

1 Genomic signatures of experimental adaptation to 2 antimicrobial peptides in *Staphylococcus aureus*

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16 **Running title:** Genomics of AMP resistance in *S. aureus*

17 **Keywords:** *Staphylococcus aureus*, antimicrobial peptide resistance, experimental evolution

18
19 **Objectives:** The evolution of resistance against antimicrobial peptides has long been considered
20 unlikely due to their mechanism of action, yet experimental selection with AMPs results in rapid
21 evolution of resistance in several species of bacteria. Although numerous studies have utilized
22 mutant screens to identify loci that determine AMP susceptibility, there is a dearth of data
23 concerning the genomic changes which accompany experimental evolution of AMP resistance.

24 **Methods:** Using genome re-sequencing we analysed the mutations which arise during experimental
25 evolution of resistance to the cationic AMPs iseganan, melittin and pexiganan, as well as to a
26 combination of melittin and pexiganan, or to the aminoglycoside antibiotic streptomycin.

27 **Results:** Analysis of 17 independently replicated *Staphylococcus aureus* selection lines, including
28 unselected controls, showed that each AMP selected for mutations at distinct loci. We identify
29 mutations in genes involved in the synthesis and maintenance of the cell envelope. This includes
30 genes previously identified from mutant screens for AMP resistance, and genes involved in the
31 response to AMPs and cell-wall-active antibiotics. Furthermore, transposon insertion mutants were
32 used to verify that a number of the identified genes are directly involved in determining AMP
33 susceptibility.

34 **Conclusions:** Strains selected for AMP resistance under controlled experimental evolution
35 displayed consistent AMP-specific mutations in genes which determine AMP susceptibility. This
36 suggests that different routes to evolve resistance are favored within a controlled genetic
37 background.

38 39 **Introduction**

40 Antimicrobial peptides (AMPs), ubiquitous in multicellular organisms¹, are considered to be a
41 promising source of new and potent antibiotics². Current research on AMPs mostly focuses on the
42 mechanisms of action and on the development of therapeutics whereas only a small number of
43 studies have addressed the important problem of bacterial resistance evolution. Resistance against
44 cationic AMPs evolves readily *in vitro* in *Escherichia coli* and *Pseudomonas aeruginosa*³,
45 *Salmonella enterica*⁴, and *Staphylococcus aureus*^{5,6}. Experimentally evolved strains of *S. aureus* that
46 were selected successfully for resistance against the cationic protegrin-1 analog iseganan⁶ survive
47 better in a model host⁷, which relies heavily on AMPs to deal with long-lasting infections⁸. *S.*
48 *aureus* populations selected for resistance to pexiganan and mellitin also show a trend towards

49 increased survival in the host⁷. Here we present a genomic analysis of *S. aureus* strains from these
50 populations⁶ together with susceptibility data from transposon insertion mutants that show a number
51 of the identified genes are directly involved in mediating AMP susceptibility.

