1 **High level methicillin resistance correlates with reduced**

2 ***Staphylococcus aureus* endothelial cell damage**

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4 **Running title: MRSA and MSSA EC damage**

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Abstract

There has been controversy about the intrinsic virulence of methicillin-resistant *Staphylococcus aureus* (MRSA) as compared to methicillin-susceptible *S. aureus* (MSSA). To address this discrepancy, the intrinsic virulence of 42 MRSA and 40 MSSA clinical isolates was assessed by testing endothelial cell (EC) damage, a surrogate marker for virulence in blood stream infections. Since these clinical isolates represent a heterogeneous group well characterized *S. aureus* laboratory strains with SCCmec loss- and gain-of-function mutations were used in addition. The clinical MRSA isolates carrying typical hospital acquired SCCmec types (I, II or III) induced significantly less damage (47.8%) as compared to isolates with other SCCmec types (62.3%, $p = 0.03$) and MSSA isolates (64.2%, $p < 0.01$). There was a strong inverse correlation between high-level oxacillin resistance and low EC damage induction ($R^2 = 0.4464$, $p < 0.001$). High-level oxacillin resistant strains (MIC $> 32\mu\text{g/ml}$) grew significantly slower as compared to isolates with low-level resistance ($p = 0.047$). The level of EC damage positively correlated with α - and δ -toxin production ($p < 0.0001$ and $p < 0.05$, respectively) but not with β -toxin production. Invasive MRSA isolates ($n=21$, 56.3%) were significantly less cytotoxic as compared to invasive MSSA isolates ($n=20$, 68.0%, $p < 0.05$). There was no difference between EC damage induced by superficial versus invasive isolates in either MRSA or MSSA strains. Our data suggest that the intrinsic virulence of MRSA is similar or even reduced as compared to MSSA strains but is linked to the level of methicillin resistance.

Key words: *Staphylococcus aureus*, methicillin resistance, virulence, endothelial cell damage

Introduction:

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a leading cause of nosocomial infections worldwide and has emerged as a community-associated pathogen (Chambers and DeLeo, 2009). Resistance to methicillin and oxacillin (the clinical derivative of methicillin) is characterized by the acquisition of the staphylococcal cassette chromosome *mec* (SCC*mec*) element (Katayama et al., 2000), which harbors the *mecA* gene. The *mecA* gene codes for the penicillin-binding protein (PBP) 2a characterized by a low affinity to β -lactams rendering MRSA strains resistant to all β -lactam antibiotics.

MRSA strains are currently grouped according to their core genetic background, for example in terms of sequence type, and the kind of SCC*mec* elements they carry. So far, eleven major SCC*mec* types have been described (http://www.sccmec.org/Pages/SCC_TypesEN.html) and several variants thereof. MRSA have been grouped into community-associated (CA-MRSA) and health-care-associated MRSA (HA-MRSA, reviewed by (DeLeo et al., 2010)). HA-MRSA usually carry SCC*mec* elements I-III, which are larger than the other SCC*mec* elements and provide resistance to several classes of antibiotics in addition to methicillin. CA-MRSA on the other hand, usually carry smaller SCC*mec* elements and are characterized by resistance to only a limited set of antibiotics. Another important subset of MRSA are sequence type (ST) 398 strains which represent a pandemic live-stock associated lineage (Reviewed by (Fluit, 2012)).

Antibiotic resistance in general and methicillin resistance in particular are often associated with a fitness burden. The limited antibiotic resistance of CA-MRSA has been suggested to only result in a negligible reduction in fitness as compared to methicillin-susceptible *S.*

aureus (MSSA) strains in terms of the growth rate. This might explain the wide dissemination of CA-MRSA in the community. This was underlined by the fact that no reduction in fitness was found when a small SCC*mec* IV element was introduced into a MSSA strain (Lee et al., 2007). In contrast, the introduction of a large SCC*mec* I, which is typical for HA-MRSA into the same MSSA strain was associated with a reduced growth rate (Ender et al., 2004). In addition to a fitness cost in terms of growth, high-level oxacillin resistance has been previously associated with reduced toxin production: Collins et al. 2010 (Collins et al., 2010) showed that the introduction of type II but not of type IV SCC*mec* element caused the bacteria to reduce their levels of costly toxin expression. This reduction in toxin expression has been suggested to be mediated by the interference of *mecA* with the *agr* quorum sensing system (Rudkin et al., 2012). *agr* is a well-characterized quorum-sensing system that regulates many adhesins and secreted virulence factors, such as α -toxin. In line with these findings, CA-MRSA has been shown to be more virulent in animal infection models (Li et al., 2009; Voyich et al., 2005) and more cytotoxic towards T-cells as compared to HA-MRSA (Rudkin et al., 2012). Together, these findings suggest that at least HA-MRSA are less virulent as compared to MSSA strains. However, several clinical studies suggest the opposite, namely that infections caused by MRSA are associated with worse clinical outcome, longer hospitalization, and higher net cost than similar infections caused by MSSA (Engemann et al., 2003; Engemann et al., 2005; Hardy et al., 2004; Khatib et al., 2006a; Melzer et al., 2003; Reed et al., 2005; Shelby D. Reed et al., 2005). This controversy might be due to the heterogeneity of clinical *S.aureus* isolates in general and of MRSA strains in particular.

