Interference with lipoprotein maturation sensitizes methicillin-resistant *Staphylococcus aureus* to human group IIA secreted phospholipase A_2 and daptomycin.

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Running title

Staphylococcus aureus sensitization to innate and antibiotic killing

Keywords

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been classified as a high priority pathogen by the World Health Organization underlining the high demand for new therapeutics to treat infections. Human group IIA secreted phospholipase A_2 (hGIIA) is among the most potent bactericidal proteins against Gram-positive bacteria, including *S. aureus*. To determine hGIIA-resistance mechanisms of MRSA we screened the Nebraska Transposon Mutant Library using a sublethal concentration of recombinant hGIIA. We identified and confirmed the role of *lspA,* encoding the lipoprotein signal peptidase LspA, as a new hGIIA resistance gene in both *in vitro* assays and an infection model in hGIIA-transgenic mice. Increased susceptibility of the *lspA* mutant was associated with faster and increased cell wall penetration of hGIIA. Moreover, *lspA* deletion also increased susceptibility to daptomycin, a last-resort antibiotic to treat MRSA infections. Exposure of MRSA wild-type to the LspA-specific inhibitors globomycin and myxovirescin A1 induced a *lspA* mutant phenotype with regard to hGIIA and daptomycin killing. Analysis of >26,000 *S. aureus* genomes showed that LspA is highly sequence-conserved, suggesting that LspA inhibition could be applied universally. The role of LspA in hGIIA resistance was not restricted to MRSA since *Streptococcus mutans* and *Enterococcus faecalis* were also more hGIIA-susceptible after *lspA* deletion or LspA inhibition, respectively. Overall, our data suggest that pharmacological blocking of LspA may disarm Gram-positive pathogens, including MRSA, to enhance clearance by innate host defense molecules and clinically-applied antibiotics.

52 ## Introduction

53 Infectious diseases are a significant cause of morbidity and mortality worldwide and are estimated to
54 increase tremendously in the coming decades due to the rise of antimicrobial resistance [1]. The rapid
55 development of antibiotic resistance does not just limit the success of treatment but also of prophylaxis
56 of infections. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a prominent example of a bacterium
57 that has developed rapid antibiotic resistance over the past decades [2, 3]. Indeed, MRSA is ranked as
58 one of the high priority pathogens by the World Health Organization with regard to the need for new
59 therapeutic strategies [4]. While this bacterium is a common member of the human microbiota and
60 asymptomatically colonizes the skin, gut, and nasal cavity, it can cause a wide spectrum of clinical
61 diseases both in the hospital and in the community once *S. aureus* breaches host barriers.

62 The discovery of new antibiotics is slower than the emergence of new resistance mechanisms
63 of pathogens [5-7]. Antibiotics are classified as substances that are able to kill bacteria (bactericidal) or
64 inhibit their growth (bacteriostatic) [5]. Consequently, antibiotics target molecules or processes in the
65 cell that are either essential or at least critical for the growth of bacteria. An alternative strategy to
66 target bacterial pathogens could include anti-virulence or sensitizing drugs. These drugs may not affect
67 bacterial viability or growth under laboratory conditions, but would affect bacterial fitness or even allow
68 killing of bacteria in the context of specific host immune components, thereby clearing the infection.
69 Indeed, *S. aureus* expresses a wide array of virulence molecules allowing for persistence in different
70 host compartments through interference with a range of immune defense mechanisms and molecules
71 [8].

72 The human group IIA secreted phospholipase A2 (hGIIA, also known as $sPLA_2$-IIA) is a bactericidal
73 enzyme that represents an important innate host defense molecule [9, 10]. hGIIA is highly cationic and
74 effectively kills Gram-positive bacteria through hydrolysis of bacterial membrane phospholipids [11].
75 The enzyme is constitutively present at low levels (<5 ng/mL) in the blood circulation and its
76 concentration increases rapidly to levels as high as 1000 ng/mL upon bacterial infection associated with
77 sepsis [12, 13]. hGIIA requires anionic structures in the bacterial cell wall for binding to and penetration
78 of the Gram-positive cell wall [14, 15]. Once at the membrane, hGIIA hydrolyzes membrane
79 phospholipids resulting in bacterial lysis. hGIIA has been implicated in host defense against *S. aureus.*
80 First, blocking hGIIA in acute phase serum results in loss of bactericidal effects against *S. aureus*, whereas
81 addition of hGIIA to normal serum conferred anti-staphylococcal activity [16]. A bactericidal role of hGIIA
82 has also been observed at barrier sites for example in human tears [17]. Second, hGIIA-transgenic (Tg)
83 mice show higher survival rates compared to control littermates that are naturally $sPLA_2$-IIA-deficient,
84 after an experimental lethal dose of *S. aureus* [18, 19]. As a result, *S. aureus* has evolved resistance
85 strategies against hGIIA-mediated killing, which are geared towards changing the overall charge of the

86 membrane or cell wall. For example, *S. aureus* increases its surface charge by adding D-alanine residues
87 to teichoic acids through the DltABCD machinery and L-lysine residues to membrane phospholipids
88 through the activities of the enzyme MprF [14, 20]. The two-component regulatory system GraRS
89 controls the expression of both *mrpF* and *dltABCD*, thereby controlling *S. aureus* resistance to cationic
90 antimicrobial peptides and proteins such as hGIIA [21, 22]. Interestingly, the same bacterial genes are
91 involved in *S. aureus* resistance to daptomycin, the antibiotic of last-resort to treat MRSA infections.
92 Indeed, increased expression or gain-of-function mutations in *mprF* and *dltABCD* confer daptomycin
93 non-susceptibility to *S. aureus* [23, 24]. Therefore, insight into hGIIA resistance mechanisms could
94 provide new clues for the resistance against clinically-important antibiotics.

95 *S. aureus* is predicted to express between 50 to 70 lipoproteins, many of unknown function [25,
96 26]. Some lipoproteins are involved in antibiotic resistance, for example the beta-lactamase BlaZ and
97 Dsp1 [27-29]. Before lipoproteins are considered mature, they need to be sequentially processed by the
98 prolipoprotein diacylglyceryl transferase (Lgt) and lipoprotein signal peptidase II (LspA) enzymes. Lgt
99 anchors prolipoproteins into the cell membrane through diacylglycerol and LspA subsequently
100 generates the mature lipoprotein by removal of the signal peptide [30]. Both enzymes are conserved in
101 all bacteria and marked as essential in Gram-negative but not Gram-positive bacteria [30]. Nonetheless,
102 incorrect processing of lipoproteins changes the immune interaction of *S. aureus*; the deletion of *lgt*
103 results in hypervirulence, whereas mutation of *lspA* attenuates virulence in a murine systemic infection
104 model [31]. In addition, two screens, one designed to identify virulence genes and the other to identify
105 MRSA resistance mechanisms to polymyxin B-mediated killing, identified *lspA* as a resistance
106 determinant [32, 33].

107 The mechanisms by which *S. aureus* or MRSA resist hGIIA-mediated killing have never been
108 studied in a comprehensive unbiased manner. Here, we screened the Nebraska Transposon Mutant
109 Library (NTML) to identify hGIIA-susceptible mutants [34]. In addition to previously implicated genes
110 [14, 35], we identified and confirmed that deletion of *lspA*, which we show to be extremely sequence-
111 conserved, sensitizes *S. aureus* to hGIIA-mediated killing both *in vitro* and *in vivo*. Moreover, LspA
112 confers resistance to the last-resort antibiotic daptomycin. Both hGIIA- and daptomycin susceptibility
113 could be induced by treatment of MRSA with the LspA inhibitors globomycin and myxovirescin A1. The
114 contribution of LspA to hGIIA resistance was not *S. aureus*-specific but was also observed in
115 *Streptococcus mutans* (*S. mutans*) and in *Enterococcus faecalis* (*E. faecalis*). In conclusion, we identify
116 LspA as a possible new therapeutic target to break resistance of *S. aureus* and possibly other Gram-
117 positive pathogens to both endogenous antimicrobials and antibiotics routinely used in clinic.

118 ## Materials and Methods

119

120 ### Materials

121 Recombinant hGIIA was produced as described previously [36]. HEPES and $CaCl_2$ were purchased from
122 Sigma Aldrich and Merck, respectively. Albumin Bovine Fraction V, pH 7.0 (BSA) was purchased from
123 Serva. SYTOX Nucleic acid stain was purchased from ThermoFisher and $DiOC_2(3)$ was obtained at
124 Promokine / Bio-Connect B.V.. All antibiotics (chloramphenicol, erythromycin, daptomycin, gentamicin,
125 and globomycin) were purchased from Sigma Aldrich.

126

127 ### Bacterial culture

128 The NTML [34] was grown in Tryptic Soy Broth (TSB, Oxoid) supplemented with 5 µg/mL erythromycin.
129 All other *S. aureus* strains and *Enterococcus* species (*E. faecalis* and *E. faecium*) used in this study (Table
130 1) were grown in Todd-Hewitt Broth (THB, Oxoid) with continuous shaking at 37°C. After overnight
131 culture, strains were sub-cultured to an optical density at 600 nm (OD_{600}) of 0.4 (early logarithmic phase;
132 ≈$1x10^8$ colony-forming units (CFU)/mL). The plasmid complemented strains were grown in THB
133 supplemented with 20 µg/mL chloramphenicol. *S. mutans* was grown statically in Brain Heart Infusion
134 (BHI) at 37 °C with 5% CO_2. The following day, sub-cultures were grown to OD_{600} of 0.2 (early logarithmic
135 phase). Plasmid complemented *S. mutans* strains were grown in the presence of 3 µg/mL
136 chloramphenicol. *Escherichia coli* (*E. coli*) strains were grown in Lysogeny broth (LB) medium
137 supplemented with appropriate antibiotics with continuous shaking.

138

Table 1. Overview of strains and plasmids used in this study.

Strains/plasmids	Description	Reference
E. coli		
DC10b	Host strain for cloning vectors *S. aureus*	[37]
MC1061	Host strain for cloning vectors *S. mutans*	
S. aureus		
NRS384	Wild-type, USA300-0114, CA-MRSA	NARSA strain collection
NRS384 Δ*lspA*	NRS384 background with a deletion of *lspA*	This study
NRS384 Δ*lspA*+p*lspA*	NRS384 Δ*lspA* background complemented with *lspA*	This study
S. mutans		
UA159	Wild-type, ATCC 700610, serotype c	[38]
UA159 Δ*lspA*	UA159 background with a deletion of *lspA*	This study
UA159 Δ*lspA*+p*lspA*	UA159 Δ*lspA* background complemented with *lspA*	This study
E. faecalis		
V583	Clinical isolate, ATCC 700802	[39]

E. faecium		
U0317	Clinical isolate	[40]
Plasmids		
pKOR1-MCS	Temperature-sensitive shuttle vector for allelic exchange in *S. aureus*	[41]
pDC123	Complementation vector for gene *lspA*	[42]

139

140 Screening the NTML for MRSA hGIIA resistance genes

141 All 1,920 mutants of the NTML were grown overnight in 96-well round bottom plates. After overnight
142 culture, all transposon-mutant cultures were diluted 20 times in TSB supplemented with 5 µg/mL
143 erythromycin and grown to early exponential phase. Cultures were subsequently diluted 20-fold in
144 HEPES solution (20 mM HEPES, 2 mM $CaCl_2$, pH=7.4) and exposed to 1.25 µg/mL recombinant hGIIA.
145 After incubation for 1 hour at 37 °C, 5 µL droplets were plated on TS agar plates. Mutants with visibly
146 reduced number of CFU were identified as putative hGIIA sensitive mutants.