52

53 **Materials and methods**

54 Strains were isolated from populations which were created by selecting *S. aureus* JLA513⁹ (*hla-*
55 *lacZ hla+*, derived from SH1000, from Simon Foster, University of Sheffield) for 28 days with
56 increasing concentrations of AMPs or with the aminoglycoside antibiotic streptomycin⁶.
57 Streptomycin-selected strains are included here as a positive control since the genetic basis of
58 streptomycin resistance is well-characterized in *S. aureus*. Briefly, to ensure adaptation to the
59 culture medium 50 µl of *S. aureus* JLA513 culture was passaged serially every 24 h for 10 days in 5
60 ml Müller-Hinton Broth (MHB). Subsequently, 5 parallel selection lines were established in each
61 treatment at MIC₅₀ (as well as unselected controls) by inoculating 5 µl of serially-passaged culture
62 into 500 µl of MHB containing the cognate selective agent. 5 µl of 24 h cultures were passaged
63 daily to fresh MHB. The concentrations of the selective agents were doubled each week for a total
64 of four weeks. See Dobson et al. 2013 Table S1 for full details and precise concentrations⁶. Strains
65 were isolated from each of three independently selected replicate populations per selective agent
66 (with the exception of iseganan-selected populations where only 2 frozen population stocks
67 remained viable), as well as from unselected controls and the ancestral strain JLA513. Minimum
68 inhibitory concentrations (MIC) were calculated for the selective agents (Table S1) in 96-well plates
69 as previously described¹⁰ and DNA was isolated from each strain using a Roboklon DNA extraction
70 kit (Roboklon GmbH, Germany). Genomic DNA from each strain was sequenced for 180 cycles
71 using a HiSeq2000 by the Beijing Genomics Institute (BGI), resulting in 90-bp paired-end reads.
72 Sequence data are available from the NCBI SRA under BioProject ID PRJNA291485. Strain
73 JLA513⁹ was constructed using strain SH1000, which is a derivative of strain 8325. The genetic
74 differences between SH1000 and other members of the 8325 lineage have been described using
75 both array-based resequencing¹¹ and subsequently by de novo genome sequencing¹². The differences
76 comprise: the excision of three prophages from strain 8325 (Φ11, 12, 13), 13 single-nucleotide
77 polymorphisms (2 synonymous, 11 non-synonymous), a 63-bp deletion in the *spa-sarS* intergenic
78 region, and an 11-bp deletion in *rsbU*¹². Therefore a consensus reference genome was first produced
79 to account for these differences. Reads from JLA513 were assembled using SPAdes¹³ and the
80 resulting contigs were used to correct for the 3 phage excision sites in the 8325 reference genome.
81 JLA513 reads were then mapped to the resulting sequence and bcftools consensus¹⁴ was used to
82 correct the remaining 13 SNPs and 2 indels. To identify mutations in the selection lines, reads were
83 mapped to this reference genome using BWA¹⁵ and sorted, deduplicated (to account for optical- and
84 PCR-duplicates) and indexed using SAMtools¹⁴ and Picard (<http://broadinstitute.github.io/picard>).
85 Average coverage was 134-fold (range 110-144 fold). Variants were called using FreeBayes version
86 v0.9.14-8-g1618f7e¹⁶ and coverage was calculated across 25-bp windows using IGVtools¹⁷. All
87 variants were independently verified using a second computational pipeline, breseq¹⁸. Insertion
88 mutants were obtained from the Nebraska Transposon Mutant Library¹⁹ in order to test if the
89 identified genes were directly involved in AMP resistance. MICs were calculated for each mutant
90 and the wild type strain USA300_FPR3757 as described above.

91

92 **Results and discussion**

93 Between one and four mutations were identified per strain after accounting for differences between
94 the JLA513 ancestor and the 8325 reference genome, and for mutations arising over the course of
95 the experiment across treatments and unselected controls. In total, 28 mutations were identified
96 across the 17 strains including 24 nonsynonymous mutations affecting 13 genes, a segmental
97 duplication of 124-kb region containing an entire *rrn* operon (Table 1, Table S2) as well as 1
98 synonymous mutation and 2 intergenic indels (Table S2).

99

100 Pexiganan resistance was characterized by distinct nonsense mutations in the gene encoding the
101 XRE-family transcriptional regulator XdrA in strains PG2.2 and PG4.2 (Table 1, Table S2). XdrA
102 was recently shown to activate transcription of *spa*²⁰, which encodes the protein A virulence factor,
103 and deletion mutants show increased β -lactam resistance²¹. Here, a transposon mutant with an
104 insertion in *xdrA* showed decreased pexiganan susceptibility (Table 1, Table S3) indicating that
105 XdrA is directly involved in pexiganan resistance. In addition to a mutation in *xdrA*, strain PG4.2
106 also carried a nonsynonymous substitution in *wcaG*, which encodes a putative UDP-glucose-4
107 epimerase (Table 1). Only a single mutation was observed in strain PG1.1, introducing a frameshift
108 into *mgt* (*sgtB*), which encodes a monofunctional peptidoglycan glycosyltransferase (Table 1). A
109 distinct nonsense in *mgt* was also identified in one pexiganan-melittin-selected strain (see below).
110 An *mgt* transposon mutant was also found to be less susceptible to pexiganan (Table 1, Table S3). As
111 part of the cell wall stimulon²², *mgt* is positively regulated by cell wall stress and participates in the
112 polymerization of lipid II into nascent peptidoglycan²³. Recent work has shown that *mgt* mutations
113 cause peptidoglycan chain length reduction as well as alterations in cellular morphology and
114 division site placement²⁴.