To dissect the role of resistance per se on the intrinsic virulence of MRSA and MSSA strains, we tested their ability to induce endothelial cell (EC) damage using a

88 complementary set of clinical isolates ($n=82$) plus a set of well-defined isogenic loss- and
89 gain-of-function *S. aureus* laboratory strains.

Materials and Methods:

Identification of clinical *S. aureus* strains and Methicillin resistance detection.

Forty MSSA and 40 MRSA isolates were obtained from the Department of Medical Microbiology of the University of Zurich, Zurich, Switzerland, which serves the University Hospital Zurich, or from the laboratory of Hospital Epidemiology, University Hospital Zurich, Switzerland. All strains were unique isolates from different patients. Identification of *S. aureus* was done by standard methods: colony morphology, Gram staining, catalase test, Staphaurex (Murex Diagnostics) and/or coagulase plasma (BD) or API Staph (bioMerieux), where required. Methicillin resistance was determined by testing cefoxitin resistance using the Kirby-Bauer disc diffusion method (EUCAST, 2013 Version 3.1) and confirmed by *mecA* PCR and/or with the MRSA screen from Denka Seiken (Denka Seiken Co., Ltd., Tokyo, Japan) where required. In order to include SCC*mec* I two ST228-SCC*mec* I MRSA isolates were added (kind gift of Dr. Patrice Francois, University Hospital Geneva, Switzerland).

Laboratory *S. aureus* strains. Laboratory strains are listed in Table 1. SCC*mec* positive and negative variants of strains BB255 (Ender et al., 2004; Lee et al., 2007) and strain COLn (Ender et al., 2008) have been previously described. A SCC*mec* negative variant of MRSA strain N315 (N315EX) was a kind gift of Dr. Philippe Moreillon, University of Lausanne, Switzerland (Stojanov et al., 2012). *agr*, *hla*, and complemented *hla* mutants in the NCTC8325-4 background have been previously described (Fairweather et al., 1983; Novick et al., 1993; O'Reilly et al., 1986). These mutations were transduced into MRSA strain JE2. The *agr* locus of strain 300-169 (*agr* group IV) (Seidl et al., 2011b) was cloned

into strain RN4220, which is also a NCTC8325-4 derivative that is phenotypically *agr* negative.

To express the *hly* gene in strain JE2, *hly* of strain RN6390 was cloned into pCN34 (Charpentier et al., 2004). A 1.75kb segment containing the *hly* open reading frame and native promoter was amplified from *S. aureus* RN6390 DNA using primers 5'-TATAAGGTACCCGTATTTGCAACTTAATTATAGCC-3' and 5'-ATTATGAATTCAATAGATTGGAATCGCCATGG-3'. The PCR product was cloned into pCN34 generating pCN34*hly*⁺ using KpnI and EcoRI restriction sites. The resulting plasmid was electroporated into strain RN4220 and subsequently transduced into strain JE2 using phage 80α. JE2 was cured from its β-toxin converting phage using mitomycin C as previously described (Huseby et al., 2010).

Molecular Typing. PFGE typing was performed with SmaI digests of genomic DNA according to standard protocols and analyzed using the BioNumerics software package (Applied Maths, Belgium). The dice coefficient was used with 1.25% optimization and 1% tolerance to calculate similarities between PFGE patterns. Isolates with >80% identity were considered identical. Sequence typing of the polymorphic repeat region of the protein A gene (*spa*) (Shopsin et al., 1999) was performed for MRSA isolates using the Ridom StaphType (Ridom GmbH, Würzburg, Germany). Clonal complexes were predicted based on our previous findings (Seidl et al., 2015) and based on the Ridom SpaServer (<http://spa.ridom.de/spatypes.shtml>).

Excision of SCCmec. SCCmec excision in several clinical isolates was performed as previously described (Ito et al., 2001). The absence of SCCmec was confirmed by PCR (Diep et al., 2008) and PFGE of SmaI digests according to standard protocols.

E-tests. To determine the minimal inhibitory concentration (MIC) Oxacillin E-tests (Biomérieux, France) were performed according to the manufacturer's instructions.

Tissue culture and MTT assays. *S. aureus* damage in primary human umbilical cord vein ECs (HUVECs) was measured using an MTT assay after adding the bacteria at a multiplicity of infection (MOI) of 20 unless otherwise indicated (Quiblier et al., 2011; Seidl and Zinkernagel, 2013). After 3h invasion, extracellular bacteria were killed by adding lysostaphin (10 μ g/ml). After 19h, 100 μ l of 5mg/ml MTT in HBSS were added and 2h later the medium was replaced with 150 μ l of 0.04M HCl in absolute isopropanol to stop the reaction and lyse the cells. Uninfected HUVEC coated wells served as 0%-damage control and wells containing medium alone were used for background correction. Strain JE2 was included in each run as positive internal control. Absorbance of 100 μ l of the solution was measured at 570nm (Hansen et al., 1989). Damage was calculated using the following formula: $1 - (A_{570} \text{ of test well} / A_{570} \text{ of 0\% damage control well})$. Growth during invasion was assessed by plating serial fold dilutions of the inoculum and after 3 hours. The growth index was then calculated using the following formula: total number of bacteria at 3 h/number of bacteria initially added.

