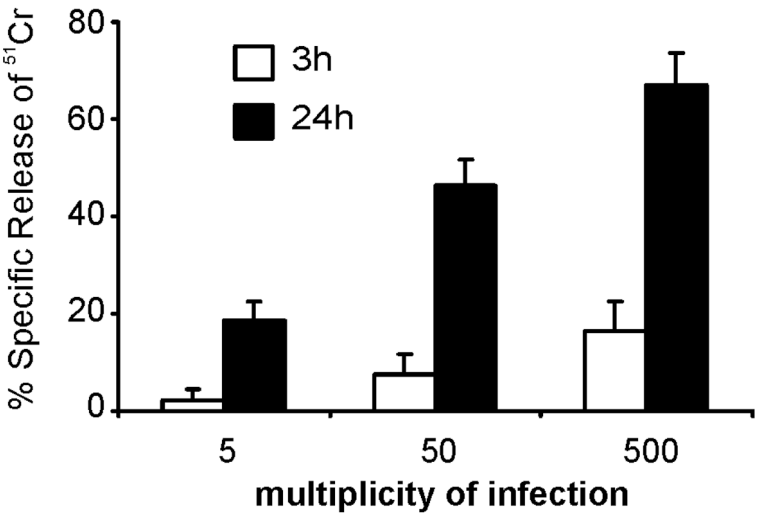

Figure 1. Endothelial cell injury by strain 6850 as determined by ^{51}Cr release

Staphylococcus aureus strain 6850 induces EC damage in an inoculum-dependent manner. Data are shown as means + SD of three independent experiments performed in triplicate.