115

116 All 3 melittin-resistant strains were found to carry missense mutations resulting in either A35T or
117 A35D substitutions in a gene encoding a putative RluD-like pseudouridylate synthase with no
118 known role in antimicrobial susceptibility. A transposon mutant from the Nebraska Transposon
119 Mutant Library with an insertion in this gene showed no change in melittin susceptibility (Table 1,
120 Table S3). One melittin-resistant strain carried a L93I missense mutation in a region encoding an
121 alpha helix immediately adjacent to the conserved active site quintet in the response regulator WalR
122 (Table 1). WalR regulates cell wall metabolism and is ubiquitous in the *Firmicutes* where it is the
123 only known essential two-component system²⁵. *walKR* mutations, including those affecting the
124 WalR active site, arise during persistent clinical *S. aureus* infections and are known to confer
125 resistance to vancomycin and the lipopeptide antibiotic daptomycin by increasing the thickness of
126 the cell wall²⁶. Identical nonsense mutations were identified in two melittin-resistant strains at the
127 extreme 5' end of the *ytrA* open reading frame, which encodes a winged helix-turn-helix GntR-
128 family repressor (Table 1). Similar to its *B. subtilis* ortholog, *ytrA* is the first gene of an operon
129 which encodes two putative ABC transporters. In *B. subtilis*, YtrA binds specifically to an inverted
130 repeat in the *ytrA* and *ywoB* promoters, and transcription of the *ytr* and *ywo* operons is induced by
131 cell-wall-active antibiotics including the peptide antibiotics bacitracin, vancomycin and
132 ramnoplanin, with *ytrA* null mutations causing constitutive expression of both operons²⁷. Notably,
133 the entire *ytrA* operon has been shown to be induced by cationic AMPs in *S. aureus*, where it is
134 under negative regulation by the AMP sensing system *aps*²⁸ and has also been implicated in nisin
135 susceptibility in *S. aureus* SH1000²⁹. Although *ytrA* insertions are not present in the Nebraska
136 Transposon Mutant Library we were able to obtain 2 independent *ytr* operon transposon mutants
137 with insertions downstream of *ytrA* which did not show any detectable difference in AMP
138 susceptibility relative to the wild type (Table S3). This raises the possibility that the *ytrA*-null
139 mutations observed here may mediate AMP susceptibility via derepression of the *S. aureus* *ywo*
140 ortholog.

141

142 Iseganan resistance was associated with an identical 5-bp deletion in the extreme 3' end of the *yjbH*
143 gene in each of two strains from independent iseganan-selected lines (Table 1). YjbH controls the
144 disulfide stress response by binding to the oxidative burst-specific transcriptional regulator Spx, and
145 thereby controlling its degradation by the ClpXP protease³⁰, a role which is conserved in *Bacillus*
146 *subtilis*³¹. YjbH also modulates β -lactam susceptibility, with deletion mutants showing moderate
147 resistance to various β -lactams but not to the glycopeptide antibiotic vancomycin³⁰. The precise
148 mechanism by which YjbH modulates susceptibility is unknown but is proposed to be a

149 consequence of upregulation of PBP4 which results in increased peptidoglycan cross-linking³⁰.

150

151 There were no common mutations identified in the genomes of three strains which were selected
152 with a 1:1 wt/wt combination of pexiganan and melittin (Table 1). However there were
153 commonalities with strains that were selected with either melittin or pexiganan. A single missense
154 mutation was identified in strain PGML3.2 which substitutes a conserved threonine residue in the
155 winged helix-turn-helix DNA binding domain of YtrA (note that *ytrA* nonsense mutations were
156 identified in 2 melittin-resistant strains described above). Similarly, a single nonsense mutation was
157 identified in strain PGML5.1 in *mgt* (also mutated in 1 pexiganan-resistant strain described above).
158 In contrast, three missense mutations were identified in the genome of a second pexiganan-melittin-
159 selected strain. Interestingly this included *dak2* which encodes a dihydroxyacetone kinase
160 responsible for incorporation of diphosphatidylglycerol into the cell membrane³². *dak2* was
161 previously identified in a high throughput mutant screen for loci affecting susceptibility to the
162 anionic human AMP dermcidin in *S. aureus*³². Mutations affecting the non-essential C-terminal
163 DegV superfamily domain of Dak2 result in altered membrane phospholipid composition and
164 decreased binding and activity of dermcidin but not of the cationic human AMPs LL-37 or human
165 β -defensin-3³². Given this lack of cross-resistance to cationic AMPs in *dak2* mutants, Dak2-
166 mediated susceptibility was thought to be specific to anionic AMPs such as dermcidin³². It is
167 therefore surprising to find *dak2* mutation in response to selection with a combination of the
168 cationic AMPs melittin and pexiganan. Further evidence of the role of Dak2 in susceptibility to
169 pexiganan and melittin was shown by increased susceptibility to both AMPs by a *dak2* transposon
170 mutant (Table 1, Table S3).