Hemolysis. Screening for toxin production was carried out on tryptic soy agar plates containing 5% human blood (for α -toxin production assessment) or on 5% sheep blood for β - and δ -toxin production assessment. δ -hemolytic activities were determined by cross streaking test strains perpendicularly to RN4220, a strain that is a hyperproducer of β -toxin but does not produce α -hemolysin (Traber and Novick, 2006). β - and δ -toxins of *S. aureus* act synergistically in the lysis of red blood cells and δ -toxin produced by a test strain results in a zone of enhanced hemolysis where this lysis overlaps with the β -toxin zone of

158 RN4220. δ -toxin production was categorized into negative (no activity) and positive (low,
159 strong or very strong activity).
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Results and Discussion:

Characterization of clinical MRSA and MSSA isolates.

To delineate the role of oxacillin resistance on EC cytotoxicity in the clinical setting, we determined the cytotoxicity of 82 *S. aureus* clinical isolates (40 MSSA and 42 MRSA). Isolates were selected based on the clinical presentation. Because *in vitro* EC damage induction has been previously shown to positively correlate with virulence in an infective endocarditis model (Seidl et al., 2011a) and seems to be relevant within the context of intravascular infection, we included bacteremia isolates ($n=42$) and isolates from asymptomatic carriers ($n=40$, Tables 2 and 3).

To exclude any bias due to clonality of the strains, we used strains with distinct genetic backgrounds. The 40 MSSA isolates belonged to 39 distinct PFGE patterns and were of different *agr* groups (Table 2, Supplementary Figure 1). One Isolate (CI111) could not be PFGE-typed. Because of the limited diversity of MRSA isolates at our hospital (Seidl et al., 2015), we were not able to obtain 42 MRSA isolates with distinct PFGE restriction patterns that also fulfilled the clinical criteria. The 42 MRSA isolates had 28 different PFGE restriction patterns and included SCCmec types I ($n=2$), II ($n=10$), III ($n=1$) and IV ($n=21$) and N1 ($n=5$) (Table 3, Supplementary Figure 2). Three isolates could not be SCCmec typed using our methods. *Spa*-typing revealed 21 different *spa*-types and one new *spa*-type. Forty-one isolates could be assigned to 5 clonal complexes (CC) and three sequence types based on their *spa*-type, PFGE-ID and SCCmec type (Table 3). For isolate CI143 no CC could be assigned. Isolates were of different *agr* groups and oxacillin MICs of MRSA strains ranged from 0.75 to >256 µg/ml (Table 3).

The average cytotoxicity of all clinical isolates (including MSSA and MRSA strains) was 60.9% and ranged from 1.0% to 85.3% (Tables 2 and 3) as compared to the EC damage of several laboratory strains that ranged from 2.0% to 76.5% (Table 1). Of the four clinical isolates that induced less than 20% EC damage, two were the highly resistant and relatively slow growing ST228 MRSA isolates (CI556 and CI559) and two were MSSA isolates (CI77 and CI116). Three of these isolates were non-hemolytic which might explain their low cytotoxicity as previously shown for laboratory MSSA strain Cowan (Haslinger-Löffler et al., 2005). The low cytotoxicity of CI77, which exhibits strong hemolytic activity might be due to other reasons such as poor invasion as previously described for MSSA strain Newman (Quiblier et al., 2013). Of the nine strains that induced more than 80 % EC damage, four were MRSA and five were MSSA strains.

Correlation of methicillin resistance with *S. aureus*-induced EC damage.

There was a trend that MSSA isolates induced more EC damage than MRSA isolates without reaching statistical significance (Table 4). When assessing the MRSA isolates carrying typical hospital SCCmec types (I, II or III) significantly less damage was observed as compared to MRSA isolates carrying other SCCmec types or MSSA strains ($p = 0.0034$, Table 4). More isolates with SCCmec type I, II, and III elements exhibited oxacillin resistances of $\geq 256 \mu\text{g/ml}$ as compared to isolates with other SCCmec elements (Table 3, 42.9% vs. 7.1%, $p = 0.0105$). Isolates with high level oxacillin resistance ($>32 \mu\text{g/ml}$) induced significantly less damage than isolates with relatively low level oxacillin resistance ($\text{MIC} \leq 32 \mu\text{g/ml}$, $p < 0.0001$, Table 4). The level of oxacillin resistance negatively correlated with the ability to induce EC damage in the clinical isolates (Fig. 1A, $R^2 = 0.4464$, $p < 0.001$) as well as in the six laboratory MRSA strains tested ($R^2 = 0.7235$, $p < 0.05$). We are aware that the number of tested strains was relatively low. However, our

findings showing that high level methicillin resistance correlated with low endothelial cell toxicity are in accordance with previous findings showing that high level methicillin resistance correlated with low toxicity towards T-cells (Rudkin et al., 2012).

Correlation of clinical outcome and genetic background with EC damage.