147

148 Construction of *lspA* deletion and *lspA* complemented strains

149 The markerless *lspA* (SAUSA300_1089) deletion mutant (MRSA Δ*lspA*) was generated in *S. aureus* strain
150 USA300 NRS384. The temperature-sensitive and modified pKOR1 plasmid was used as described earlier
151 [41, 43]. A fusion PCR of the upstream region of 1,008 base pairs (bp) and downstream region of 986
152 bp flanking the *lspA* gene was generated using NRS384 genomic DNA as template. The fusion PCR
153 product was ligated into the pKOR1-MCS plasmid and amplified in *E. coli* DC10b before electroporation
154 into *S. aureus* NRS384. Allelic exchange was performed through temperature shifts and counter
155 selection [43].

156 To generate a *lspA* (SMU_853) deletion mutant in *S. mutans* strain UA159, the flanking regions
157 (upstream fragment of 635 bp, downstream fragment of 574 bp) were fused with an erythromycin
158 cassette into a single PCR product. For transformation, *S. mutans* was grown in BHI supplemented with
159 heat-inactivated horse serum and the PCR fusion construct was added at 0.5 µg/mL.

160 Complementation of both *S. aureus* (MRSA Δ*lspA*::p*lspA*) and *S. mutans* strains was performed
161 with pDC123 containing the full length *lspA* (SAUSA300_1089 for *S. aureus* or SMU_853 for *S. mutans*,
162 respectively). Successful transformation was checked with chloramphenicol resistance and colony PCR.
163 An overview of all strains, plasmids and primers used in this study are shown in Tables 1 and 2. All
164 transformants were plated on selective plates containing appropriate antibiotics and successful
165 transformation was checked with PCR and sequencing.

Table 2. Overview of primers used in this study.			
Primers	Orientation	RE site	Sequence
S. aureus NRS384			
lspA up	Forward	KpnI	GCG**GGTACC**GAATGGCTATTATCAACATTTGGC
lspA up	Reverse		GGAAGTATCCTTTAATAAGGCGCATTTTCGTTCCTCCAATCAATC
lspA down	Forward		GATTGATTGGAGGAACGAAAATGCGCCTTATTAAAGGATACTTCC
lspA down	Reverse	EcoRI	GCG**GAATTC**CGTAATTATAGCACGACACAATTATGCATC
Complementation *lspA*	Forward	EcoRI	GCG**GAATTC**CATGGACGATTGATTGGAG
Complementation *lspA*	Reverse	BglII	GCG**AGATCT**CATTACTTAACCTCCTTCTCC
S. mutans UA159			
lspA up	Forward		GCCAGTCAGCACTATGATTTCTTACCGCC
lspA up	Reverse		GTTTTGAGAATATTTTATATTTTTGTTCATAAGATCTCCTAAGGCTTATAAGTTTC
lspA down	Forward		*AGTTATCTATTATTTAACGGGAGGAAATAA*GTGTTGGTGTAGCACTTC
lspA down	Reverse		GGTCATTTGGCAAGTTGCCGTGTACAAGGG
Erythromycin cassette	Forward		ATGAACAAAAATATAAAATATTCTCAAAACTTTTTAACG
Erythromycin cassette	Reverse		*TTATTTCCTCCCGTTAAATAATAGATAACT*
Complementation *lspA*	Forward	XbaI	GC**TCTAGA**GCCTTAGGAGATCTTATGCG
Complementation *lspA*	Reverse	BamHI	CGC**GGATCC**GCCTTATCCAGACGCACTCCTGC
Underlined and italic bases indicate overlapping sequences to generate fusion construct. Bases in bold indicate either restriction enzyme (RE) sites.			

166

167 CFU killing assay

168 Survival after hGIIA, daptomycin, or gentamicin exposure was determined by quantifying CFU on TH
169 agar. Early log-phase bacteria (OD_{600} of 0.2 for S. *mutans* or 0.4 for *S. aureus* and *Enterococcus* spp.)
170 were washed and resuspended in HEPES solution supplemented with 1% BSA (HEPES 1% BSA) and cell
171 density was adjusted to the original OD_{600}. Bacterial suspensions (containing 10^3 CFU of *S. aureus*, $2x10^3$
172 CFU of *S. mutans* or 10^5 CFU of *Enterococcus* spp.) were mixed 1:1 with increasing concentrations of
173 recombinant hGIIA, daptomycin, or gentamicin in HEPES 1% BSA and incubated for 1 hour at 37°C.
174 Samples were then serially diluted in phosphate buffered saline (PBS, pH 7) and plated on TH agar plates.
175 After overnight incubation at 37°C, CFU were counted and bacterial survival was calculated compared
176 to untreated bacteria. To investigate the effect of the LspA inhibitor globomycin or myxovirescin A1 on
177 hGIIA- or daptomycin-mediated killing, the compounds were added to wild-type (WT) bacteria during
178 sub-culturing to early exponential phase at a concentration of 100 µg/mL for globomycin and 10 µg/mL
179 for myxovirescin A1, which were produced and purified as previously described [44] and dissolved in
180 DMSO. The maximum concentration of DMSO was 1%, which was also added to other bacterial cultures
181 as a control.

182 Scanning Electron Microscopy (SEM)

183 MRSA WT, MRSA Δ*lspA*, and MRSA Δ*lspA*::p*lspA* at stationary phase and early exponential phase (OD_{600}
184 0.4) were washed, fixed, and dehydrated as described previously [45]. Samples were mounted on 12.5
185 mm specimen stubs (Agar scientific, Stansted, Essex, UK) and coated with 1 nm gold using the Quorum
186 Q150R S sputter coater at 20 mA. Microscopy was performed with a Phenom PRO desktop SEM
187 (Phenom-World BV) operating at an acceleration voltage of 10 kV.

188

189 Growth curve

190 MRSA WT, MRSA Δ*lspA*, and MRSA Δ*lspA*::p*lspA* were grown overnight and sub-cultured the following
191 day to an OD_{600} of 0.4 in THB supplemented with antibiotics when appropriate. The early exponential
192 phase bacteria were diluted to OD_{600} 0.025 in THB. OD_{600} was measured every 5 minutes over 20 hours
193 (shaking) in a Biotek Synergy H1.

194

195 MRSA infection experiment in hGIIA-Tg mice

196 Tg mice overexpressing hGIIA were from Taconic (Denmark). They were generated by inserting the
197 6.2 kb full-length of human gene (*PLA_2G2A*) into the mouse genome and were bred to a $sPLA_2$-IIA
198 naturally-deficient C57BL/6 female mouse that lacks the functional mouse homologue (Pla2g2a) [19,
199 46]. The animals were housed at Institut Pasteur animal facility accredited by the French Ministry of
200 Agriculture for performing experiments on live rodents. The study on animals was performed in
201 compliance with the French and European regulations on care and protection of laboratory animals (EU
202 Directive 2010/63, French Law 2013-118, February 6th, 2013). The experimental protocol was approved
203 by the Institut Pasteur Ethics Committee and registered under the reference 2014–0014 with the
204 infection protocol 21.185 (AC 0419).

205 Mice, both males and females (Supplementary Table 1) of 7–9 weeks old, were bred at Institut
206 Pasteur animal facility and infected intra-peritoneally with MRSA WT or the isogenic Δ*lspA* mutant
207 ($1x10^7$ or $5x10^7$ CFU) suspended in 100 µL PBS. Mortality and weight loss of mice were monitored twice
208 daily up to 5 days after infection.

209

210 Surface charge

211 Bacterial surface charge was determined as previously described [47]. Briefly, early-exponential phase
212 bacteria (OD_{600} = 0.4) were washed twice in 20 mM MOPS buffer (pH 7.0, Sigma-Aldrich) and adjusted
213 to OD_{600} 0.7. Bacteria were concentrated 10 times, of which 200 µL aliquots were added to 0.5 mg/mL
214 cytochrome c (from Saccharomyces cerevisiae, Sigma-Aldrich) in a sterile 96 well round-bottom plate.
215 Suspensions were incubated for 10 minutes at room temperature and subsequently centrifuged at
216 3,500 rpm for 8 minutes. Supernatant was transferred to a sterile 96 well flat-bottom plate and

217 absorbance was recorded at 530 nm. The percentage of residual cytochrome c was calculated using
218 samples containing MOPS buffer only (100% binding) and samples containing MOPS buffer and
219 cytochrome c (0% binding).
220
221 Membrane potential and permeability assays
222 Changes in hGIIA-dependent membrane potential were determined using the membrane potential
223 probe $DiOC_2(3)$ (PromoKine) [15, 48]. Bacterial suspensions (OD_{600} of 0.4) were diluted 100 times
224 (~$1x10^6$ CFU/mL) and incubated with serial dilutions of hGIIA. After incubation at 37˚C, 3 mM $DiOC_2(3)$
225 was added and incubated at room temperature for 5 minutes in the dark. Changes in green and red
226 fluorescence emissions were analyzed by flow cytometry. Bacterial staining with the DNA stain SYTOX
227 Green (Invitrogen) is a measurement for membrane permeabilization and an indication of bacterial cell
228 death [49]. Serial dilutions of hGIIA in HEPES solutions were added to wells of a sterile flat-bottom 96
229 well plate. Bacteria were resuspended in HEPES solution containing 1 µM SYTOX green (OD_{600} of 0.4)
230 and added to hGIIA dilutions in a final volume of 100 µL. Fluorescence over time was recorded using
231 Optima Fluostar (green fluorescence 520 nm emission and excitation 485 nm) at 37˚C.
232
233 PubMLST database analysis of *S. aureus lspA*
234 The PubMLST database, assessed at https://pubmlst.org/organisms/staphylococcus-aureus [50] was
235 used to analyze the presence and sequence conservation of *lspA* (SAUR1197) across the *S. aureus*
236 population. Alignments were made using the locus explorer of the PubMLST database and
237 nucleotide and amino acid identity was calculated using the NCBI BLAST tool
238 (https://blast.ncbi.nlm.nih.gov/blast.cgi). *LspA* gene sequences of 26,036 *S. aureus* strains were
239 downloaded from the database in February 2021. We excluded whole genome sequences for data
240 analysis that were unlikely to be *S. aureus*, contained > 300 contigs or an N50 contig length shorter than
241 20,000 bp, contained an internal stop codon rendering a truncated LspA or when *lspA* was located at
242 the end of a contig.
243
244 Statistical analysis
245 Statistical analysis was performed using GraphPad Prism 9. We used the Student's *t* test and one- and
246 two-way ANOVA's with Bonferroni statistical hypothesis testing to correct for multiple comparisons. All
247 values are reported as mean with standard error of the mean of three biological replicates unless
248 indicated otherwise. A p value of < 0.05 was considered statistically significant.

249 ## Results

250

251 ### Identification of hGIIA resistance genes in MRSA.