171

172 Mutations identified in streptomycin-selected strains mostly occurred in genes with known roles in
173 streptomycin susceptibility (Table 1). Frameshift mutations in *gidB*, which encodes a 16S rRNA-
174 specific 7-methylguanosine methyltransferase, were identified in all three streptomycin-selected
175 strains (Table 1). In each case, the frameshift occurs within the region encoding the GidB
176 methyltransferase domain. Mutations in *gidB* (*rsmG*) are associated with low-level streptomycin
177 resistance in several species of bacteria including *S. aureus*³³⁻³⁶ and it is speculated that loss of 16S
178 methylation lowers the binding affinity of streptomycin thus conferring the resistance phenotype³⁵.
179 Here, a *gidB* transposon mutant was found to be 4-fold less susceptible to streptomycin (Table 1,
180 Table S3). Two further mutations were identified which potentially affect ribosomal RNA. A 124-kb
181 region containing an entire *rrn* operon appears to have been duplicated in a strain STR3.2 whereas
182 strain STR1.1 carries a non-synonymous substitution in the essential gene encoding NusA, which
183 acts as an antiterminator for 16S rRNA transcription, as well as a chaperone for 16S rRNA folding³⁷
184 (Table S2). Mutations were also identified in the glycerol kinase gene *glpK* in two strains (Table 1)
185 however a transposon insertion did not detectably alter streptomycin susceptibility (Table 1, Table
186 S3).

187

188 Numerous studies have utilized mutant screens to identify loci that determine AMP susceptibility^{32,38}
189 but with the exception of a single study⁴, there is a dearth of data concerning the genomic changes
190 which accompany experimental evolution of AMP resistance. Here, genome sequencing of strains
191 isolated from independently replicated AMP selection lines identified mutations associated with
192 AMP resistance evolution and showed that each AMP selected for mutations at distinct loci. These
193 mutations affected genes with known roles in susceptibility to AMPs and/or cell-wall-active
194 antibiotics, as well as cell wall stress stimulon genes. All cationic AMPs used here form toroidal
195 pores, yet there was little evidence of cross resistance or for mutations that were common across all
196 AMP-selected strains. There is limited evidence of AMP-specific responses. For example, the
197 staphylococcal virulence factor MprF determines susceptibility towards protegrins (e.g. iseganan)
198 but has little effect on magainin (pexiganan analog) or melittin susceptibility³⁹. Also, little is known

199 about AMP interactions with other constituents of the cell membrane and whether these may
200 contribute to the specificity observed here. A small number of mutations occurred in genes with no
201 known role in antimicrobial susceptibility, such as the gene encoding the RluD-like pseudouridylate
202 synthase, and may represent compensatory adaptations that warrant further study. Furthermore,
203 mutations in the *walR* gene such as that described here are known to increase multidrug resistance
204 and to arise during clinical *S. aureus* infections²⁶. This is consistent with the notion that the
205 evolution of resistance to AMPs may compromise host defences against infection^{5,40}.

206

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214

215 **Transparency declarations**

216 None to declare.

217

218 **Supplementary data**

219 Table S1. MICs for various antimicrobials against 18 strains of *S. aureus*.

220 Table S2. Summary of all mutations.

221 Table S3. MICs for various antimicrobials against transposon insertion mutants of *S. aureus* strain

222 USA300_FPR3757 from the Nebraska Transposon Mutant Library.

223 Table S4. Details of antimicrobial peptides used.

224

225 **References**

226 1. Zasloff M. Antimicrobial peptides of multicellular organisms. *Nature* 2002; **415**: 389–95.

227 2. Nguyen LT, Haney EF, Vogel HJ. The expanding scope of antimicrobial peptide structures and
228 their modes of action. *Trends Biotechnol* 2011; **29**: 464–72.

229 3. Perron GG, Zasloff M, Bell G. Experimental evolution of resistance to an antimicrobial peptide.
230 *Proc Biol Sci* 2006; **273**: 251–6.

231 4. Lofton H, Pr anting M, Thulin E, Andersson DI. Mechanisms and fitness costs of resistance to
232 antimicrobial peptides LL-37, CNY100HL and wheat germ histones. *PLoS One* 2013; **8**: e68875.

233 5. Habets MGJL, Brockhurst MA. Therapeutic antimicrobial peptides may compromise natural
234 immunity. *Biol Lett* 2012; **8**: 416–8.

235 6. Dobson AJ, Purves J, Kamysz W, Rolff J. Comparing Selection on *S. aureus* between
236 Antimicrobial Peptides and Common Antibiotics de Lencastre H, ed. *PLoS One* 2013; **8**: e76521.

237 7. Dobson AJ, Purves J, Rolff J. Increased survival of experimentally evolved antimicrobial
238 peptide-resistant *Staphylococcus aureus* in an animal host. *Evol Appl* 2014; **7**: 905–12.