Together, invasive isolates (MRSA and MSSA) induced similar EC damage as compared to superficial isolates. Invasive MSSA strains were significantly more cytotoxic vs. invasive MRSA strains (68.0% vs. 56.3% cytotoxicity, $p = 0.0147$, Table 4). Invasive MRSA isolates included SCCmec I ($n=1$), II ($n=7$), III ($n=1$), IV ($n=5$), N1 ($n=4$), and three non-SCCmec-typable isolates. One MRSA isolate was a USA300 strain (CI160) (Seidl et al., 2014) and induced similar EC damage as compared to USA300 laboratory strain JE2 (Table 1). Together, CC8 isolates ($n = 7$) induced significantly more damage as compared to other MRSA isolates ($n=35$, $p < 0.05$, Supplementary Figure 3A). There was no correlation between *agr*-groups and the level of EC cytotoxicity (Supplementary Figure 3B).

Impact of fitness on *S. aureus* induced EC damage.

High-level oxacillin resistance has been associated with reduced fitness in terms of growth (Collins et al., 2010; Ender et al., 2004; Lee et al., 2007). In line with these findings, MRSA isolates with low level oxacillin resistance had a higher growth index as compared to high level resistant strains (Table 4). To further test the impact of fitness on EC damage we compared EC damage induced by laboratory MSSA strain BB255 and its methicillin-resistant variant carrying the SCCmec I element of MRSA strain COLn. This strain has been previously shown to exhibit reduced fitness in terms of growth (Ender et al., 2004). MSSA strain BB255 carrying SCCmec I, which is highly resistant to oxacillin (MIC $> 256 \mu\text{g/ml}$), exhibited a ~90% lower cytotoxicity vs. the parental MSSA strain. EC

cytotoxicity was restored when BB255 SCC*mec* I + was cured from its SCC*mec* element (Fig. 1B). In contrast, strain BB255 carrying the SCC*mec* IV element of strain WSPP, which displayed intermediate resistance levels (oxacillin MIC 24 µg/ml) and no fitness reduction (Lee et al., 2007) exhibited an intermediate (~35%) reduction in cytotoxicity as compared to the parental strain (Fig. 1B). The findings using our well defined laboratory strains showing that fitness plays a role in *S. aureus* induced EC damage corroborated our previous similar observations in the heterogenous set of clinical isolates.

Role of *agr*-activity in *S. aureus* induced EC damage.

High-level oxacillin resistance has been previously associated with lower *agr*-activity and reduced toxin production (Collins et al., 2010; Rudkin et al., 2012). *agr* is a well-characterized quorum-sensing system that regulates many adhesins and secreted virulence factors. The effector molecule of *agr*, RNAIII, has been shown to be inhibited in the presence of the *mecA* gene (Rudkin et al., 2012). To assess the role of *agr* in *S. aureus* induced EC damage in our clinical isolates, we determined the δ-toxin production, which is a surrogate marker for *agr* activity (Sakoulas et al., 2002). δ-toxin production was categorized into negative (no activity) and positive (low, strong or very strong activity). Thirteen isolates produced no δ-toxin, eight of which were MRSA and five MSSA isolates. Clinical isolates with δ-toxin production induced significantly more damage than isolates without δ-toxin production (Table 4). To further confirm the role of *agr* in EC cytotoxicity, we tested the impact of *agr* in laboratory strains MRSA JE2 and MSSA RN6390, a NCTC8325-4 derivative. Inactivation of *agr* lead to significant EC damage reductions in both strains (Fig. 2A). Introducing the *agr* locus into laboratory strain RN4220, which is a derivative of NCTC8325-4 and phenotypically *agr* negative lead to a significant increase in cytotoxicity ($p < 0.05$, Fig. 2B).

Our findings confirm that *agr* is needed for maximum damage induction, which is in accordance with previous studies (Seidl et al., 2011a; Seidl et al., 2012). However, several clinical isolates without δ -toxin production were still able to induce substantial EC damage (e.g. CI70 and CI63) while several isolates that were highly hemolytic and produced δ -toxin (e.g. CI77) induced only very little EC damage (Table 2). This underlines that EC damage induction is multifactorial and several other factors, such as invasiveness, fitness, or the presence of other toxins can influence EC damage induction (Haslinger-Löffler et al., 2005).

Role of α -toxin production in *S. aureus* induced EC damage.

As a second marker for *agr* activity, we tested hemolytic activity. Hemolysis by *S. aureus* is attributable to α -toxin, one of the most prominent *S. aureus* toxins that is positively regulated by *agr* (Novick et al., 1993). We noted that only ten δ -toxin negative strains were also α -toxin negative (Tables 2 and 3). Three strains were δ -toxin negative but α -toxin positive (CI70, CI108, and CI138). This is possible because not δ -toxin itself is the regulator of *agr* but RNAIII, which encodes for *hld*, the δ -toxin gene (Novick et al., 1993). A strain can therefore be δ -toxin negative but still have an active *agr* system if RNAIII is present, as previously shown (Novick et al. 1993). Three of the tested strains were δ -toxin positive but α -toxin negative (CI141, CI174, and CI175). These strains might have an active *agr* system but an intact or lacking α -toxin (Sharma-Kuinkel et al., 2015) (O'Reilly et al., 1990). Of the ten strains that were both α -toxin and δ -toxin-negative, three induced more than 50% EC damage (CI63, CI113, and CI161). These strains might have other, not yet identified toxins/mechanisms that are responsible for damage induction.