252 To unravel new hGIIA resistance mechanisms of MRSA, we screened 1,920 individual MRSA mutants of
253 the Nebraska Transposon Mutant Library (NTML). Exponentially-grown transposon mutants were
254 exposed to recombinant hGIIA for one hour and subsequently spotted on agar plates for semi-
255 quantitative assessment of survival (Supplementary Figure 1A). In total, 39 mutants were identified with
256 potential increased susceptibility to hGIIA-mediated killing (Supplementary Table 2). These hits included
257 the transposon mutant NE1360 (*mrpF*), which displays an increased positive charge of membrane
258 phospholipids and was previously linked to hGIIA resistance [14]. Additionally, transposon insertion in
259 genes encoding the two-component system GraRS and its ABC-transporter VraFG also rendered MRSA
260 more susceptible to hGIIA. These genes are important for the regulation of the before mentioned *mprF*
261 and *dltABCD* operon [22], which has a known role in hGIIA resistance [14]. Transposon mutants in
262 individual genes of the *dltABCD* operon were not identified since these mutants are absent in the NTML
263 [34].

264 To confirm the phenotype of individual transposon mutants identified in our screen, we
265 assessed their susceptibility in a quantitative killing assay across a hGIIA concentration range. As
266 expected, disruption of previously-identified genes *graR*, *graS*, and *mprF* rendered MRSA more
267 susceptible to hGIIA-mediated killing (Supplementary Figure 1B). In contrast, mutants with transposons
268 inserted in the genes *esaC*, *srtB*, *ltaA*, and *asp1* were not differently affected by hGIIA (Supplementary
269 Figure 1B). Interestingly, the *lspA* transposon mutant (NE1757), showed increased susceptibility to hGIIA
270 (Supplementary Figure 1B). *LspA* is conserved among Gram-positive and Gram-negative bacteria and
271 encodes the lipoprotein signal peptidase A, an enzyme involved in the lipoprotein maturation pathway
272 [30, 51].

273

274 ### Deletion of *lspA* attenuates MRSA resistance to hGIIA *in vitro* and virulence in a hGIIA-Tg mouse model.

275 To verify the contribution of LspA to hGIIA resistance, we constructed a markerless *lspA* deletion mutant
276 in the MRSA strain NRS384 (MRSA Δ*lspA*) and a plasmid complemented mutant strain (MRSA
277 Δ*lspA*::p*lspA*). In accordance to results from our NTML screen, MRSA Δ*lspA* was 5 to 10-fold more
278 susceptible to hGIIA-mediated killing and the phenotype was rescued by complementation with the full
279 length *lspA* gene (Fig. 1A). Deletion of *lspA* in the MRSA background did not result in morphological
280 differences as assessed by scanning electron microscopy (Fig. 1B). Moreover, in accordance with
281 previous literature of other Gram-positive bacteria [52-55], growth of MRSA in bacterial broth was not
282 affected in the *lspA* deletion mutant (Fig. 1C).

283 It was previously shown that mutation of *lspA* resulted in attenuated virulence of *S. aureus* but
284 had no effect on median lethal dose (LD_{50}) values in a mouse infection model [31]. Interestingly, the
285 mouse strain used in this study was C57BL/6, which lacks a functional mouse $sPLA_2$-IIA homologue due
286 to a natural frameshift mutation [19]. Although hGIIA-Tg mice, generated in this naturally-deficient
287 strain background, showed enhanced survival compared to control littermates after infection with WT
288 *S. aureus* [18], it has not yet been determined how *lspA* mutation affects *S. aureus* virulence in a mouse
289 strain with a functional GIIA gene. Therefore, we infected hGIIA-Tg C57BL/6 mice with MRSA WT or its
290 isogenic mutant Δ*lspA* at 2 different doses ($1x10^7$ or $5x10^7$ CFU/mouse). All mice survived the challenge.
291 However, as judged by weight loss, mice infected with either $1x10^7$ or $5x10^7$ MRSA WT bacteria showed
292 significantly more weight loss compared to mice infected with Δ*lspA* bacteria (Fig. 1D). This suggests
293 that in the presence of a functional hGIIA enzyme, LspA contributes to MRSA virulence in this infection
294 model.
295
296 hGIIA shows faster cell wall penetration and membrane permeabilization in the absence of LspA.
297 To gain further insights into the underlying mechanisms of hGIIA susceptibility in the absence of LspA,
298 we assessed the effects of *lspA* deletion on hGIIA binding and cell wall penetration. Since charge-
299 dependent binding is an important first step in hGIIA's mechanism of action, we determined the surface
300 charge of the three strains using the cationic compound cytochrome c [47]. Equal binding levels of
301 cytochrome c was observed for all three strains (Fig. 2A), suggesting that *lspA* does not affect surface
302 charge. However, we did observe that MRSA Δ*lspA* was not only more sensitive to hGIIA, but that killing
303 kinetics were also faster for the mutant compared to WT (Fig. 2B). To assess whether hGIIA trafficking
304 across the cell wall was different, we compared how hGIIA affected membrane depolarization (early
305 effect of hGIIA activity) and membrane permeabilization (late effect of hGIIA activity). Membrane
306 depolarization was measured with the fluorescent voltage-sensitive dye $DiOC_2(3)$ that exhibits green
307 fluorescence (FITC) in all bacterial cells dependent on cell size and red fluorescence (PerCP) dependent
308 on membrane potential. Deletion of *lspA* resulted in a faster and more extensive membrane
309 depolarization (Fig. 2C, Supplementary Figure 2). Loss of LspA also caused increased SYTOX intensity, an
310 indication of membrane permeabilization [15, 47], compared to MRSA WT and complemented strain
311 starting from 9 min (Fig. 2D).
312
313 Interruption of lipoprotein maturation sensitizes MRSA towards daptomycin.
314 The antibiotic daptomycin is clinically important to treat MRSA infections. Interestingly, the mechanism
315 of action of daptomycin displays similarities with hGIIA, since it is dependent on its positive charge and
316 targets the cell membrane [9, 56]. Correspondingly, the identified *S. aureus* resistance genes, i.e.
317 *dltABCD*, *graRS*, and *mprF* overlap for daptomycin and hGIIA [14, 22-24, 57]. We therefore investigated

318 whether *lspA* deletion affected daptomycin resistance. Indeed, MRSA Δ*lspA* was about 5-fold more
319 susceptible to daptomycin killing, whereas the *lspA* plasmid complemented strain became even more
320 resistant compared to WT (Fig. 3A). As comparison, we assessed whether an intracellular acting
321 antibiotic, gentamicin, was differentially effective in the presence and absence of LspA. Only at one
322 concentration did we observe that loss of *lspA* rendered MRSA more susceptible to gentamicin killing
323 (Fig. 3B), indicating that LpsA has minimal impact on gentamicin-mediated killing.
324
325 LspA inhibitors sensitize MRSA towards hGIIA and daptomycin.
326 The antibiotics globomycin and myxovirescin A1 are directly bactericidal towards Gram-negative
327 bacteria with minimum inhibitory concentration values of 12.5 and 1 µg/mL for *E. coli*, respectively [58,
328 59]. Interestingly, both compounds are LspA inhibitors [60, 61] and do not kill *S. aureus* growth even at
329 concentrations of 30 µg/mL myxovirescin A1 and >100 µg/mL globomycin [58, 59]. The co-crystal
330 structures of *S. aureus* LspA with both of these inhibitors were recently published [44]. We assessed
331 whether MRSA could also be sensitized to hGIIA and daptomycin through pharmacological inhibition of
332 LspA. To this end, we pre-incubated MRSA WT with either of these compounds during growth to
333 exponential phase and subsequently exposed the bacterial culture to hGIIA or daptomycin. Indeed,
334 pharmacological interference with LspA by either compound rendered MRSA more susceptible to killing
335 by hGIIA and daptomycin compared to untreated bacteria (Fig. 4A, B). This suggests that these
336 compounds may be interesting sensitizing agent in the context of *S. aureus* infections.
337
338 LspA is highly sequence-conserved within the *S. aureus* population.
339 In considering LspA as a drug target, it is important to assess the sequence conservation over bacterial
340 species. LspA contains five conserved domains, including the catalytic residues, across several bacteria
341 [62]. Moreover, LspA amino acid sequence identity is in-between 35% and 95% across 485 different
342 bacterial species [63].
343 To investigate the presence and sequence conservation of *lspA*, the genomes of 25,243 *S.*
344 *aureus* isolates were surveyed using PubMLST [50]. These isolates originated from different continents
345 and from a wide variety of hosts as well as human patients and carriers. A *lspA* gene was present in all
346 isolates examined. Only 5 isolates contained a gene with an internal stop codon rendering a truncated
347 LspA. In total, 141 *lspA* alleles were observed.
348 The majority of the isolates contained *lspA*4 (14,000 isolates, 54%), *lspA*5 (6,000 isolates, 23%)
349 or *lspA*1 (4,000 isolates, 16%). All other *lspA* alleles were at frequencies < 2.5% (Table 3). Interestingly,
350 specific clonal complexes were associated with a single dominant allele (Table 3). Among 141 *lspA* alleles
351 110 polymorphic positions out of a total gene length of 492 nucleotides were found. These 110
352 polymorphic sites represented 124 single nucleotide polymorphisms (SNPs). None of the SNPs found in

353 critical residues were synonymous, emphasizing the high degree of conservation. The most frequently
354 observed SNPs were found at nucleotide positions 331 and 402 (Fig. 5A). Only one of these, at
355 nucleotide position 331, and present in *lspA*1, *lspA*3, *lspA*7 and *lspA*26, results in an amino acid
356 substitution (Ile111Val). A second non-synonymous SNP at nucleotide position 230 is found in *lspA*3,
357 but this allele is present in only 1% of the isolates (Table 3 and Fig. 5B). All other SNPs, as found in the
358 most frequently observed *lspA* alleles among the *S. aureus* population studied are synonymous (Fig. 5B).
359 Thus, only two amino acid differences are found when comparing the protein sequences
360 encoded by the six most frequently found alleles among a total population of 25,243 isolates analyzed.
361

Table 3. Distribution of *lspA* among 25,243 *S. aureus* isolates.

lspA allele	# isolates	Percentage [a]	Dominant in cc [b]
4	14,000	53.7%	1, 8, 15, 22, 97
5	6,000	22.6%	5
1	4,000	15.7%	30
7	500	2.2%	45
26	400	1.7%	93
3	300	1.1%	-
Other	700	2.9%	

[a]Alleles with at least 1% occurrence in isolates are shown

[b]The allele was considered most dominant in the clonal complex (cc) with an occurrence percentage of 92% or higher.

Numbers are rounded off to thousands and to tenths for number of isolates and percentage, respectively.