239 8. Johnston PR, Makarova O, Rolff J. Inducible defenses stay up late: temporal patterns of immune
240 gene expression in *Tenebrio molitor*. *G3 (Bethesda)* 2014; **4**: 947–55.

241 9. Shaw LN, Aish J, Davenport JE, *et al.* Investigations into σ B-mediated regulatory pathways
242 governing extracellular virulence determinant production in *Staphylococcus aureus*. *J Bacteriol*
243 2006; **188**: 6070–80.

244 10. Andrews JM. Determination of minimum inhibitory concentrations. *J Antimicrob Chemother*
245 2001; **48 Suppl 1**: 5–16.

246 11. O'Neill AJ. *Staphylococcus aureus* SH1000 and 8325-4: Comparative genome sequences of key
247 laboratory strains in staphylococcal research. *Lett Appl Microbiol* 2010; **51**: 358–61.

248 12. B ak KT, Frees D, Renzoni A, *et al.* Genetic variation in the *Staphylococcus aureus* 8325 strain

- 249 lineage revealed by whole-genome sequencing. *PLoS One* 2013; **8**: e77122.
- 250 13. Bankevich A, Nurk S, Antipov D, *et al.* SPAdes: A New Genome Assembly Algorithm and Its
251 Applications to Single-Cell Sequencing. *J Comput Biol* 2012; **19**: 455–77.
- 252 14. Li H, Handsaker B, Wysoker A, *et al.* The Sequence Alignment/Map format and SAMtools.
253 *Bioinformatics* 2009; **25**: 2078–9.
- 254 15. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform.
255 *Bioinformatics* 2009; **25**: 1754–60.
- 256 16. Garrison E, Marth G. Haplotype-based variant detection from short-read sequencing. *arXiv*
257 2012; **1207.3907**.
- 258 17. Thorvaldsdóttir H, Robinson JT, Mesirov JP. Integrative Genomics Viewer (IGV): high-
259 performance genomics data visualization and exploration. *Brief Bioinform* 2013; **14**: 178–92.
- 260 18. Deatherage DE, Barrick JE. Identification of mutations in laboratory-evolved microbes from
261 next-generation sequencing data using breseq. *Methods Mol Biol* 2014; **1151**: 165–88.
- 262 19. Fey PD, Endres JL, Yajjala VK, *et al.* A genetic resource for rapid and comprehensive
263 phenotype screening of nonessential *Staphylococcus aureus* genes. *MBio* 2013; **4**: 1–8.
- 264 20. McCallum N, Hinds J, Ender M, Berger-Bächli B, Stutzmann Meier P. Transcriptional profiling
265 of XdrA, a new regulator of *spa* transcription in *Staphylococcus aureus*. *J Bacteriol* 2010; **192**:
266 5151–64.
- 267 21. Ender M, Berger-Bächli B, McCallum N. A novel DNA-binding protein modulating methicillin
268 resistance in *Staphylococcus aureus*. *BMC Microbiol* 2009; **9**: 15.
- 269 22. Wang Q, Peery R. Identification and characterization of a monofunctional glycosyltransferase
270 from *Staphylococcus aureus*. *J Bacteriol* 2001; **183**: 4779–85.
- 271 23. Lovering AL, Safadi SS, Strynadka NCJ. Structural perspective of peptidoglycan biosynthesis
272 and assembly. *Annu Rev Biochem* 2012; **81**: 451–78.
- 273 24. Rebets Y, Lupoli T, Qiao Y, *et al.* Moenomycin resistance mutations in *Staphylococcus aureus*
274 reduce peptidoglycan chain length and cause aberrant cell division. *ACS Chem Biol* 2014; **9**: 459–
275 67.
- 276 25. Dubrac S, Bisicchia P, Devine KM, Msadek T. A matter of life and death: cell wall homeostasis
277 and the WalKR (YycGF) essential signal transduction pathway. *Mol Microbiol* 2008; **70**: 1307–22.
- 278 26. Howden BP, McEvoy CRE, Allen DL, *et al.* Evolution of multidrug resistance during
279 *Staphylococcus aureus* infection involves mutation of the essential two component regulator
280 WalKR. *PLoS Pathog* 2011; **7**: e1002359.
- 281 27. Salzberg LI, Luo Y, Hachmann A-B, Mascher T, Helmann JD. The *Bacillus subtilis* GntR family
282 repressor YtrA responds to cell wall antibiotics. *J Bacteriol* 2011; **193**: 5793–801.
- 283 28. Li M, Cha DJ, Lai Y, Villaruz AE, Sturdevant DE, Otto M. The antimicrobial peptide-sensing
284 system *aps* of *Staphylococcus aureus*. *Mol Microbiol* 2007; **66**: 1136–47.
- 285 29. Blake KL, O’Neill AJ. Transposon library screening for identification of genetic loci
286 participating in intrinsic susceptibility and acquired resistance to antistaphylococcal agents. *J*
287 *Antimicrob Chemother* 2013; **68**: 12–6.
- 288 30. Göhring N, Fedtke I, Xia G, *et al.* New role of the disulfide stress effector YjbH in β -lactam
289 susceptibility of *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2011; **55**: 5452–8.
- 290 31. Larsson JT, Rogstam A, von Wachenfeldt C. YjbH is a novel negative effector of the disulfide
291 stress regulator, Spx, in *Bacillus subtilis*. *Mol Microbiol* 2007; **66**: 669–84.
- 292 32. Li M, Rigby K, Lai Y, *et al.* *Staphylococcus aureus* mutant screen reveals interaction of the
293 human antimicrobial peptide dermcidin with membrane phospholipids. *Antimicrob Agents*
294 *Chemother* 2009; **53**: 4200–10.
- 295 33. Mikheil DM, Shippy DC, Eakley NM, Okwumabua OE, Fadl AA. Deletion of gene encoding
296 methyltransferase (*gidB*) confers high-level antimicrobial resistance in *Salmonella*. *J Antibiot*
297 (*Tokyo*) 2012; **65**: 185–92.
- 298 34. Verma JS, Gupta Y, Nair D, *et al.* Evaluation of *gidB* alterations responsible for streptomycin