Isolates with α -toxin production induced significantly more EC damage as compared to isolates without α -toxin production (Table 4). MRSA isolates carrying typical

hospital SCCmec types (I, II or III) were less hemolytic as compared to MRSA isolates carrying other SCCmec types and to MSSA strains (Table 4, $p < 0.01$). To further evaluate the role of α -toxin in *S. aureus* induced EC damage, we confirmed the impact of α -toxin-gene (*hla*)-inactivation in the two laboratory strains (Figure 2C). Inactivation of the *hla*-gene in these two strains lead to significant reductions in EC cytotoxicity that could be restored by introducing the *hla*-gene on a plasmid confirming that α -toxin contributes to *S. aureus* EC cytotoxicity. However, the fact that several clinical isolates without α -toxin production could still induce cytotoxicity again suggests that EC damage induction by *S. aureus* is multifactorial and that other factors can compensate for an inactive α -toxin.

Role of β -toxin production in EC damage induced by *S. aureus*.

Another toxin that has been associated with virulence and cytotoxicity is β -toxin. This toxin has been previously shown to act synergistically with δ -toxin to mediate phagolysosomal escape (Giese et al., 2011). However, the role of phagolysosomal escape in cytotoxicity has not been clearly defined. On the one hand, phagolysosomal escape has been suggested to be a prerequisite for host cell death in HUVECs (Menzies and Kourteva, 1998). On the other hand, phagolysosomal escape was not associated with increased host cell death in HeLA cells (Giese et al., 2011). Similarly, the presence of β -toxin did not lead to higher cytotoxicity in bovine aortic endothelial cells (Vann and Proctor, 1988) and β -toxin negative strains have been previously shown to induce damage to HUVECs (Seidl et al., 2011a).

Our strain collection only contained seven β -toxin positive isolates that induced similar cytotoxicity as compared to β -toxin negative strains (Table 4). To further delineate the role of β -toxin in EC damage, we cured strain JE2 from its β -toxin converting phage. The generated strain was strongly β -toxin positive but exhibited similar cytotoxicity as

compared to the parental strain. The β -toxin converting phage of USA300 also encodes for other factors that might influence cytotoxicity, such as chemotaxis inhibitory protein or staphylokinase and might mimic any effect of β -toxin itself (Diep et al., 2006). We therefore introduced the β -toxin gene *hly* of strain RN6390 into strain JE2 on a plasmid, which resulted in strong β -toxin activity. However, introducing the *hly*-plasmid did not lead to an increase in damage vs. the strain carrying the empty plasmid (not shown). This is in contrast to a previous report in which β -toxin contributed to T-cell toxicity, indicating that different mechanisms are involved in T-cell and EC cytotoxicity (Collins et al., 2008).

Role of SCCmec in *S. aureus* induced EC damage.

To test whether the SCCmec element itself has an impact on EC damage induction, we excised the SCCmec elements of different MRSA strains. MRSA strains carrying different SCCmec types and exhibiting different resistance levels were included. No significant effect on EC damage was observed despite complete abolishment of oxacillin resistance by SCCmec excision suggesting that SCCmec itself has no impact on EC damage (Fig. 3). Only excision of the SCCmec I element of MRSA strain COLn increased cytotoxicity, without reaching statistical significance ($p = 0.23$). Importantly, SCCmec excision had no significant impact on the growth index or hemolysis in either of the isolates tested (not shown).

Even though the presence of SCCmec carrying *mecA* is a prerequisite for methicillin resistance, the level of resistance is influenced by many other factors such as genes affecting autolytic activity (*atl*, genes encoding for teichoic acids), transposable elements, genes involved in cell wall biosynthesis, or regulatory genes (Berger-Bächi and Tschierske, 1998; De Lencastre et al., 1999; Rohrer et al., 2003). These factors are present in MRSA and MSSA strains and might in turn affect the ability to induce EC damage by altering

fitness, adhesion and invasion, and/or toxin production. This most likely explains why the level of resistance inversely correlated with the level of EC damage while SCC_{mec} excision had no impact on EC damage. The fact that the introduction of SCC_{mec} into strain BB255 had an impact on damage induction (Fig. 1B) further suggests that EC damage induction by *S. aureus* depends on the genetic background.

Conclusion:

We found a strong inverse correlation between high-level oxacillin resistance and low EC damage induction in clinical isolates. Reduced EC damage also correlated with lower toxin production and reduced fitness in terms of growth, both factors that have been associated with high level oxacillin resistance. However, the fact that several isolates without *agr* activity and α -toxin production were still able to induce substantial EC damage underlines that *S. aureus* induced EC damage is multifactorial and dependent on the genetic background. Using well-defined gain- and loss-of-function *S. aureus* strains in addition to the clinical isolates we confirmed that MRSA virulence is dependent on fitness and toxin production. This suggests that the estimation of *S. aureus* induced EC damage represents a relevant method to estimate *S. aureus* virulence at least within the context of endovascular infections. Our data further underline that the intrinsic virulence of MRSA is similar or even reduced as compared to MSSA strains and the extent of virulence is linked to the level of methicillin resistance. Worse clinical outcomes associated with MRSA are most likely due to other confounding factors including ineffective initial antimicrobial treatment and comorbidities as previously suggested (Khatib et al., 2006b; Yaw et al., 2014) and are not due to a higher intrinsic virulence.