362

363 LspA contribution to hGIIA resistance is not restricted to *S. aureus*.

364 *Streptococcus mutans* is a Gram-positive bacterium that resides in the human oral cavity and is the
365 major cause of dental caries [64]. To assess whether LspA-mediated resistance to hGIIA is restricted to
366 *S. aureus* or more widespread, we created a *lspA* deletion mutant in *S. mutans* strain UA159 by replacing
367 the *lspA* gene with an erythromycin cassette. Complementation of this deletion mutant was
368 accomplished by introducing the plasmid pDC123 containing the full *lspA* gene of *S. mutans*. Results
369 from the killing assay revealed that *lspA* deletion renders *S. mutans* more susceptible to hGIIA and
370 complementation fully restored this phenotype (Fig. 6A).
371 In addition, we tested two clinical isolates of the enterococcal strains *E. faecalis* V583 and *E.*
372 *faecium* U0317. These species are part of a group that consists of clinically-relevant and antibiotic-
373 resistant pathogens, collectively called ESKAPE pathogens (*Enterococcus* spp.*, Staphylococcus aureus,*
374 *Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp.) [65,
375 66]. Of the Gram-positive *Enterococci*, the species *E. faecalis* and *E. faecium* are most abundant and are

376 responsible for 75% of all enterococcal infections [67]. We observed that *E. faecalis* was 5-fold more
377 sensitive to hGIIA compared to *E. faecium* (Supplementary Figure 3). Pretreating the clinical
378 enterococcal isolates with 10 µg/mL myxovirescin A1 sensitized *E. faecalis*, but not *E. faecium,* to hGIIA
379 killing compared to the untreated bacteria (Fig. 6B, C). Also, higher concentrations of myxovirescin A1
380 (i.e. 50 µg/mL) did not increase hGIIA killing of *E. faecium*.

381 ## Discussion

382

383 New treatment strategies against MRSA are in high demand due to the rise of antibiotic resistance even
384 against the last-resort antibiotic daptomycin. The current antibiotic arsenal as well as many therapeutic
385 agents under development aim to be directly bactericidal or stop bacterial growth [6]. The drawback of
386 these compounds is the high selective pressure leading to antimicrobial resistance. Non-traditional
387 antibacterial agents, such as anti-virulence drugs, can offer new therapies in the race against
388 antimicrobial resistance by interfering with bacterial strategies that normally allow survival in the
389 context of immune defenses [68]. Such strategies are expected to be less affected by resistance
390 development as there is no direct pressure on survival [69]. Although sensitizing agents still have to
391 prove their clinical use, the concept is appealing. Many of these strategies against *S. aureus* are under
392 active investigation and some are already in preclinical development [70]. For example, inhibition of
393 staphyloxanthin production increased susceptibility to killing in human blood and decreased the
394 virulence of *S. aureus* in mouse infection models [71, 72]. The present work shows that interfering with
395 lipoprotein maturation by inhibition of LspA enhances innate immune killing of MRSA through the
396 modulation of the bactericidal effects of hGIIA. LspA inhibition also enhances daptomycin-mediated
397 killing, which may provide an add-on strategy in antibiotic treatment.

398 To identify resistance genes against hGIIA in MRSA, we screened the NTML and confirmed
399 increased susceptibility for hits in *graR*, *graS*, and *mprF*. These three genes have previously been linked
400 to cationic antimicrobial resistance [22], and also specifically to hGIIA resistance [14]. We also identified
401 *vraF* and *vraG*, which is also in line with expectations, since these genes encode the ABC-transporter
402 linked to the GraRS two-component system [73]. This confirms that the screen, although semi-
403 quantitative, does allow the identification of hGIIA-susceptible mutants. However, the screen likely lacks
404 sensitivity to provide a comprehensive list of hGIIA-susceptible mutants. This is illustrated by the fact
405 that we did not identify *graX*, the gene encoding GraX, which was shown to be involved in cationic
406 antimicrobial peptide resistance and interacts with the GraRS system [73, 74]. Therefore, additional
407 hGIIA sensitive mutants are likely to be identified using another set-up of the screening assay.

408 In our unbiased genetic screen, we identified the transposon mutant NE1757 (*lspA*) to be more
409 susceptible to hGIIA-mediated killing. To exclude the possibility that the *lspA* transposon mutant was
410 identified as a result of growth defects or polar effects of the transposon insertion, we constructed a
411 *lspA* deletion strain in the MRSA background NRS384 that was exposed to a hGIIA concentration range
412 and quantified for bacterial survival. With this quantitative killing assay as well as an infection model in
413 hGIIA-Tg mice, we confirmed *lspA* as a novel hGIIA resistance determinant. Additionally, MRSA Δ*lspA*
414 was also more effectively killed by daptomycin compared to WT. This makes LspA an interesting

415 therapeutic target as its inhibition would simultaneously increase susceptibility to endogenous and
416 specific clinically-used antibiotics.

417 Indeed, we provided proof-of-principle that inhibition of LspA by two known pharmacological
418 inhibitors, globomycin and myxovirescin, renders MRSA more susceptible to hGIIA and daptomycin
419 killing. A previous study has shown that the *S. aureus* LspA enzyme is inhibited by these compounds, but
420 has no direct bactericidal effects [44]. This is in line with the observation that deletion of *lspA* does not
421 affect growth and morphological appearance of MRSA. Hence, selective pressure of this anti-virulence
422 strategy is likely to be minimal. LspA inhibition as a therapeutic strategy may have other advantages.
423 For example, the extracellular location of LspA makes it accessible to drug while no LspA analogs are
424 found in eukaryotic cells, thereby reducing the risk of off-target effects [44, 62, 63]. In addition, we
425 showed that LspA is highly conserved among *S. aureus* strains with only 1 amino acid substitution in
426 >96% of the *S. aureus* collection in the PubMLST database (>26,000 isolates at the time of this analysis).
427 Conserved proteins are less likely to mutate, making them ideal targets as the inhibitor compounds are
428 longer lasting and more effective [75]. The natural antibiotics globomycin and myxovirescin A1
429 specifically inhibit LspA and have similar binding sites on LspA, docking to the catalytic dyad and
430 clustering around 14 conserved residues [44, 60, 61, 63]. Although they have a distinct chemical
431 structure and biosynthesis, there is a remarkable similarity in their mode of action. This might point
432 towards a co-evolution that advanced to prevent resistance [44].

433 LspA processes prolipoproteins that are anchored into the cell membrane by the enzyme Lgt
434 [30]. The mechanism by which LspA mediates hGIIA and daptomycin resistance is currently not clear.
435 We explored the possibility that LspA deletion altered surface charge, thereby facilitating hGIIA binding.
436 However, no difference in binding of the cationic protein cytochrome c was observed, suggesting no
437 large effects on the net charge. Since hGIIA binding to bacteria is based on electrostatic interactions
438 [76], we expect that hGIIA binds similar to WT and *lspA* knock-out strains. On the other hand, we did
439 observe that loss of LspA affected both kinetics and concentration-dependent effects on membrane
440 depolarization and membrane permeabilization, with Δ*lspA* mutants showing faster disruption after
441 exposure to hGIIA. Since LspA is a transmembrane protein [62], lack of LspA may change membrane
442 properties such as membrane fluidity. However, since we observed the same effects in MRSA WT after
443 pretreatment with globomycin or myxovirescin, which inhibit LspA enzymatic activity, this explanation
444 is unlikely. Nonetheless, the presence of multiple immature lipoproteins that still carry the signal
445 peptide may affect membrane characteristics as these prolipoproteins likely accumulate in the
446 membrane. In some Gram-positive bacteria other putative signal peptidases are present that could take
447 over the role of LspA [26], but it is not known if this is the case in *S. aureus*. Another explanation could
448 be that the function of a single lipoprotein is abolished by deletion of *lspA*, resulting in the observed
449 phenotypes. However, our screen did not identify mutants in individual lipoprotein-encoding genes. In

450 addition, lipoproteins may retain their function even without proper processing by LspA [77]. Based on
451 these considerations and observations, we currently favor the hypothesis that differences in membrane
452 composition due to the presence of the signal peptide are responsible for the observed phenotypes.

453 We observed that *lspA* deletion affected antibiotic susceptibility, most pronounced for
454 daptomycin and marginally for gentamicin. In addition, daptomycin susceptibility could also be
455 conferred by pharmacological inhibition of LspA. These findings suggest that LspA is involved in
456 daptomycin resistance. However, the role of LspA in daptomycin-resistance is not necessarily
457 straightforward, since *lspA* was not identified in two previous screens aimed at identifying daptomycin
458 resistance determinants [78, 79]. The study using the same NTML as we did here [78], only identified a
459 single daptomycin-susceptible mutant (SAUSA300_1003). This may indicate that the assay set up was
460 unable to identify all susceptible mutants, since even *mprF*, a well-known daptomycin resistance
461 determinant [24], was not identified. The second study used methicillin-sensitive *S. aureus* instead of
462 MRSA to screen for antibiotic susceptibility, including daptomycin [79]. It may well be that strain
463 background affects the contribution of *lspA* to daptomycin susceptibility. This is illustrated by a recent
464 comparative transposon sequencing (Tn-seq) screen where only one of five *S. aureus* strains showed
465 significant changes in *lspA* insertions after daptomycin exposure [80]. This observation may suggest that
466 despite high protein sequence conservation, therapeutic efficacy of LspA inhibition may be strain-
467 specific. This should be addressed in future studies when considering anti-virulence strategies.

468 Earlier *in vivo* experiments performed with a *S. aureus lspA* deletion strain showed that the
469 mutant was less virulent [31, 32]. Interestingly, these experiments were performed in inbred C57BL/6
470 mice or outbred CD-1 mice, which carry a natural homozygous or heterozygous inactivating mutation in
471 the mouse $sPLA_2$-IIA-encoding gene, respectively [19]. Thus, to assess the contribution of *lspA* mutation
472 to *S. aureus* virulence in an animal with a functional $sPLA_2$-IIA enzyme, we performed a mouse infection
473 experiment using hGIIA-Tg C57BL/6 mice [46]. These hGIIA-Tg mice have increased resistance to lethal
474 *S. aureus* infection compared to control littermates [18]. In this hGIIA-Tg background, mice infected with
475 MRSA Δ*lspA* did not display weight loss whereas mice infected with MRSA WT showed on average 5 to
476 10% weight loss depending on the infectious dose. Altogether, we conclude that LspA-dependent
477 virulence occurs in a hGIIA-dependent and -independent manner as the effects are observed in
478 naturally-deficient C57BL/6 mice and hGIIA-Tg mice.

479 The hGIIA susceptibility phenotype was not only observed in *S. aureus*, but also in *S. mutans*
480 after *lspA* deletion or *E. faecalis* upon LspA inhibition. LspA inhibitors can bind LspA from multiple Gram-
481 positive bacteria [44, 63], which may broaden the scope of therapeutic application. However, LspA
482 inhibition does not universally sensitize Gram-positive bacteria to hGIIA killing, since hGIIA killing of *E.*
483 *faecium* was not affected by myxovirescin A1 pretreatment. It is possible that myxovirescin could not
484 reach LspA in sufficient amounts due differences in cell wall architecture between species and strains.

485 Alternatively, LspA has no role in hGIIA resistance in the *E. faecium* strain, therefore inhibition had no
486 effect on susceptibility. Similar differences have been observed with regard to daptomycin resistance
487 mechanisms, where mutations in the LiaFSR system caused a rearrangement of anionic membrane
488 phospholipids in *E. faecalis* and daptomycin resistance but this was not observed for *E. faecium* [81].
489 More research is needed to clarify the potential application of LspA inhibitors as therapeutic add on for
490 different Gram-positive pathogens.