- 299 resistance in *Mycobacterium tuberculosis*. *J Antimicrob Chemother* 2014; 1–7.
- 300 35. Wong SY, Lee JS, Kwak HK, Via LE, Boshoff HIM, Barry CE. Mutations in *gidB* confer low-
- 301 level streptomycin resistance in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2011;
- 302 **55**: 2515–22.
- 303 36. Okamoto S, Tamaru A, Nakajima C, *et al.* Loss of a conserved 7-methylguanosine modification
- 304 in 16S rRNA confers low-level streptomycin resistance in bacteria. *Mol Microbiol* 2007; **63**: 1096–
- 305 106.
- 306 37. Bubunenko M, Court DL, Al Refaii A, *et al.* Nus transcription elongation factors and RNase III
- 307 modulate small ribosome subunit biogenesis in *Escherichia coli*. *Mol Microbiol* 2013; **87**: 382–93.
- 308 38. Peschel A, Otto M, Jack RW, Kalbacher H, Jung G, Götz F. Inactivation of the *dlt* operon in
- 309 *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides.
- 310 *J Biol Chem* 1999; **274**: 8405–10.
- 311 39. Peschel A, Jack RW, Otto M, *et al.* *Staphylococcus aureus* resistance to human defensins and
- 312 evasion of neutrophil killing via the novel virulence factor MprF is based on modification of
- 313 membrane lipids with l-lysine. *J Exp Med* 2001; **193**: 1067–76.
- 314 40. Bell G, Gouyon P. Arming the enemy: the evolution of resistance to self-proteins. *Microbiology*
- 315 2003; **149**: 1367–75.
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349 **Table 1.** Mutations identified in strains selected for resistance to different antimicrobials.
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Selection	No. of strains ^a	Gene	Function	Locus tag ^b	Susceptibility of Tn mutant ^c
IG	2	<i>yjbH</i>	Disulfide stress response	SAOUHSC_00938	not tested
ML	1	<i>walR (yycG)</i>	Cell envelope biogenesis	SAOUHSC_00020	not tested
ML	3	<i>rluA</i>	Pseudouridine synthase	SAOUHSC_00944	unchanged
ML/PGML	3(2ML/1PGL)	<i>ytrA</i> ortholog	Cell wall stimulon	SAOUHSC_02155	not tested
PG	1	<i>wcaG</i>	Nucleoside-diphosphate-sugar epimerase	SAOUHSC_00664	unchanged
PG	2	<i>xdrA</i>	Xenobiotic response element	SAOUHSC_01979	decreased
PG	2(1PG/1PGL)	<i>mgt (sgtB)</i>	Cell wall stimulon	SAOUHSC_02012	decreased
PGML	1	<i>hpr</i>	Carbohydrate transport	SAOUHSC_01028	not tested
PGML	1	<i>dak2</i>	Cell envelope biogenesis	SAOUHSC_01193	increased
PGML	1	<i>putA (fadM)</i>	Amino acid metabolism	SAOUHSC_01884	unchanged
STR	1	<i>nusA</i>	Transcription antitermination	SAOUHSC_01243	not tested
STR	2	<i>glpK</i>	Glycerol kinase	SAOUHSC_01276	unchanged
STR	1	<i>rrn</i> operons	Ribosome biogenesis	124-kb <i>rrn</i> region	not tested
STR	3	<i>gidB (rsmG)</i>	Ribosome biogenesis	SAOUHSC_03051	decreased

351 ^a Number of strains with a mutation in a given gene.