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550

Figure legends

FIG. 1. (A) Intercorrelation between the level of oxacillin resistance and EC damage in clinical isolates. Clinical isolates were tested in triplicate in two independent assays using ECs isolated from different donors. Because oxacillin MICs are highly skewed, a log transformation was carried out for MIC values. Oxacillin MICs $> 256\mu\text{g/ml}$ were assumed to be $512\mu\text{g/ml}$, Oxacillin MICs of $0.75\mu\text{g/ml}$ were set to $1\mu\text{g/ml}$ for illustrating purposes. (B) Impact of fitness on EC damage. EC damage of the wt strain was set to 100%. *, $p < 0.05$; **, $p < 0.01$. Each strain was tested in triplicate in three independent experiments. Results are shown as means and error bars indicated the standard error of the mean. p -values were determined using the paired rank sum test.

Fig. 2. (A) Impact of *agr* inactivation on EC cytotoxicity. (B) Impact of *agr* introduction into the phenotypically *agr* negative NCTC8325-4 derivative RN4220. (C) Impact of *hla* inactivation and complementation on EC cytotoxicity. Cytotoxicity of the wt strains was set to 100%. Each strain was tested in triplicate in three independent experiments. Results are shown as means and error bars indicated the standard error of the mean. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. p -values were determined using the paired rank sum test.

Fig. 3. Effect of *SCCmec* excision on EC damage. EC damage of the according parental strains was set to 100%. Each strain was tested in triplicate in three independent experiments. Results are shown as means and error bars indicated the standard error of the mean. For strains COLn and CI140 and their corresponding *SCCmec*- mutants, an MOI of 200 was used.

574 **Tables**

575 **Table 1.** Endothelial cell damage induced by different *S.aureus* laboratory strains.

Strain	Properties and reference	SCCmec type	Oxacillin MIC (µg/ml)	Endothelial cell damage (%)
MRSA				
JE2	CA-MRSA USA300 isolate (Fey et al., 2013)	Iva	2	76.5±13.4
MW2	CA-MRSA USA400 (CDC, 1999)	Iva	32	36.5±7.6
WSPP	West Samoan clone, ST92/398, (Adhikari et al., 2002)	IV	12	57.7±0.6
N315	HA-MRSA (Kuroda et al., 2001)	II	32	33.9±7.8
COLn	MRSA strain COL derivative (Katayama et al., 2004)	I	>256	2.0±2.0
BB1310	First MRSA in England 1961, NCTC10442 (Ito et al., 2001)	I	2	42.8±11.9
MSSA				
RN4220	NCTC8325-4, phenotypically <i>agr</i> - (Peng et al., 1988)	-	-	23.2±10.6
RN6390	NCTC8325-4 (Peng et al., 1988)	-	-	47.1±7.9
BB255	NCTC8325 (Ender et al., 2004)	-	-	46.1±13.1

576

577 **Table 2.** Characteristics of clinical MSSA isolates used in this study.

ID	Clinical presentation	Year	PFGE-ID	<i>agr</i> group ^a	EC Damage	Hemolysis ^b	β - toxin	δ - toxin	Growth index
Invasive MSSA isolates (blood culture)									
CI52	STI	2011	singleton	II	47.44%	0.68	-	+	12.7
CI53	Endocarditis	2011	singleton	I	73.13%	1.71	-	+	11.2
CI54	Bone and joint infection	2011	singleton	II	60.10%	1.34	-	+	6.4
CI55	Parotitis	2011	singleton	I	63.48%	1.37	-	+	7.0
CI56	STI	2011	singleton	I	72.89%	1.63	-	+	6.9
CI59	Endocarditis	2011	singleton	II	58.40%	0.67	-	+	5.0
CI60	Endocarditis	2011	singleton	I	80.47%	1.45	+	+	5.8
CI61	Bone and joint infection	2011	singleton	I	79.63%	1.57	-	+	7.8
CI63	Endocarditis	2011	singleton	II	72.07%	0.00	-	-	11.3
CI68	Catheter-related BSI	2012	singleton	I	71.39%	1.88	-	+	3.3
CI70	Catheter-related BSI	2012	singleton	III	85.33%	1.21	-	-	6.2
CI85	Catheter-related BSI	1997	singleton	III	60.17%	1.23	-	+	8.1
CI86	STI	2002	singleton	II	64.78%	1.52	-	+	5.3
CI95	Pneumonia	2006	singleton	II	77.19%	1.84	-	+	11.9