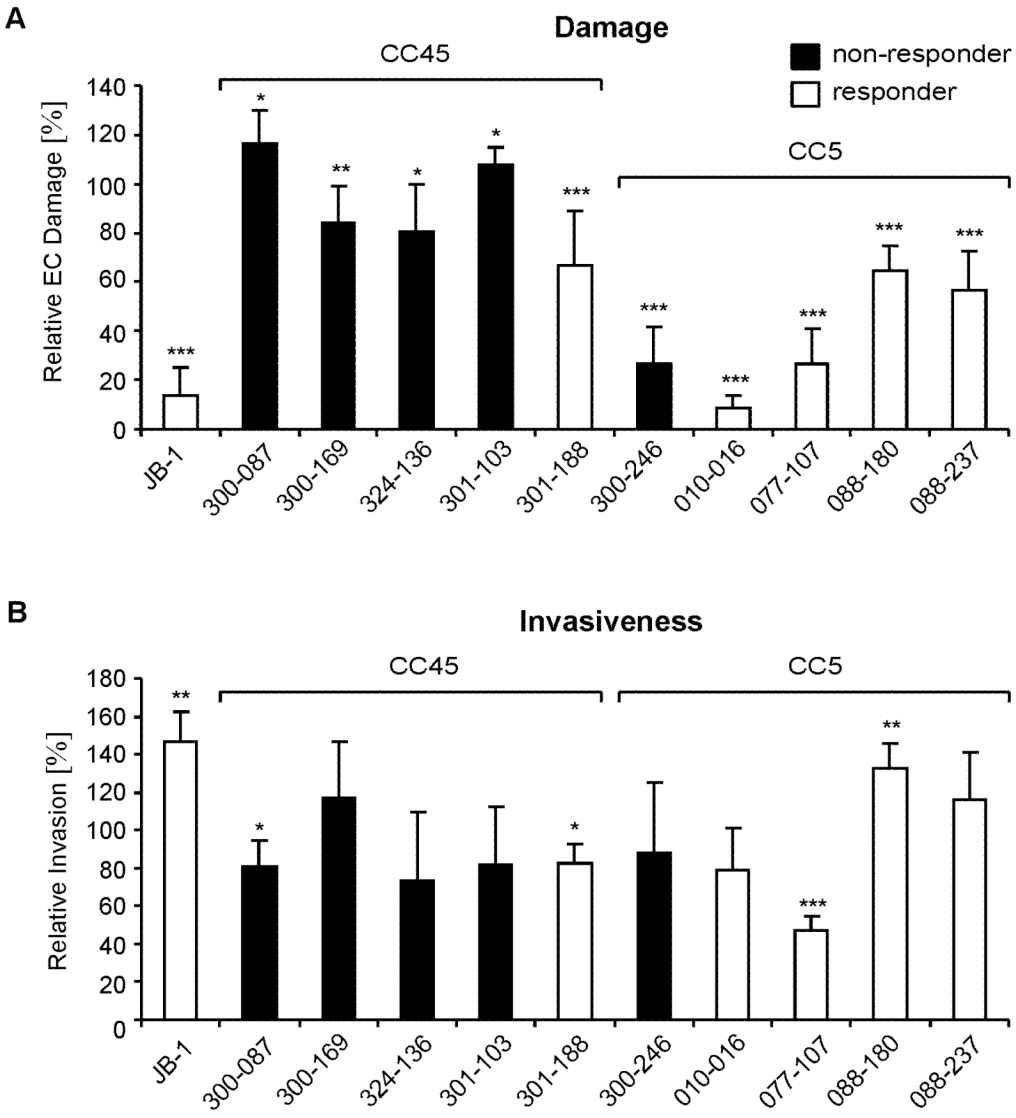

Figure 2. Endothelial cell invasion and damage

A, Endothelial cell damage as determined by specific ^{51}Cr release 24h after infection. Data are shown as means + SD of three independent experiments performed in triplicate and expressed as the percentage of damage caused by strain 6850 (set up as 100%). **B,** Corrected invasiveness shown as means + SD of three independent experiments performed in duplicate and expressed as the percentage of invasiveness of strain 6850 (set up as 100%). Responder: MRSA strains that responded to vancomycin treatment in the experimental IE model; non-responder: MRSA strains that did not respond to vancomycin treatment in the IE model (see Table 1 for detailed descriptions). *; $P < 0.05$; ** $P < 0.01$; ***, $P < 0.001$ as compared to strain 6850.

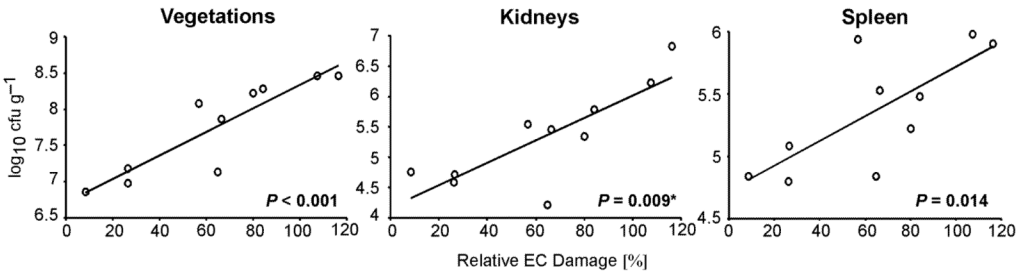

Figure 3. Relationship between EC injury and virulence in experimental IE

EC damage induced by ten clinical MRSA isolates was plotted against average bacterial densities in vegetations, kidneys, and spleen. Simple linear regression and Spearman rank correlation (*) showed a direct relationship, whereby higher EC damage was associated with higher bacterial density in all target tissues. The results do not change significantly when two *agr* mutant strains are included in the analysis. EC damage is expressed as the percentage of damage induced by strain 6850.