352 ^b Identifier in *Staphylococcus aureus* NCTC 8325 reference genome.

353 ^c Susceptibility of transposon insertion mutants from the Nebraska Transposon Mutant Library to
354 the cognate selective agent. Not tested, transposon mutant not available. See Table S3 for full
355 details.

356 IG, iseganan; ML, melittin; PG, pexiganan; PGML, 1:1 wt/wt combination of melittin and
357 pexiganan; STR, streptomycin. See Table S4 for further details on AMPs used.

TABLE S1. MICs for various antimicrobials against 18 strains of *S. aureus*.

Strain	MIC(ug/ml) ^a				
	Melittin	Pexiganan	Pex-Mel ^b	Streptomycin	Vancomycin
JLA513	8	8	8	4	2
IG1.2	4	8	8	4	2
IG2.1	4	8	8	4	2
ML1.1	32	8	32	8	4
ML4.2	32	8	16	4	2
ML5.2	32	16	16	4	2
PG1.1	8	16	8	4	2
PG2.2	4	16	8	4	2
PG4.2	4	16	8	8	2
PGML3.2	16	16	16	2	2
PGML4.4	8	32	16	4	2
PGML5.1	8	16	16	2	2
STR1.1	8	8	8	32	2
STR2.2	8	8	8	>64	2
STR3.2	8	16	8	>64	2
Uns1.1	4	4	4	4	2
Uns3.4	4	4	4	4	2
Uns4.2	8	8	8	8	2

^aMIC, minimum antimicrobial concentration necessary to inhibit the growth of *S. aureus*.

^bEqual quantities of pexiganan and melittin.

ML, melittin; PG, pexiganan; PGML, 1:1 wt/wt combination of melittin and pexiganan; STR, streptomycin; Uns, unselected control strain.

TABLE S2. Summary of all mutations.

Strain	Mutation	Locus tag ^a	Annotation	Function
IG1.2	p.S266IfsX45	SAOUHSC_00938	<i>yjbH</i>	Disulfide stress response
IG2.1	p.S266IfsX45	SAOUHSC_00938	<i>yjbH</i>	Disulfide stress response
ML1.1	p.L93I	SAOUHSC_00020	<i>walR/yycG</i>	Cell envelope biogenesis; response regulator
ML1.1	p.A35T	SAOUHSC_00944	<i>rluD</i> -like	Pseudouridylate synthase
ML1.1	g.2101984_2101985insT	SAOUHSC_02270	intergenic	-
ML4.2	p.A35D	SAOUHSC_00944	<i>rluD</i> -like	Pseudouridylate synthase
ML4.2	p.L5X	SAOUHSC_02155	<i>ytrA</i>	Cell wall stimulon; repressor
ML5.2	p.A35D	SAOUHSC_00944	<i>rluD</i> -like	Pseudouridylate synthase
ML5.2	p.L5X	SAOUHSC_02155	<i>ytrA</i>	Cell wall stimulon; repressor
PG1.1	p.P39XfsX3	SAOUHSC_02012	<i>mgt/sgtB</i>	Cell wall stimulon; peptidoglycan glycosyltransferase
PG2.2	p.Q40RfsX24	SAOUHSC_01979	<i>xdrA</i>	Xenobiotic response element
PG4.2	p.M280V	SAOUHSC_00664	<i>wcaG</i>	Nucleoside-diphosphate-sugar epimerase; oxidoreductase
PG4.2	p.Q30X	SAOUHSC_01979	<i>xdrA</i>	Xenobiotic response element
PGML3.2	p.T74A	SAOUHSC_02155	<i>ytrA</i>	Cell wall stimulon; repressor
PGML4.4	p.A16D	SAOUHSC_01028	<i>hpr</i>	Carbohydrate transport
PGML4.4	p.G341D	SAOUHSC_01193	<i>dak2</i>	Cell envelope biogenesis; dihydroxyacetone kinase
PGML4.4	p.S138I	SAOUHSC_01884	<i>putA/fadM</i>	Amino acid metabolism; proline dehydrogenase
PGML5.1	p.Q251X	SAOUHSC_02012	<i>mgt/sgtB</i>	Cell wall stimulon; peptidoglycan glycosyltransferase
STR1.1	p.A227E	SAOUHSC_01243	<i>nusA</i>	Transcription antitermination; antiterminator
STR1.1	p.H87L	SAOUHSC_02727	NC_007795.1	Hypothetical protein; peptidase
STR1.1	p.R218DfsX75	SAOUHSC_03051	<i>gidB/rsmG</i>	Ribosome biogenesis; 16S rRNA methyltransferase
STR2.2	c.63A>G ^b	SAOUHSC_00489	<i>folP</i>	Dihydropteroate synthase
STR2.2	g.1090526_1090533del	intergenic	-	-
STR2.2	p.A332E	SAOUHSC_01276	<i>glpK</i>	Glycerolipid metabolism; glycerol kinase
STR2.2	p.S115EfsX12	SAOUHSC_03051	<i>gidB/rsmG</i>	Ribosome biogenesis; 16S rRNA methyltransferase
STR3.2	p.G251X	SAOUHSC_01276	<i>glpK</i>	Glycerolipid metabolism; glycerol kinase
STR3.2	g.2122437_2246248dup	segmental duplication	-	Encodes rRNA and ribosomal protein genes
STR3.2	p.S115EfsX12	SAOUHSC_03051	<i>gidB/rsmG</i>	Ribosome biogenesis; 16S rRNA methyltransferase