491 hGIIA is considered as an acute phase protein [82]. It is strongly expressed by innate immune
492 cells upon infection [10] and rises high levels in blood and organs that could be exploited for the
493 development of new treatment strategies for MRSA infections. Deletion of *lspA* or its pharmacological
494 inhibition renders MRSA more susceptible to hGIIA-mediated killing possibly due to altered membrane
495 properties. Moreover, hGIIA resistance mechanisms overlap partially with daptomycin resistance
496 mechanisms and indeed interference with LspA enhanced MRSA susceptibility to daptomycin. We only
497 focused on hGIIA and clinically-relevant antibiotics, but it is possible that LspA inhibition has broader
498 effects on virulence. We provided proof-of-concept for this potential add-on therapy by demonstrating
499 that the antibiotics globomycin and myxovirescin A1 sensitizes MRSA for hGIIA-mediated killing,
500 although strain-specific effects should be investigated. In addition to MRSA, *S. mutans* and *E. faecalis*
501 were sensitized by pharmacological inhibition of LspA, increasing the impact of LspA as an sensitizing
502 target. Therefore, interference with lipoprotein maturation through LspA inhibition is a strategy that
503 warrants further exploration.
504

505 ## Statements

506

507 ### Conflict of interest

508 The authors have no conflicts of interest to declare.
509

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Author contributions

M.M.K., Y.W., V.P.v.H., G.S., C.P., and J.H. carried out the experiments. Y.W., C.P., G.L., J.H., R.M., and L.T. provided essential reagents. M.K. and V.H. took the lead in writing the manuscript. Y.W., G.L., J.H., R.M., Y.P., and L.T. revised the manuscript. N.S conceptualized the study and acquired funding. N.M.v.S., Y.P., and J.A.G.v.S. supervised the project.

Data availability statement

Data and resources are available upon request from the corresponding author.

References

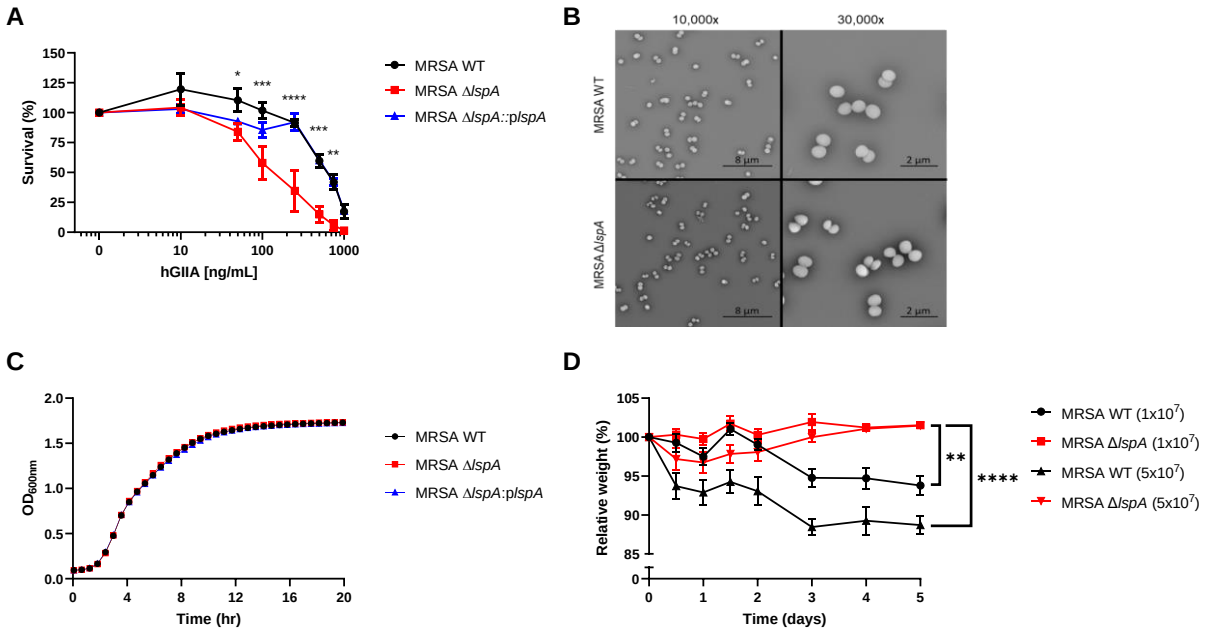
1. O'Neill, J., *Tackling drug-resistant infections globally: final report and recommendations*. 2016.
2. Enright, M.C., et al., *The evolutionary history of methicillin-resistant Staphylococcus aureus (MRSA).* Proc Natl Acad Sci U S A, 2002. **99**(**11**): p. 7687-92.
3. Lee, A.S., et al., *Methicillin-resistant Staphylococcus aureus.* Nat Rev Dis Primers, 2018. **4**: p. 18033.
4. Tacconelli, E., et al., *Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis.* Lancet Infect Dis, 2018. **18**(3): p. 318-327.
5. Ferri, M., et al., *Antimicrobial resistance: A global emerging threat to public health systems.* Crit Rev Food Sci Nutr, 2017. **57**(13): p. 2857-2876.
6. Theuretzbacher, U., et al., *Critical analysis of antibacterial agents in clinical development.* Nat Rev Microbiol, 2020. **18**(5): p. 286-298.
7. Miethke, M., et al., *Towards the sustainable discovery and development of new antibiotics.* Nature Reviews Chemistry, 2021. **5**(10): p. 726-749.
8. Koymans, K.J., et al., *Staphylococcal Immune Evasion Proteins: Structure, Function, and Host Adaptation.* Curr Top Microbiol Immunol, 2017. **409**: p. 441-489.
9. van Hensbergen, V.P., et al., *Type IIA Secreted Phospholipase A2 in Host Defense against Bacterial Infections.* Trends Immunol, 2020. **41**(4): p. 313-326.
10. Dore, E. and E. Boilard, *Roles of secreted phospholipase A2 group IIA in inflammation and host defense.* Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids, 2019. **1864**(6): p. 789-802.
11. Lambeau, G. and M.H. Gelb, *Biochemistry and physiology of mammalian secreted phospholipases A2.* Annu Rev Biochem, 2008. **77**: p. 495-520.
12. Rintala, E.M. and T.J. Nevalainen, *Group II phospholipase A2 in sera of febrile patients with microbiologically or clinically documented infections.* Clin Infect Dis, 1993. **17**(5): p. 864-70.
13. Nevalainen, T.J., et al., *Time-resolved fluoroimmunoassays of the complete set of secreted phospholipases A2 in human serum.* Biochim Biophys Acta, 2005. **1733**(2-3): p. 210-23.
14. Koprivnjak, T., et al., *Role of charge properties of bacterial envelope in bactericidal action of human group IIA phospholipase A2 against Staphylococcus aureus.* J Biol Chem, 2002. **277**(49): p. 47636-44.
15. van Hensbergen, V.P., et al., *Streptococcal Lancefield polysaccharides are critical cell wall determinants for human Group IIA secreted phospholipase A2 to exert its bactericidal effects.* PLoS Pathog, 2018. **14**(10): p. e1007348.
16. Weinrauch, Y., et al., *Mobilization of potent plasma bactericidal activity during systemic bacterial challenge. Role of group IIA phospholipase A2.* J Clin Invest, 1998. **102**(3): p. 633-8.
17. Qu, X.D. and R.I. Lehrer, *Secretory phospholipase A2 is the principal bactericide for staphylococci and other gram-positive bacteria in human tears.* Infect Immun, 1998. **66**(6): p. 2791-7.
18. Laine, V.J., D.S. Grass, and T.J. Nevalainen, *Protection by Group II Phospholipase A2 against Staphylococcus aureus.* J Immunol, 1999. **162**(12): p. 7402-8.
19. Kennedy, B.P., et al., *A natural disruption of the secretory group II phospholipase A2 gene in inbred mouse strains.* J Biol Chem, 1995. **270**(38): p. 22378-85.
20. Slavetinsky, C.J., et al., *Sensitizing Staphylococcus aureus to antibacterial agents by decoding and blocking the lipid flippase MprF.* Elife, 2022. **11**.
21. Kraus, D., et al., *The GraRS regulatory system controls Staphylococcus aureus susceptibility to antimicrobial host defenses.* BMC Microbiol, 2008. **8**: p. 85.
22. Yang, S.J., et al., *The Staphylococcus aureus two-component regulatory system, GraRS, senses and confers resistance to selected cationic antimicrobial peptides.* Infect Immun, 2012. **80**(1): p. 74-81.
23. Bayer, A.S., et al., *Dysregulation of mprF and dltABCD expression among daptomycin-non-susceptible MRSA clinical isolates.* J Antimicrob Chemother, 2016. **71**(8): p. 2100-4.

24. Ernst, C.M. and A. Peschel, *MprF-mediated daptomycin resistance.* Int J Med Microbiol, 2019. **309**(5): p. 359-363.
25. Babu, M.M., et al., *A database of bacterial lipoproteins (DOLOP) with functional assignments to predicted lipoproteins.* J Bacteriol, 2006. **188**(8): p. 2761-73.
26. Kovacs-Simon, A., R.W. Titball, and S.L. Michell, *Lipoproteins of bacterial pathogens.* Infect Immun, 2011. **79**(2): p. 548-61.
27. Zhang, H.Z., et al., *A proteolytic transmembrane signaling pathway and resistance to beta-lactams in staphylococci.* Science, 2001. **291**(5510): p. 1962-5.
28. Foster, T.J., *Antibiotic resistance in Staphylococcus aureus. Current status and future prospects.* FEMS Microbiol Rev, 2017. **41**(3): p. 430-449.
29. Jousselin, A., et al., *The posttranslocational chaperone lipoprotein PrsA is involved in both glycopeptide and oxacillin resistance in Staphylococcus aureus.* Antimicrob Agents Chemother, 2012. **56**(7): p. 3629-40.
30. Nguyen, M.T. and F. Gotz, *Lipoproteins of Gram-Positive Bacteria: Key Players in the Immune Response and Virulence.* Microbiol Mol Biol Rev, 2016. **80**(3): p. 891-903.
31. Bubeck Wardenburg, J., W.A. Williams, and D. Missiakas, *Host defenses against Staphylococcus aureus infection require recognition of bacterial lipoproteins.* Proc Natl Acad Sci U S A, 2006. **103**(37): p. 13831-6.
32. Mei, J.M., et al., *Identification of Staphylococcus aureus virulence genes in a murine model of bacteraemia using signature-tagged mutagenesis.* Mol Microbiol, 1997. **26**(2): p. 399-407.
33. Vestergaard, M., et al., *Inhibition of the ATP Synthase Eliminates the Intrinsic Resistance of Staphylococcus aureus towards Polymyxins.* mBio, 2017. **8**(5).
34. Fey, P.D., et al., *A genetic resource for rapid and comprehensive phenotype screening of nonessential Staphylococcus aureus genes.* mBio, 2013. **4**(1): p. e00537-12.
35. Koprivnjak, T., et al., *Wall teichoic acid deficiency in Staphylococcus aureus confers selective resistance to mammalian group IIA phospholipase A(2) and human beta-defensin 3.* Infect Immun, 2008. **76**(5): p. 2169-76.
36. Ghomashchi, F., et al., *Preparation of the Full Set of Recombinant Mouse- and Human-Secreted Phospholipases A2.* Methods Enzymol, 2017. **583**: p. 35-69.
37. Monk, I.R., et al., *Transforming the untransformable: application of direct transformation to manipulate genetically Staphylococcus aureus and Staphylococcus epidermidis.* mBio, 2012. **3**(2).
38. Ajdic, D., et al., *Genome sequence of Streptococcus mutans UA159, a cariogenic dental pathogen.* Proc Natl Acad Sci U S A, 2002. **99**(22): p. 14434-9.
39. Paulsen, I.T., et al., *Role of mobile DNA in the evolution of vancomycin-resistant Enterococcus faecalis.* Science, 2003. **299**(5615): p. 2071-4.
40. van Schaik, W., et al., *Pyrosequencing-based comparative genome analysis of the nosocomial pathogen Enterococcus faecium and identification of a large transferable pathogenicity island.* BMC Genomics, 2010. **11**(1): p. 239.
41. Stapels, D.A., et al., *Staphylococcus aureus secretes a unique class of neutrophil serine protease inhibitors.* Proc Natl Acad Sci U S A, 2014. **111**(36): p. 13187-92.
42. Chaffin, D.O. and C.E. Rubens, *Blue/white screening of recombinant plasmids in Gram-positive bacteria by interruption of alkaline phosphatase gene (phoZ) expression.* Gene, 1998. **219**(1-2): p. 91-9.
43. Bae, T. and O. Schneewind, *Allelic replacement in Staphylococcus aureus with inducible counter-selection.* Plasmid, 2006. **55**(1): p. 58-63.
44. Olatunji, S., et al., *Structures of lipoprotein signal peptidase II from Staphylococcus aureus complexed with antibiotics globomycin and myxovirescin.* Nat Commun, 2020. **11**(1): p. 140.
45. van der Beek, S.L., et al., *GacA is essential for Group A Streptococcus and defines a new class of monomeric dTDP-4-dehydrorhamnose reductases (RmlD).* Mol Microbiol, 2015. **98**(5): p. 946-62.