CI179	Catheter-related BSI	2001	singleton	I	61.68%	1.06	-	+	9.5
CI180	Sepsis with pneumonia	2002	singleton	III	61.98%	1.27	-	+	9.2
CI181	Catheter-related BSI	2003	singleton	I	56.87%	1.74	-	+	8.2
CI182	Unknown	2004	singleton	I	56.56%	1.69	-	+	5.6
CI183	Endocarditis	1997	singleton	I	74.75%	1.68	-	+	14.5
CI184	Bone and joint infection	1997	singleton	IV	81.30%	2.07	-	+	9.0
Superficial MSSA isolates (nose swabs)									
CI75	none	2012	singleton	II	71.81%	1.20	-	+	4.8
CI77	none	2012	singleton	IV	13.43%	1.34	-	+	7.4
CI80	none	2012	singleton	I	72.16%	1.45	-	+	8.6
CI104	none	2012	singleton	I	52.09%	1.76	-	+	9.7
CI105	none	2012	singleton	III	67.39%	0.85	-	+	12.5
CI106	none	2012	singleton	I	57.15%	2.09	-	+	10.4
CI107	none	2012	singleton	I	66.57%	1.81	-	+	6.8
CI108	none	2012	singleton	I	74.20%	1.78	+	-	5.3
CI109	none	2012	singleton	I	79.44%	1.50	-	+	8.6
CI110	none	2012	singleton	I	68.37%	1.27	-	+	11.6
CI111	none	2012	nt	I	75.21%	1.86	-	+	5.1
CI112	none	2012	singleton	IV	50.88%	1.37	-	+	9.5

CI113	none	2012	singleton	III	66.18%	0.00	-	-	6.7
CI114	none	2012	singleton	I	82.88%	1.83	-	+	5.2
CI115	none	2012	singleton	II	22.40%	1.73	+	+	6.3
CI116	none	2012	singleton	I	1.07%	0.00	-	-	6.7
CI119	none	2012	singleton	III	66.42%	1.66	-	+	6.4
CI120	none	2012	singleton	I	79.75%	1.61	-	+	11.0
CI121	none	2012	singleton	II	81.22%	1.29	-	+	6.3
CI122	none	2012	singleton	II	60.48%	1.57	-	+	11.5

578 BSI, blood stream infection; STI, soft tissue infection; nt, non-typable

579 ^a, *agr* grouping was performed as described (Lina et al., 2003).

580 ^b, radius in mm.

581

582 **Table 3.** Characteristics of clinical MRSA isolates used in this study.

ID	Clinical presentation	Year	PFGE- ID	Spa- type	Pred. CC	agr ^a	SCCmec type ^b	Oxacillin MIC	EC Damage	Hemolysis ^c	β- toxin	δ- toxin	Growth index
Invasive MRSA isolates (blood culture)													
CI175	Bone and joint infection	2002	19	t004	CC45	I	N1 ^d	0.75	50.53%	0.00	-	+	9.3
CI176	Endocarditis	2002	19	t004	CC45	I	N1 ^d	1	81.83%	1.50	-	+	7.1
CI169	Bone and joint infection	2005	19	t004	CC45	I	N1 ^d	1.5	67.66%	1.42	-	+	12.2
CI151	Endocarditis	2011	19	t282	CC45	I	N1 ^d	1.5	56.71%	1.16	-	+	5.1
CI174	Endocarditis	2004	20	t1771	CC22	I	IV	24	36.11%	0.44	-	+	8.0
CI166	Catheter-related BSI	2008	20	t032	CC22	I	IV	48	53.97%	1.31	-	+	2.2
CI171	Catheter-related BSI	2003		t002	CC5	II	II	>256	36.99%	0.00	-	-	9.7
CI173	Vascular Graftinfection	2004		t062	CC5	II	II	256	48.88%	0.74	-	+	9.4
CI170	Pneumonia and STI	2006	43	t002	CC5	I	nt	0.75	46.18%	0.00	-	-	12.0
CI167	Urosepsis	2008		t002	CC5	II	II	24	60.43%	0.51	+	+	9.6
CI154	Catheter-related BSI	2010		t954	CC5	II	nt	1.5	69.57%	1.55	-	+	7.0
CI152	STI	2011	21	t002	CC5	II	II	4	65.44%	1.47	-	+	9.3

CI150	Shunt-associated infection	2011	21	t586	CC5	II	II	16	46.18%	0.75	-	+	6.2
CI153	Bone and joint infection	2011	74	t002	CC5	II	nt	1.5	64.67%	1.20	-	+	7.3
CI177	STI	2002		t018	CC30	II	II	12	76.82%	0.84	-	+	4.8
CI178	Bone and joint infection	2002	08	t018	CC30	III	IV	2	74.89%	1.30	-	+	8.9
CI164	Pneumonia	2011	08a	t1752	CC30	III	IV	6	63.64%	0.80	-	+	9.6
CI168	Endocarditis	2007		t014	ST225	II	II	8	59.04%	0.62	-	+	15.7
CI165	Thrombophlebitis	2009		t394	CC8	I	IV	2	76.23%	1.74	-	+	12.0
CI559	Unknown	unknow n		t041	ST228	II	I	>256	3.02%	0.00	-	-	6.6
CI172	Soft-tissue infection	2004		t037	ST239	I	III	>256	42.38%	0.27	-	-	7.6
Superficial MRSA isolates (nose swabs)													
CI185	none	2005	19	t282	CC45	I	N1 ^d	1.5	73.30%	1.39	-	+	13.9
CI161	none	2008	47	t015	CC45	I	IV	1.5	71.48%	0.00	-	-	18.6
CI186	none	2006	20	t223	CC22	I	IV	1.5	80.25%	1.72	-	+	5.3
CI162	none	2009		t032	CC22	I	IV	24	77.36%	1.52	+	+	9.3
CI159	none	2010	20	t032	CC22	I	IV	>256	30.65%	1.64	-	+	2.3
CI144	none	2011	20	t3212	CC22	I	IV	64	45.07%	1.84	-	+	3.2
CI142	none	2011	20	t032	CC22	I	IV	32	51.64%	1.69	-	+	7.9