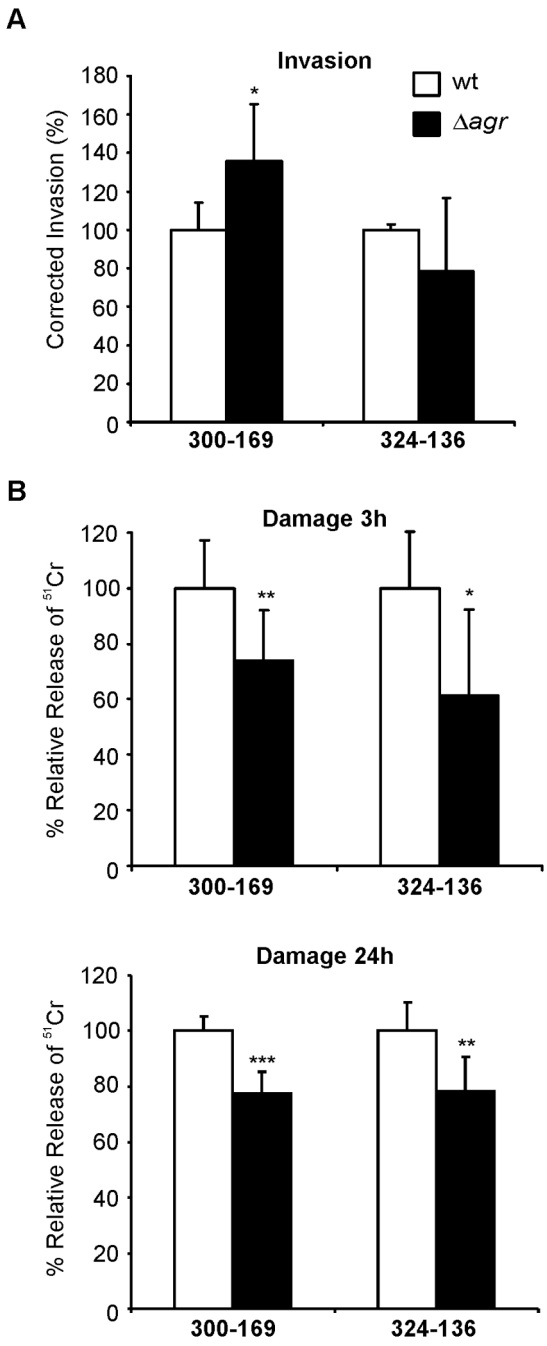

Figure 4. Influence of *agr* on EC invasion and damage

(A) Relative EC invasiveness of and **(B)** damage induced by two clinical MRSA isolates and their corresponding *agr* mutants. Data for the mutants are expressed as percentage of the corresponding parental strains. Data represent the mean of three independent experiments carried out in duplicate and triplicate for invasion and damage, respectively. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