^aIdentifier in *Staphylococcus aureus* NCTC 8325 reference genome.

^bSynonymous.

IG, iseganan; ML, melittin; PG, pexiganan; PGML, 1:1 wt/wt combination of melittin and pexiganan; STR, streptomycin.

TABLE S3. MICs for various antimicrobials against transposon insertion mutants of *Staphylococcus aureus* strain USA300_FPR3757 from the Nebraska Transposon Mutant Library.

Strain	Locus tag ^b	Annotation	MIC(ug/ml) ^a			
			Melittin	Pexiganan	Pex-Mel ^c	Streptomycin
USA300	-	-	8	16	16	4
NE229	SAUSA300_1119	<i>dak2</i>	8	8	8	4
NE239	SAUSA300_1711	<i>putA (fadM)</i>	8	16	16	4
NE249	SAUSA300_2644	<i>gidB (rsmG)</i>	8	16	16	16
NE467	SAUSA300_0644	<i>wcaG</i>	8	16	16	4
NE596	SAUSA300_1855	<i>mgt (sgtB)</i>	8	32	16	4
NE822	SAUSA300_0909	<i>rluD</i> -like	8	16	16	4
NE896	SAUSA300_0903	<i>yjbH</i>	8	16	16	4
NE1023	SAUSA300_0984	<i>ptsI</i>	8	16	16	4
NE1445	SAUSA300_1797	<i>xdrA</i>	8	32	8	4
NE1587	SAUSA300_1192	<i>glpK</i>	8	16	16	4
NE1908 ^d	SAUSA300_1911	ABC transporter	8	16	16	4
NE1188 ^d	SAUSA300_1912	ABC transporter	8	16	16	4

^aMIC(minimum inhibitory concentration), minimum antimicrobial concentration necessary to inhibit the growth of *S. aureus*.

^bIdentifier in *S. aureus* USA300_FPR3757 reference genome.

^cEqual quantities of pexiganan and melittin.

^dInsertions in the *ytr* operon downstream of *ytrA*. Insertions in *ytrA* are not present in the Nebraska Transposon Mutant Library.

ML, melittin; PG, pexiganan; PGML, 1:1 wt/wt combination of melittin and pexiganan; STR, streptomycin.

TABLE S4. Details of antimicrobial peptides used.

AMP	Length (aa)	Net charge	Origin	Reference
Iseganan	17	+	Pig	Mosca et al. (2000)
Melittin	26	+	Honey bee	Raghuraman and Chattopadhyay (2007)
Pexiganan	22	+	Frog	Ge et al. (1999)

References

Mosca, D.; Hurst, M.; So, W. *Antimicrobial agents and chemotherapy* **2000**, *44*, 1803–1808.

Raghuraman, H.; Chattopadhyay, A. *Bioscience reports* **2007**, *27*, 189–223.

Ge, Y.; Macdonald, D. L.; Holroyd, K. J.; Thornsberry, C.; Wexler, H.; Zasloff, M.; Ge, Y.; Donald, D. L. M. A. C.; Holroyd, K. J.; Thornsberry, C.; Wexler, H.; Zasloff, M. *Antimicrobial agents and chemotherapy* **1999**, *43*, 782–788.