46. Grass, D.S., et al., *Expression of human group II PLA2 in transgenic mice results in epidermal hyperplasia in the absence of inflammatory infiltrate.* J Clin Invest, 1996. **97**(10): p. 2233-41.
47. Carvalho, F., et al., *L-Rhamnosylation of Listeria monocytogenes Wall Teichoic Acids Promotes Resistance to Antimicrobial Peptides by Delaying Interaction with the Membrane.* PLoS Pathog, 2015. **11**(5): p. e1004919.
48. Shapiro, H.M., *Membrane potential estimation by flow cytometry.* Methods, 2000. **21**(3): p. 271-9.
49. Saar-Dover, R., et al., *D-alanylation of lipoteichoic acids confers resistance to cationic peptides in group B streptococcus by increasing the cell wall density.* PLoS Pathog, 2012. **8**(9): p. e1002891.
50. Jolley, K.A., J.E. Bray, and M.C.J. Maiden, *Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications.* Wellcome Open Res, 2018. **3**: p. 124.
51. Braun, V. and K. Hantke, *Lipoproteins: Structure, Function, Biosynthesis.* Subcell Biochem, 2019. **92**: p. 39-77.
52. Khandavilli, S., et al., *Maturation of Streptococcus pneumoniae lipoproteins by a type II signal peptidase is required for ABC transporter function and full virulence.* Mol Microbiol, 2008. **67**(3): p. 541-57.
53. Weston, B.F., A. Brenot, and M.G. Caparon, *The metal homeostasis protein, Lsp, of Streptococcus pyogenes is necessary for acquisition of zinc and virulence.* Infect Immun, 2009. **77**(7): p. 2840-8.
54. Reglier-Poupet, H., et al., *Maturation of lipoproteins by type II signal peptidase is required for phagosomal escape of Listeria monocytogenes.* J Biol Chem, 2003. **278**(49): p. 49469-77.
55. de Greeff, A., et al., *Lipoprotein signal peptidase of Streptococcus suis serotype 2.* Microbiology (Reading), 2003. **149**(Pt 6): p. 1399-1407.
56. Taylor, S.D. and M. Palmer, *The action mechanism of daptomycin.* Bioorg Med Chem, 2016. **24**(24): p. 6253-6268.
57. Slavetinsky, C.J., et al., *Sensitizing Staphylococcus aureus to antibacterial host defense by decoding and blocking the lipid flippase MprF.* bioRxiv, 2021: p. 2020.11.12.379776.
58. Gerth, K., et al., *The myxovirescins, a family of antibiotics from Myxococcus virescens (Myxobacterales).* J Antibiot (Tokyo), 1982. **35**(11): p. 1454-9.
59. Kiho, T., et al., *Structure-activity relationships of globomycin analogues as antibiotics.* Bioorg Med Chem, 2004. **12**(2): p. 337-61.
60. Inukai, M., et al., *Mechanism of action of globomycin.* J Antibiot (Tokyo), 1978. **31**(11): p. 1203-5.
61. Xiao, Y., et al., *Myxobacterium-produced antibiotic TA (myxovirescin) inhibits type II signal peptidase.* Antimicrob Agents Chemother, 2012. **56**(4): p. 2014-21.
62. Paetzel, M., *Bacterial Signal Peptidases.* Subcell Biochem, 2019. **92**: p. 187-219.
63. Vogeley, L., et al., *Structural basis of lipoprotein signal peptidase II action and inhibition by the antibiotic globomycin.* Science, 2016. **351**(6275): p. 876-80.
64. Nakano, K. and T. Ooshima, *Serotype classification of Streptococcus mutans and its detection outside the oral cavity.* Future Microbiol, 2009. **4**(7): p. 891-902.
65. Rice, L.B., *Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE.* J Infect Dis, 2008. **197**(8): p. 1079-81.
66. Pendleton, J.N., S.P. Gorman, and B.F. Gilmore, *Clinical relevance of the ESKAPE pathogens.* Expert Rev Anti Infect Ther, 2013. **11**(3): p. 297-308.
67. Weiner-Lastinger, L.M., et al., *Antimicrobial-resistant pathogens associated with adult healthcare-associated infections: Summary of data reported to the National Healthcare Safety Network, 2015-2017.* Infect Control Hosp Epidemiol, 2020. **41**(1): p. 1-18.
68. Theuretzbacher, U. and L.J.V. Piddock, *Non-traditional Antibacterial Therapeutic Options and Challenges.* Cell Host Microbe, 2019. **26**(1): p. 61-72.
69. Dickey, S.W., G.Y.C. Cheung, and M. Otto, *Different drugs for bad bugs: antivirulence strategies in the age of antibiotic resistance.* Nat Rev Drug Discov, 2017. **16**(7): p. 457-471.

682 70. Ford, C.A., I.M. Hurford, and J.E. Cassat, *Antivirulence Strategies for the Treatment of*
683 *Staphylococcus aureus Infections: A Mini Review.* Front Microbiol, 2020. **11**: p. 632706.
684 71. Chen, F., et al., *Small-molecule targeting of a diapophytoene desaturase inhibits S. aureus*
685 *virulence.* Nat Chem Biol, 2016. **12**(3): p. 174-9.
686 72. Liu, C.I., et al., *A cholesterol biosynthesis inhibitor blocks Staphylococcus aureus virulence.*
687 Science, 2008. **319**(5868): p. 1391-4.
688 73. Falord, M., et al., *GraXSR proteins interact with the VraFG ABC transporter to form a five-*
689 *component system required for cationic antimicrobial peptide sensing and resistance in*
690 *Staphylococcus aureus.* Antimicrob Agents Chemother, 2012. **56**(2): p. 1047-58.
691 74. Herbert, S., et al., *Molecular basis of resistance to muramidase and cationic antimicrobial*
692 *peptide activity of lysozyme in staphylococci.* PLoS Pathog, 2007. **3**(7): p. e102.
693 75. Rao, C.V.S., et al., *Antibiotic targeting of the bacterial secretory pathway.* Biochim Biophys Acta,
694 2014. **1843**(8): p. 1762-83.
695 76. Weiss, J.P., *Molecular determinants of bacterial sensitivity and resistance to mammalian Group*
696 *IIA phospholipase A2.* Biochim Biophys Acta, 2015. **1848**(11 Pt B): p. 3072-7.
697 77. Shahmirzadi, S.V., M.T. Nguyen, and F. Gotz, *Evaluation of Staphylococcus aureus Lipoproteins:*
698 *Role in Nutritional Acquisition and Pathogenicity.* Front Microbiol, 2016. **7**: p. 1404.
699 78. Vestergaard, M., et al., *Genome-Wide Identification of Antimicrobial Intrinsic Resistance*
700 *Determinants in Staphylococcus aureus.* Front Microbiol, 2016. **7**: p. 2018.
701 79. Rajagopal, M., et al., *Multidrug Intrinsic Resistance Factors in Staphylococcus aureus Identified*
702 *by Profiling Fitness within High-Diversity Transposon Libraries.* mBio, 2016. **7**(4).
703 80. Coe, K.A., et al., *Multi-strain Tn-Seq reveals common daptomycin resistance determinants in*
704 *Staphylococcus aureus.* PLoS Pathog, 2019. **15**(11): p. e1007862.
705 81. Miller, W.R., A.S. Bayer, and C.A. Arias, *Mechanism of Action and Resistance to Daptomycin in*
706 *Staphylococcus aureus and Enterococci.* Cold Spring Harb Perspect Med, 2016. **6**(11).
707 82. Crowl, R.M., et al., *Induction of phospholipase A2 gene expression in human hepatoma cells by*
708 *mediators of the acute phase response.* J Biol Chem, 1991. **266**(4): p. 2647-51.
709

710 Figures



711 **Figure 1. LspA contributes to hGIIA resistance *in vitro* as well as virulence in a hGIIA-Tg mouse model.** (A)
712 Survival of MRSA WT, MRSA Δ*lspA*, and MRSA Δ*lspA*::p*lspA* after exposure to a concentration range of
713 recombinant hGIIA. (B) Representative scanning electron microscopy (SEM) images of MRSA WT and
714 MRSA Δ*lspA* in early exponential phase. (C) Growth curves of MRSA WT, MRSA Δ*lspA*, and MRSA
715 Δ*lspA*::p*lspA*. (D) Relative weight of male and female hGIIA-Tg C57BL/6 mice injected i.p. with either
716 MRSA WT or MRSA Δ*lspA* ($1x10^7$ or $5x10^7$ CFU). Statistical significance was determined using one- or
717 two-way ANOVA + Bonferroni's Multiple Comparison Test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p <$
718 0.0001. A, D: Data represent mean with standard error of the mean of three biological replicates.