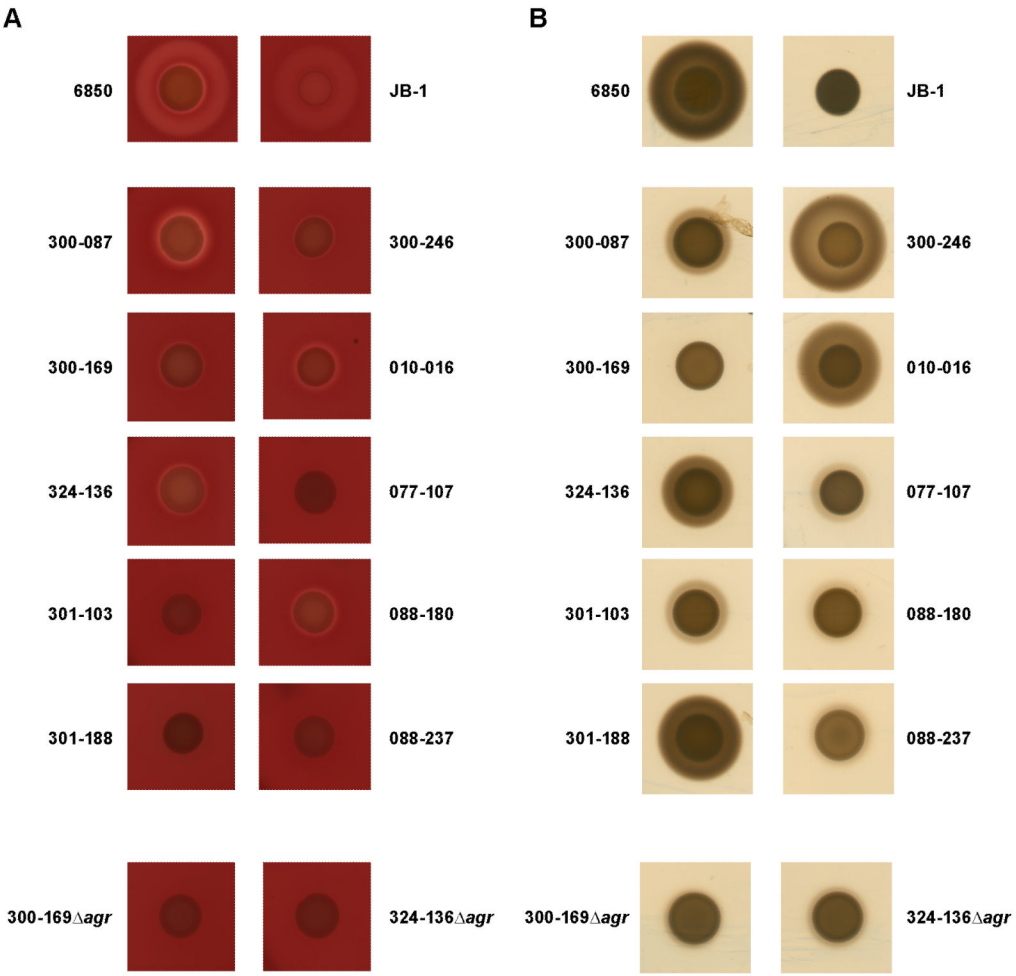

Figure 5. Hemolytic activity and protease production

A, Hemolytic activity was assessed on sheep blood agar. **B,** Protease production was assessed on caseinate agar (Ismail *et al.*, 2000).

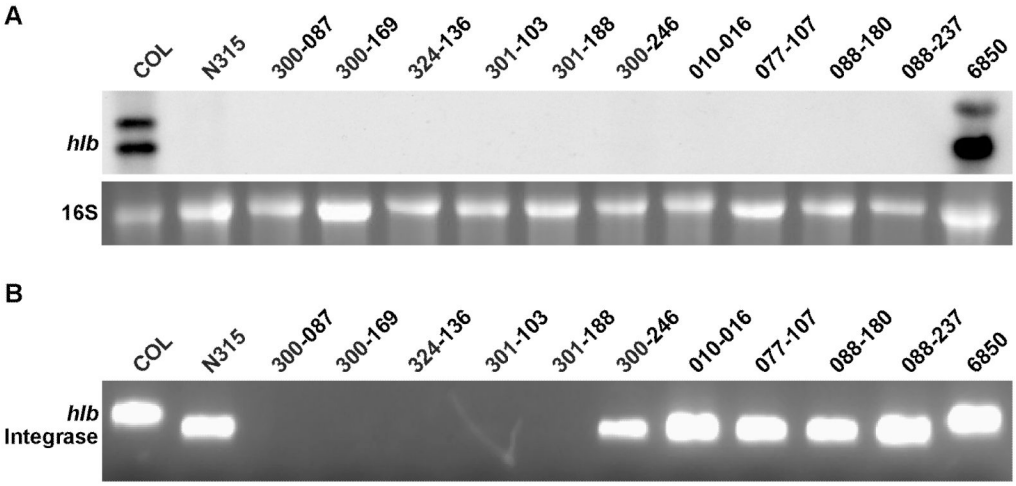

Figure 6. *hlb* expression and detection of *hlb*-converting phages

A, Northern blot analysis of *hlb* transcripts at late exponential growth phase (4h). **B,** Multiplex PCR for the detection of the intact *hlb* and phage integrase.

Table 1

Correlation of *S. aureus* - EC interactions and genetic background of MRSA strains.

