1 **Supplemental Information Inventory**

2 Title: Evolution of resistance to a last-resort antibiotic in *Staphyloccocus aureus* via bacterial

3 competition

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6 Supplemental Information of this manuscript contains:

7 - 7 Supplemental figures (from S1 to S7)

8 Supplemental figure 1: In relation to main figure 1.

9 Supplemental figure 2: In relation to main figure 1.

10 Supplemental figure 3: In relation to main figures 2 and 3.

11 Supplemental figure 4: In relation to main figure 3.

12 Supplemental figure 5: In relation to main figures 4 and 5.

13 Supplemental figure 6: In relation to main figures 5, 6 and 7.

14 Supplemental figure 7: In relation to main figure 5 and 7.

15 - 7 Supplemental tables (from S1 to S7)

16 Supplemental table S1: In relation to main figures 1 to 7.

17 Supplemental table S2: In relation to main figures 1, 3, 4, 5, and 7.

18 Supplemental table S3: In relation to main figure 2.

19 Supplemental table S4: In relation to main figures 2 and 4.

20 Supplemental table S5: In relation to main figures 2 and 5.

21 Supplemental table S6: In relation to main figure 5.

22 Supplemental table S7: In relation to main figures 6 and 7.

23 - An extended section of Experimental Procedures: In relation to the section of

24 Experimental Procedures of the main text.

25 - A section of Supplemental references: In relation to the main References section.

26

27 <u>**Supplemental Information**</u>

28 **Supplemental Figure Legends**

29 **Supplemental Figure S1 (Related to main figure 1): *Staphylococcus aureus* develops**
30 **robust multicellular aggregates in magnesium-supplemented growth medium (TSBMg**
31 **medium). (A)** Top-view pictures showing the progression of *S. aureus* microbial communities
32 when they grow in TSB and TSBMg growth media. The microbial communities were incubated at
33 37°C for five days. Scale bar is 1 mm. **(B)** (i) *S. aureus* growing in TSBMg medium developed
34 robust microbial aggregates and their dispersion resulted only with the assistance of blunt
35 objects (ii) Agr and SigB influence biofilm formation in *S. aureus* and antagonistically influence
36 the architecture of the microbial communities. (iii) Diversified communities of *S. aureus* are
37 robust communities. The dispersion of the microbial aggregates resulted only with the assistance
38 of blunt objects. Scale bar is 1 mm. **(C)** Multicellular aggregates of different staphylococcal
39 strains grown in TSBMg medium at 37°C during five days. It is possible to visualize the
40 expansion of discrete sectors in several strains. Scale bar is 1 mm. **(D)** Top-view pictures
41 showing the development of three independent diversification experiments of the multi-drug
42 resistance CA-MRSA (Sc01 derivate) strain. The strains were grown TSBMg medium at 37°C
43 during five days. Scale bar is 1 mm. **(E)** Strain diversification occurred in magnesium-
44 supplemented growth medium (TSBMg) and not in other tested growth media. Colonies were
45 grown for five days at 37°C. TSB+ Sheep Blood 5% medium (Hebert and Hancock, 1985), TSB +
46 Horse serum 10% (Orth et al., 1971) and Brain-Heart Infusion medium (Yoshida et al., 1969).
47 These growth media are routinely used to recreate acute staphylococcal infections in laboratory
48 conditions. TSB+NaCl 500Mm+glucose 0.5% medium is conventionally used to recreate
49 staphylococcal biofilm formation in laboratory conditions (i.e. chronic infections) (Beenken et al.,
50 2003; Gotz, 2002). Synthetic nasal medium is used to recreate nasal colonization of
51 *staphylococcus aureus,* in which *S. aureus* is not virulent (Krismer et al., 2014). We also tested