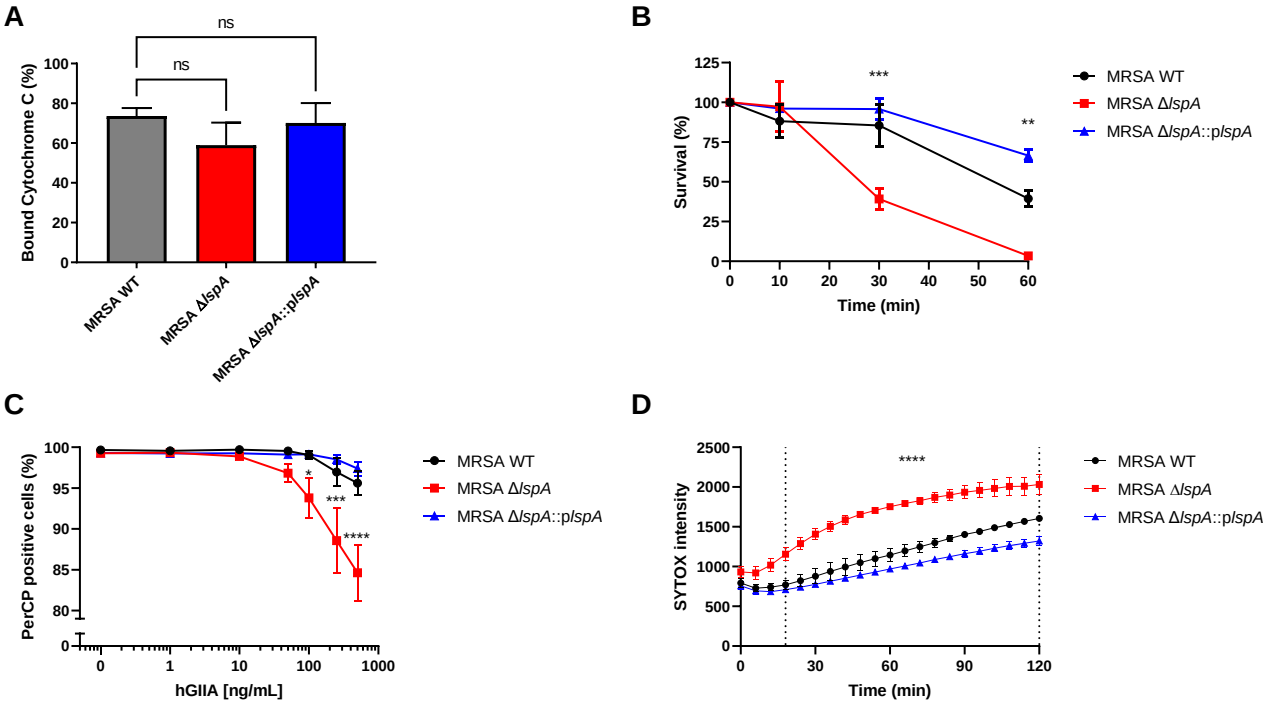
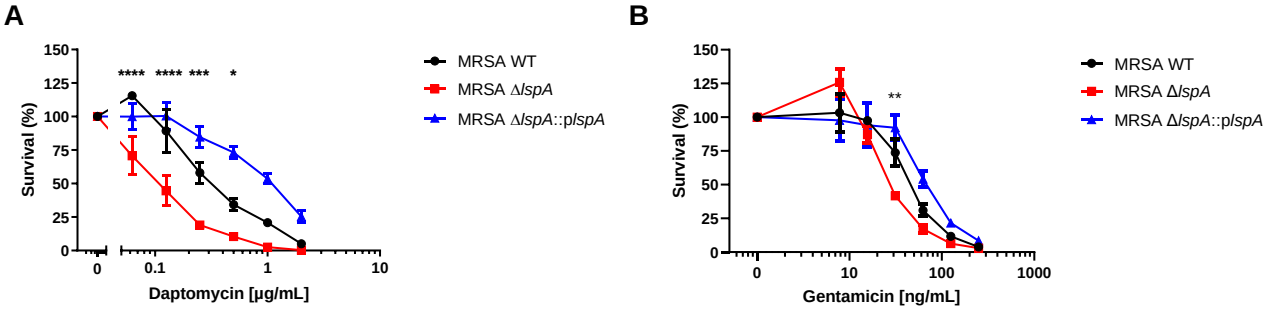


Figure 2. Deletion of *lspA* results in faster hGIIA cell wall penetration and membrane permeabilization. (A) Surface charge of MRSA WT, MRSA Δ*lspA*, and MRSA Δ*lspA*::p*lspA* as determined in a cytochrome c binding assay. (B) Survival of MRSA WT, MRSA Δ*lspA*, and MRSA Δ*lspA*::p*lspA* over time after incubation with 500 ng/mL recombinant hGIIA. (C) Flow cytometric analysis of PerCP-positive cells of MRSA WT, MRSA Δ*lspA*, and MRSA Δ*lspA*::p*lspA* stained with $DiOC_2(3)$ after exposure to a concentration range of recombinant hGIIA. (D) Kinetic analysis of SYTOX intensity for MRSA WT, MRSA Δ*lspA*, and MRSA Δ*lspA*::p*lspA* in the presence of 250 ng/mL recombinant hGIIA. Statistical significance was determined using a one- or two-way ANOVA + Bonferroni's Multiple Comparison Test. ns = not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Data represent mean with standard error of the mean of three biological replicates.



729 **Figure 3. Impact of LspA on killing by clinically-relevant antibiotics.** Survival of MRSA WT, MRSA Δ*lspA*,
730 and MRSA Δ*lspA*::p*lspA* after exposure to (A) daptomycin or (B) gentamicin. Statistical significance was
731 determined between MRSA WT and MRSA Δ*lspA* using a two-way ANOVA + Bonferroni's Multiple
732 Comparison Test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Data represent mean with
733 standard error of the mean of three biological replicates.

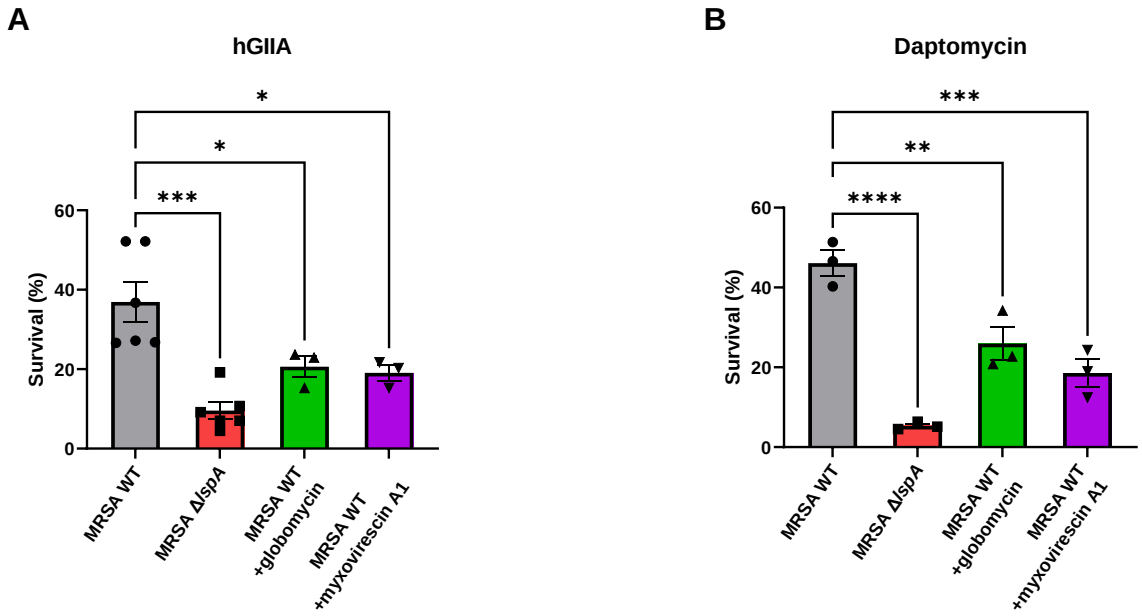
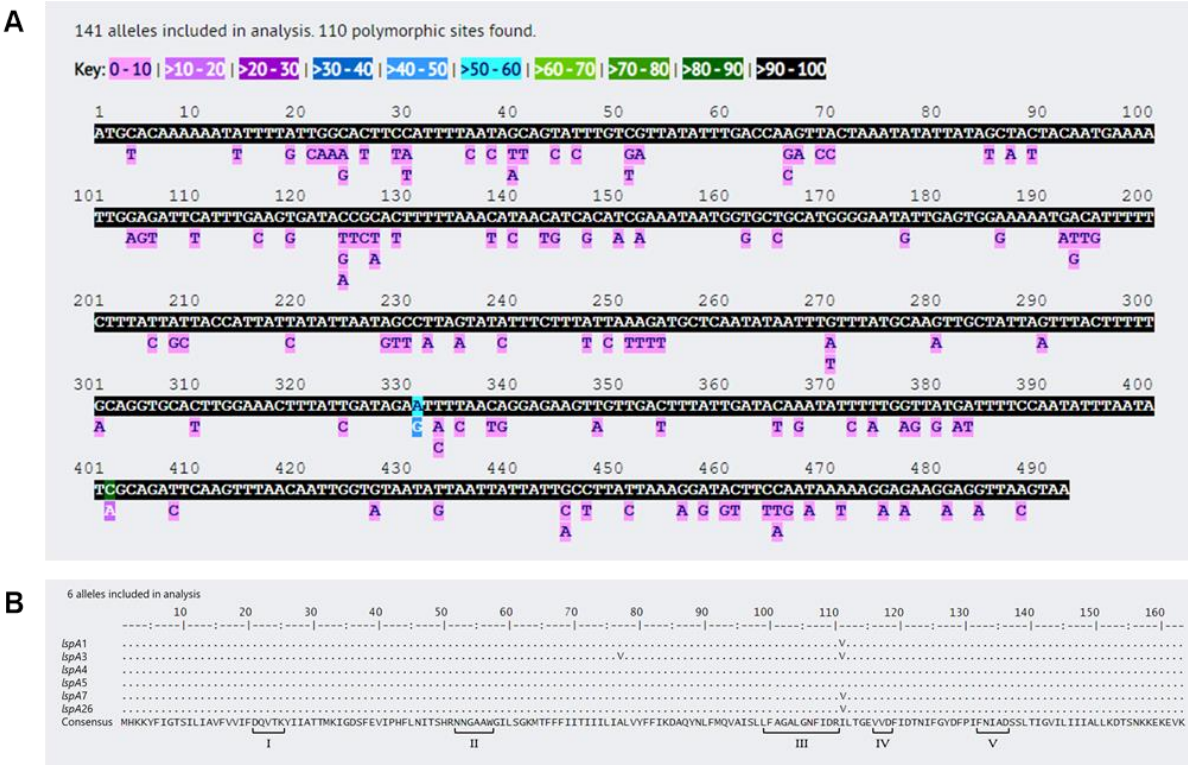


Figure 4. Globomycin and myxovirescin A1 increase MRSA killing by hGIIA and daptomycin. Survival of MRSA WT, MRSA Δ*lspA*, MRSA WT + 100 µg/mL globomycin, and MRSA WT + 10 µg/mL myxovirescin A1 after subsequent exposure to (A) recombinant hGIIA (250 ng/mL) or (B) daptomycin (1 µg/mL). Statistical significance was determined using a one-way ANOVA + Bonferroni's Multiple Comparison Test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Data represent mean with standard error of the mean of three biological replicates.



740

741 **Figure 5. *lspA* and the encoded LspA protein are highly sequence conserved across the *S. aureus* population.** (A) Polymorphic site frequencies of 141 alleles of *lspA* among 25,243 *S. aureus* genomes.
742 Consensus sequence is depicted with color coding for the occurrence in percentages. (B) Alignment and
743 consensus sequence at amino acids level encoded by the 6 most common *lspA* alleles. The five
744 conserved domains across all bacterial species are depicted below with roman numerals [62]. *LspA*4 is
745 the reference allele.