CI140	none	2012	20	t032	CC22	I	IV	>256	27.44%	1.56	-	+	6.3
CI163	none	2009	63	t005	CC5	II	IV	12	82.91%	1.80	-	+	11.5
CI146	none	2011	21	t067	CC5	II	II	256	63.08%	1.28	+	+	10.8
CI141	none	2011		t002	CC5	II	II	1.5	62.51%	0.43	-	+	6.6
CI137	none	2012	21	t586	CC5	II	II	24	48.59%	0.67	-	+	7.3
CI138	none	2012	83	t018	CC30	III	IV	3	51.22%	0.57	-	-	5.7
CI149	none	2010	69	t008	CC8	I	IV	3	65.81%	1.57	-	+	13.1
CI135	none	2012		t008	CC8	I	IV	6	60.40%	1.25	-	+	6.6
CI148	none	2010		t064	CC8	I	IV	1.5	73.18%	1.37	-	+	6.4
CI160	none	2010	41	t008	CC8	I	IV	4	80.63%	0.64	-	+	11.9
CI147	none	2011	49	t008	CC8	I	IV	2	77.54%	0.68	+	+	13.9
CI139	none	2012		t008	CC8	I	IV	1	69.66%	1.37	-	+	5.1
CI143	none	2011		New*	-	I	IV	6	45.50%	0.00	-	-	9.3
CI556	none	unkno		t041	ST228	II	I	>256	7.75%	0.00	-	-	5.3
wn													

583 BSI, blood stream infection; STI, soft tissue infection; nt, non-typable.

584 ^a, *agr* grouping was performed as described (Lina et al., 2003).

585 ^b, *SCCmec* typing was performed as previously described (Oliveira et al., 2006).

586 ^c, radius in mm.

587 ^d, The *SCCmec* type was predicted from the PFGE-ID (PFGE-19, Swiss Drug clone) (Ender et al., 2007; Seidl et al., 2015).

588 *, 07-23-12-21-12-17-20-12-12-17

589

591 **Table 4. Bacterial factors associated with *S. aureus* induced EC damage.**

Bacterial phenotype			Mean		Hemolysis		Growth	
		<i>n</i>	damage (%)	<i>P</i>	(radius in mm)	<i>P</i>	index ^b	<i>P</i>
Resistance	MSSA all	40	64.2 ¹	0.07	1.39²	0.0032	8.1	0.52
	MRSA all	42	57.8		0.97		8.6	
	Low level Oxacillin resistance (≤32μg/ml)	32	64.2	0.0001	1.00	0.6895	9.3	0.047
	High level Oxacillin resistance (>32μg/ml)	10	35.9		0.86		6.3	
	SCC <i>mec</i> I/II/III	13	47.8¹	0.03	0.58²	0.0059	8.4	0.86
	Other SCC <i>mec</i> types	29	62.3		1.14		8.6	
Toxins	α-toxin + ^a	69	64.6	0.0006	1.38	<0.0001	8.2	0.36
	α-toxin -	13	41.6		0.09		9.1	
	β-toxin +	7	65.0	0.58	1.28	0.58	8.7	0.77
	β-toxin -	75	60.5		1.16		8.3	
	δ-toxin +	69	63.6	0.0314	1.34	<0.0001	8.3	0.80
	δ-toxin -	13	46.6		0.30		8.6	
Clinical	Invasive isolates (MRSA and MSSA)	41	62.0	0.74	1.10	0.18	8.4	0.88
presentation	Superficial isoaltes (MRSA and MSSA)	41	59.9		1.24		8.3	
	Invasive MSSA	20	68.0 ³	0.66	1.40	0.92	8.2	0.81

Superficial MSSA	20	60.5		1.38		8.0	
Invasive MRSA	21	56.3 ³	0.44	0.83	0.18	8.6	0.99
Superficial MRSA	21	59.3		1.09		8.6	

592 All *p*-values were determined using the Mann-Whitney test.

593 ^a, hemolysis zones of <0.5mm were considered negative.

594 ^b, growth index = total number of bacteria at 3 h/number of bacteria initially added.

595 ¹, *p* = 0.0034 (EC damage of MRSA strains with *SCCmec* types I-III vs. MSSA isolates).

596 ², *p* < 0.0001 (Hemolysis of MRSA strains with *SCCmec* types I-III vs. MSSA isolates).

597 ³, *p* = 0.0314 (invasive MRSA vs. invasive MSSA isolates).

598

A

EC damage [%]

B

Rel. EC damage [%]