52 diverse growth media in which TSB was supplemented with several cations different from Mg^{2+}.
53 The frequency of W and Y strain diversification was tested after five days of incubation. Samples
54 were dilution plated and examined for colony heterogeneity based on differences in
55 pigmentation. To test the occurrence of VISA-like phenotypes, 88 colonies were randomly
56 selected and grown in liquid TSB medium conditioned with vancomycin 4 µg/ml (See figure S2).
57 Results are shown on the left side of the pictures. Scale bar is 1 mm.
58
59 **Supplemental Figure S2 (Related to main figure 1): Diversification of W and Y strains in**
60 **TSBMg medium. (A)** Quantification of the frequency of W and Y strains at different time points
61 during the course of a diversification experiment. We assayed biofilm formation on solid agar and
62 liquid culture using TSB and TSBMg media. We took samples at different time points during
63 growth. Samples were dilution plated and examined for colony heterogeneity based on
64 differences in the pigmentation of the resultant colonies. To perform these experiments, the
65 inoculum was plated directly from the stock glycerol and incubated in TSB agar for 12h at 37°C.
66 A sample of the inoculum was also examined for colony heterogeneity. (i) Panel shows a sector
67 of the dilution plate experiments at different incubation time points until reaching five days
68 incubation period (three days for liquid cultures). (ii) 88 colonies were randomly selected at each
69 time point and tested for intermediate-resistance to vancomycin by allowing them to grow in
70 liquid TSB cultures in the presence of vancomycin 4 µg/ml. ii panel shows the 96-well plate of the
71 last time point (120h for agar cultures and 72h for liquid cultures). The upper row of the well plate
72 contains several controls. O, W and Y strains grew in triplicate. Only Y grew in the presence of
73 vancomycin. An extra well with no inoculation (-) was placed to control growth contamination.
74 The VISA strain (Mu50) grew in the two last wells as a positive control (+). (iii) Quantitative
75 analysis of strain diversification based on the results of the assays that are shown in i and ii
76 panels. **(B)** qRT-PCR analysis of the expression of staphyloxanthin production (*crt* gene), biofilm
77 formation (*ica* and *spa* genes) and hemolytic toxins (*hla* gene) in O, W and Y strains obtained

78 from solid agar biofilm formation assay and liquid biofilm formation assay. *rpoZ* is used as control
79 gene expression to normalize results.
80
81 **Supplemental Figure S3 (Related to main figure 2 and 3): (A) Correlation of genome-wide**
82 **gene expression levels quantified by RNA-seq.** Left panel correlates expression levels of O to
83 W strains. Centre panel correlates expression levels of O to Y strains. Right panel shows
84 quantification of the functional classification of over- and under-expressed genes of W in relation
85 to O strain (upper and bottom bars of the panel, respectively). Functional classification is MOB:
86 Mobile and extrachromosomal element functions, COF: Biosynthesis of cofactors, prosthetic
87 groups, and carriers, AMI: Amino acid biosynthesis. PUR: Purines, pyrimidines, nucleosides, and
88 nucleotides, DNA: DNA metabolism, FAT: Protein fate, CEL: Cellular processes, ENE: Energy
89 metabolism, REG: Regulatory functions, SIG: Signal transduction, HYP: Hypothetical proteins,
90 TRA: Transport and binding proteins ENV: Cell envelope, UNK: Unknown function. **(B)** Read
91 coverage of 3 control genes in comparative RNA-seq in H, W and Y transcriptomic data. i, ii and
92 iii panels present the expression level of 5S rRNA, SAUSA300_0777(cold-shock protein) and
93 gmk-rpoz operon (guanilate kinase, essential for recycling GMP). **(C)** Generation of a *Bacillus*
94 *subtilis* transducer strain that senses AIP signal from *S. aureus*. The transducer strain was
95 generated using the signaling transduction pathway that triggers the differentiation of the
96 subpopulation of surfactin producers in *B. subtilis* (Lopez et al., 2009; Nakano et al., 1991). The
97 self-produced pheromone signal ComX is sensed by the sensor kinase ComP and this, in turn,
98 triggers the ComA bistable switch, which ultimately leads to the activation of surfactin expression
99 in only a fraction of cells (Lopez et al., 2009; Magnuson et al., 1994). Fluorescence microscopy
100 analysis evidenced that the fluorescence signal of a strain labeled with a transcriptional reported
101 for surfactin expression (P_{srf}-*yfp*) was activated in only a subpopulation of cells. This
102 fluorescence signal was dependent on the extracellular presence of ComX because the