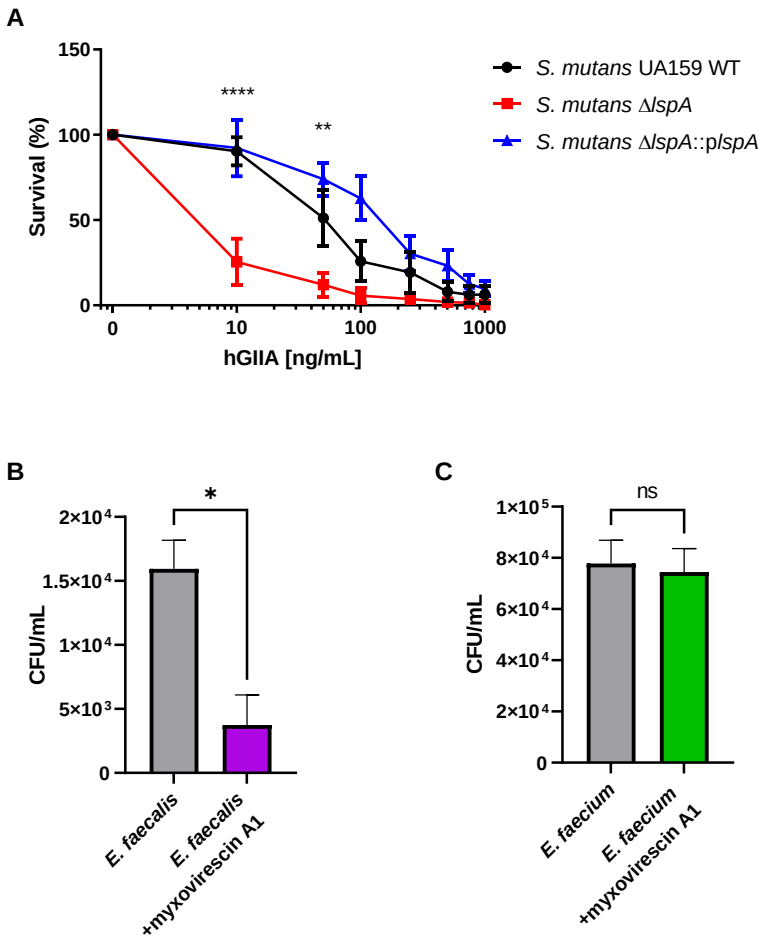


Figure 6. *S. mutans* and *E. faecalis*, but not *E. faecium*, are sensitized to hGIIA via *lspA* deletion or LspA inhibition. (A) Survival of *S. mutans* UA159 WT, *S. mutans* Δ*lspA*, and *S. mutans* Δ*lspA*::p*lspA* after exposure to concentration range of recombinant hGIIA. (B) Survival of *E. faecalis* and *E. faecalis* + 10 µg/mL myxovirescin A1 after subsequent exposure to recombinant hGIIA (0.5 ng/mL). (C) Survival of *E. faecium* and *E. faecium* + 10 µg/mL myxovirescin A1 after subsequent exposure to recombinant hGIIA (0.5 ng/mL). Statistical significance was determined using a one- or two-way ANOVA + Bonferroni's Multiple Comparison Test or an unpaired two-tailed Student's *t* test. ns = not significant, *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$. Data represent mean with standard error of the mean of three biological replicates.

757 Supplemental Figures

758

759 **Supplementary Table 1.** The four different groups including number and sex of mice used in the MRSA

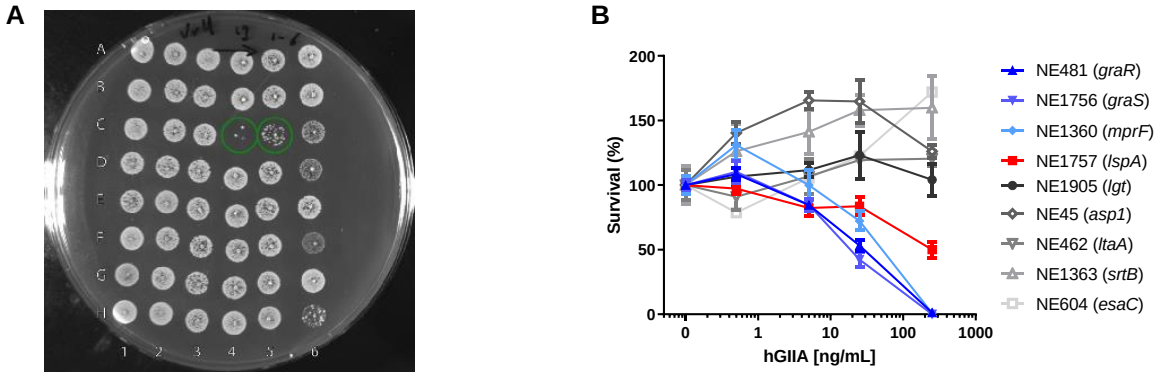
760 infection experiment using hGIIA-Tg C57BL/6 mice.

Bacteria	Infectious dose	No. of animals (male)
MRSA WT	$1x10^7$ CFU	8 (3)
MRSA Δ*lspA*	$1x10^7$ CFU	9 (4)
MRSA WT	$5x10^7$ CFU	7 (2)
MRSA Δ*lspA*	$5x10^7$ CFU	9 (4)

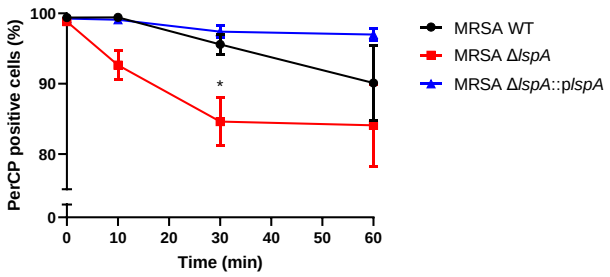
761

762 **Supplementary Table 2.** The 39 MRSA transposon mutants that showed decreased survival on plate
763 after hGIIA exposure in a non-biased genetic screen.

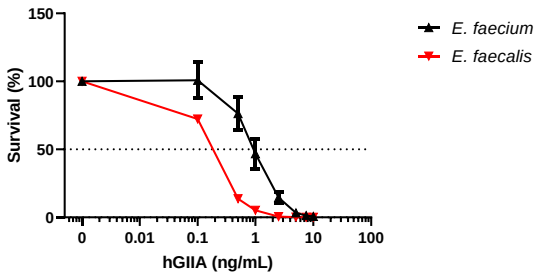
Strain name	Gene	Function	Accession number
NE43	*ureF*	urease accessory protein	SAUSA300_2242
NE45	*asp1*	accessory secretory protein	SAUSA300_2587
NE70	*vraG*	ABC transporter of GraRS	SAUSA300_0648
NE77	-	conserved hypothetical protein	SAUSA300_0465
NE150	-	conserved hypothetical protein	SAUSA300_0097
NE229	*fakA*	conserved hypothetical protein	SAUSA300_1119
NE235	-	conserved hypothetical protein	SAUSA300_0847
NE256	-	putative pyridoxal phosphate-dependent acyltransferase	SAUSA300_0535
NE257	-	conserved hypothetical protein	SAUSA300_1623
NE259	*pnpA*	polyribopolyribonucleotide nucleotidyltransferase	SAUSA300_1167
NE264	*lipA*	lipoic acid synthetase	SAUSA300_0829
NE340	*ilvA*	threonine dehydratase	SAUSA300_2014
NE352	*rsgA*	ribosome small subunit-dependent GTPase A	SAUSA300_1114
NE428	*moaE*	molybdopterin converting factor	SAUSA300_2222
NE462	*ltaA*	proton-coupled antiporter flippase	SAUSA300_0917
NE481	*graR*	part of two component system GraRS	SAUSA300_0645
NE592	*atpA*	ATP synthase F1, alpha subunit	SAUSA300_2060
NE603	*moeA*	molybdopterin biosynthesis protein A	SAUSA300_2224
NE604	*esaC*	protein within ESAT-6 gene cluster	SAUSA300_0284
NE605	*brnQ2*	branched-chain amino acid transport system II carrier protein	SAUSA300_0306
NE630	-	acetyltransferase, GNAT family	SAUSA300_0665
NE631	-	phiPVL ORF39-like protein	SAUSA300_1962
NE645	*vraF*	ABC transporter of GraRS	SAUSA300_0647
NE788	*trkA*	potassium uptake protein	SAUSA300_0988
NE883	*xerC*	tyrosine recombinase xerC	SAUSA300_1145
NE885	-	pyruvate ferredoxin oxidoreductase, alpha subunit	SAUSA300_1182
NE891	-	sodium transport family protein	SAUSA300_0924
NE1193	*sarA*	accessory regulator A	SAUSA300_0605
NE1334	-	hypothetical protein	SAUSA300_1494
NE1360	*mprF*	phosphatidylglycerol lysyltransferase	SAUSA300_1255
NE1363	*srtB*	sortase B	SAUSA300_1034
NE1371	-	conserved hypothetical phage protein	SAUSA300_1967
NE1504	-	Na+/H+ antiporter	SAUSA300_0617
NE1531	*pdxT*	glutamine amidotransferase subunit	SAUSA300_0505
NE1536	*gcvH*	glycine cleavage system protein H	SAUSA300_0791
NE1756	*graS*	part of two component system GraRS	SAUSA300_0646
NE1757	*lspA*	lipoprotein signal peptidase	SAUSA300_1089
NE1828	*pdxS*	pyridoxal biosynthesis lyase	SAUSA300_0504
NE1894	-	hypothetical membrane protein	SAUSA300_1908



Supplementary Figure 1. Identification of MRSA transposon mutants with increased susceptibility to hGIIA-mediated killing. (A) Representative image of an agar plate with spotted MRSA transposon mutants after exposure to 1.25 µg/mL recombinant hGIIA. Two transposon mutants on this plate showed decreased viability; at position C4 is NE0646 (*graS*) and at position C5 is the transposon mutant NE1757 (*lspA*). (B) Survival of potentially hGIIA susceptible mutants from the NTML using quantitative concentration-dependent killing assay. Data represent mean with standard error of the mean from three technical replicates.



Supplementary Figure 2. Faster membrane depolarization in *lspA* deletion mutant compared to WT and complemented strain after hGIIA exposure. Flow cytometric analysis of PerCP-positive cells of MRSA WT, MRSA Δ*lspA*, and MRSA Δ*lspA*::p*lspA* stained with $DiOC_2(3)$ at different time point after exposure to 500 ng/mL hGIIA. Data represent mean ± standard error of the mean of three independent experiments. Statistical significance was determined using a two-way ANOVA + Bonferroni's Multiple Comparison Test. *$p < 0.05$. Data represent mean with standard error of the mean of three biological replicates.



Supplementary Figure 3. *E. faecalis* is more sensitive to hGIIA killing compared to *E. faecium*. Survival of *E. faecalis* V583 and *E. faecium* U0317 after exposure to concentration range of recombinant hGIIA. Data represent mean with standard error of the mean of three biological replicates.