	Mean ± SD		
	CC45	**CC5**	***P***
Relative Invasion (%)[a]	86 ± 31	93 ± 39	0.48
Relative EC damage (%)[b]	86 ± 25	34 ± 24	< 0.0001

[a] Corrected invasiveness as expressed as the percentage of invasiveness of strain 6850 (set up as 100%).

[b] *S. aureus* induced EC damage as determined by specific ^{51}Cr release at 24h after infection. Data are expressed as damage induced by strain 6850 (set up as 100%).

Table 2

***Staphylococcus aureus* strains used in this study**

Strain	Description	α-hem [a]	δ-hem [a]	VAN response[b]	Reference
Clinical MRSA isolates					
300-087	*agr*-I, SCC*mec* IV, CC45	+	++	No	(Fowler *et al.*, 2006, Seidl *et al.*, 2011)
324-136	*agr*-I, SCC*mec* IV, CC45	+	++	No	as above
300-169	*agr*-I, SCC*mec* IV, CC45	+	++	No	as above
300-103	*agr*-I, SCC*mec* IV, CC45	+	+/-	No	as above
301-188	*agr*-I, SCC*mec* IV, CC45	+	+/-	Yes	as above
300-246	*agr*-II, SCC*mec* I, CC5	+/-	++	No	as above
010-016	*agr*-II, SCC*mec* II, CC5	+	+/-	Yes	as above
077-107	*agr*-II, SCC*mec* II, CC5	+	+/-	Yes	as above
088-180	*agr*-II, SCC*mec* II, CC5	+	+/-	Yes	as above
088-237	*agr*-II, SCC*mec* II, CC5	-	-	Yes	as above
Mutants					
324-136Δ*agr*	324-136 *agr*::*tet*(M), Tc^r	+/-	-	No	(Seidl *et al, submitted; Antimicrob Agents Chemother*)
300-169Δ*agr*	300-169 *agr*::*tet*(M), Tc^r	+/-	-	No	(Seidl *et al, submitted; Antimicrob Agents Chemother*)
Laboratory strains (controls)					
6850	Wild type isolate from osteomyelytis				(Balwit *et al*., 1994)
JB-1	Menadione auxotroph SCV from strain 6850				(Balwit *et al*., 1994)
COL	Homogeneous methicillin-resistant strain, *hlb*$^+$				(Tomasz *et al*., 1989)
N315	Clinical methicillin-resistant isolate, θN315, *hlb*$^-$				(Kuroda *et al*., 2001)

[a] Presence of α- and δ-hemolysin toxins as previously determined (Seidl et al, submitted). Results are listed semiquantitatively in 4 categories: -, not present; +/-, borderline; +, present, ++, strong activity.

[b] Vancomycin response in an experimental IE model as previously determined (Seidl *et al*., submitted). Yes: ≥ 5 $\log_{10}$ cfu reduction per g of vegetations, and ≥ 3 $\log_{10}$ cfu/g reductions per g of kidneys and spleen due to vancomycin treatment. No: < 1.5 $\log_{10}$ cfu reduction per g of vegetations, kidneys and spleen due to vancomycin treatment.

Tc^r, tetracyclin resistance.

Table 3

Correlation of *S. aureus* - EC interactions and response to vancomycin treatment in an experimental IE model.

	Mean ± SD		
	Responder[a]	**Non-responder[a]**	***P***
Relative Invasion (%)[b]	81 ± 35	87 ± 35	0.62
Relative EC damage (%)[c]	45 ± 29	77 ± 36	< 0.0001

[a] Responder: MRSA strains that responded to vancomycin treatment in the experimental IE model; non-responder: MRSA strains that did not respond to vancomycin treatment in the IE model (see Table 1 for detailed descriptions).

[b] Corrected invasiveness as expressed as the percentage of invasiveness of strain 6850 (set up as 100%).

[c] *S. aureus* induced EC damage as determined by specific ^{51}Cr release at 24h after infection. Data are expressed as damage induced by strain 6850 (set up as 100%).