103 expression of the fluorescence reporter was not detected in the Δ*comX* P_{srf}-*yfp* strain. Scale bar
104 is 10 μm. **(D)** We used the Δ*comX* P_{srf}-*yfp* strain to replace the ComP sensor kinase by a
105 chimeric kinase that contains the sensor domain of AgrC and the kinase domain of ComP
106 kinases. A strain of *B. subtilis* harboring this chimeric kinase is able to detect the presence of the
107 staphylococcal pheromone AIP in the extracellular milieu and respond to it by activating the
108 differentiation pathway of surfactin producers. **(E)** Calibration of the transducer strain in response
109 to different concentrations of AIP signal using fluorescence microscopy. Control with no AIP
110 added to the cells represents the fluorescence background. Scale bar is 4 μm. AIP was
111 previously purified according to the methodology published in (P et al., 2001). **(F)** Calibration of
112 the transducer strain in response to different concentrations of AIP signal using flow cytometry
113 (n=50,000). The grey profile shows control with no AIP added to the cells. **(G)** Fluorescence
114 microscopy field of a culture of the transducer strain in the presence of AIP (dilution 1:10). The
115 size of the subpopulation of surfactin producers differentiates in a direct function to the
116 concentration of AIP in the extracellular millieu. Scale bar is 10 μm.

117

118 **Supplemental Figure S4 (Related to main figure 3): The W strain showed low SigB activity.**
119 **(A)** (i) Scheme of the four-gene long *rsbU*-*sigB* operon of *S. aureus*. (ii) RsbW sequesters σ^B and
120 prevents it from biding to the RNA polymerase. In this state, the kinase activity of RsbW
121 phosphorylates and inactivates RsbV. The active, dephosphorylated form of RsbV attacks the
122 RsbW–σ^B complex to liberate σ^B (Miyazaki et al., 1999; Palma and Cheung, 2001). Mutations in
123 *rsbW* and σ^B detected in W strains are detailed. **(B)** Sequence alignment of the active site of the
124 RsbW from different bacterial species. G1 and G2 boxes are conserved motifs in all ATPases
125 and histidine kinases. The final side of the ATP binding pocket couples ATP binding to function-
126 specific interdomain associations. The integrity of this region is essential for the binding and the
127 phosphorylative activity of this enzyme (King-Scott et al., 2011). It is reported that mutations

128 similar to D105N identified in W strain are able to inactivate similar enzymes. A second mutation
129 N62K was also found in some W strains but the region where the mutation localizes is not
130 conserved in other RsbW proteins and therefore, the functionality of this region is unknown. **(C)**
131 Western blot analysis comparing the expression of SigB and RsbW proteins in W cell extracts of
132 O, W and Y strains using antibodies against SigB and RsbW. C- are the Δ*sigB* and Δ*rsbU*
133 defective mutants, respectively. C+ is a wild type strain in both experiments. SDS-PAGE are
134 shown as loading control. **(D)** Western blot analysis to detect production of SigB and RsbW
135 proteins in W cells previously treated and untreated with a protease inhibitor cocktail (Abcam®).
136 Treatment with protease inhibitors partially recovered the detection of SigB and RsbW. This is
137 consistent with the detection of transcription of the *sigB* operon that was detected in W strains
138 (See Table S4). Antibodies against SigB were used for protein detection. SDS-PAGE is shown
139 as loading control. **(E)** Semi-quantitative drop-collapse assay monitoring the concentration of
140 surfactants in the supernatants of distinct genetic backgrounds. Drop diameter represents drop
141 collapse (y axis) in control LB medium and WT, Δ*agr,* Δ*sigB* and Δ*agr* Δ*sigB* supernatants,
142 respectively. **(F)** Growth expansion of Muticellular communities of distinct strains in TSBMg for
143 five days at 37°C. The absence of *agr* prevents expansion. **(G)** The mutation detected in the
144 *rsbU-sigB* operon of W strain is responsible for the acquisition of a *sigB*-defective phenotype. A
145 SigB-deficient laboratory strain of *S. aureus* was complemented with the *rsbU-sigB* operon from
146 the O strain and W strains isolated from *in vitro* and *in vivo* experiments. The recovery of SigB
147 activity was measured in the resultant strains by monitoring staphyloxanthin production (Giachino
148 et al., 2001; Wieland et al., 1994), spreading (Tsompanidou et al., 2013; Tsompanidou et al.,
149 2011) and hemolytic activity (Herbert et al., 2010). **(H)** Complementation with the *rsbU-sigB*
150 operon from the O strain restored pigmentation and reduced the spreading and the hemolytic
151 activities in the SigB-deficient laboratory strain of *S. aureus*. However, complementation with the
152 *rsbU-sigB* operon from the W strains did not recovered staphyloxanthin production or reduced
153 the spreading and the hemolytic activity. Left column contains quantitative data of the assays

154 that were performed. Right column contains the battery of physiological assays that were
155 performed and the particular behavior of each one of the strains tested.
156
157 **Supplemental Figure S5 (Related to main figure 4 and 5): (A) VISA isolates do not have**
158 ***bsa* gene cluster.** MAUVE alignment of the genomic regions that contains the *bsa* gene cluster
159 in VISA and VSSA *Staphylococcus aureus* isolates. The reference strain for VSSA is LAC (top of
160 the panel). VISA isolates are JH1, JH9, Mu3 and Mu50 strains (this is the group of VISA isolates
161 with published genome sequences). We performed a non-progressive alignment using default
162 parameters with a seed-weight of 19. **(B)** Alignment of the whole genome sequences of 37 *S.*
163 *aureus* strains. The progressive alignment is represented in a guide tree that compares the level
164 of nucleotide similarity between genomes. A blue sector contains the region of the guide tree
165 with the group of VISA strains. A red sector contains the region of the guide tree with the
166 genomes of the strains that contains the *bsa* cluster. **(C)** Emergence of VISA-like strains from
167 Bsa+/Bsa- mixed communities. 1:1 mixtures of LAC/N315 (Bsa+/Bsa-) and LACΔ*bsa*/N315
168 (control Bsa-/Bsa-) were incubated for five days in TSB medium. After incubation, samples were
169 dilution plated in TSB agar medium supplemented with vancomycin 4 µg/ml. The colonies that
170 were able to grow in the presence of vancomycin were analyzed by PCR-amplification of the
171 *bsaA* gene or the chloramphenicol resistance cassette in the case of the LACΔ*bsa* strain. We
172 detected ≤ 1% of colonies rising on the plates supplemented with vancomycin. This effect occurs
173 in all strains tested and is attributed to the selective pressure on cells to become resistant to the
174 vancomycin in the selection plates, which leads to the possibility of producing false positives.
175 Therefore, this ≤ 1% of rising colonies was considered as background. (**D)** Subtle expression
176 changes in VISA strains. Read coverage of the transcription profile of several VISA-related
177 genes in Y strain and the parental O strain. *ssaA* encodes for an autolysin, *dltA* and *murZ* is
178 related to cell wall metabolism. **(E)** The mutations identified in the *vraRS, graRS* and *walKR*
179 operons of the Y strains confer a VISA-like phenotype. We complemented a laboratory strain of

180 *S. aureus* with the *vraRS, graRS* or *walKR* operons from the O strain or Y strains obtained from
181 *in vitro* and *in vivo* experiments. The acquisition of the VISA-like phenotype was monitored my
182 measuring the ability of the resultant strains to grow in the presence of vancomycin 4 µg/ml.
183 Strains complemented with the operons from the O strain did not acquire intermediate resistance
184 to vancomycin. However, the strains complemented with the operons from the Y strains resulted
185 in the acquisition of a VISA-like phenotype.
186
187 **Supplemental Figure S6 (Related to main figure 5, 6 and 7): Schematic representation of**
188 **the single-nucleotide point (SNP) mutations that confer a SigB-defective phenotype and**
189 **VISA phenotype to *S. aureus*. (A)** Schematic representation of the SNP mutations that confer a
190 SigB-defective phenotype to *S. aureus*. The SNPs that are represented in black and located on
191 the upper side of the operon scheme are those found in clinical isolates and reported in the
192 literature to confer a SigB-defective phenotype to *S. aureus*. (Herbert et al., 2010; Inose et al.,
193 2006; Karlsson-Kanth et al., 2006; McAdam et al., 2011; Savage et al., 2013). The SNPs that
194 were detected in our study are represented on the bottom side of the operon scheme. They are
195 colored in red if they were detected in *in vitro* experiment. They are colored in blue if they were
196 detected in *in vivo* experiments. The SNPs that were identified in this work and already reported
197 to confer a SigB-defective phenotype to *S. aureus* are highlighted with a colored frame. **(B)**
198 Schematic representation of the SNPs mutations that confer a VISA phenotype to *S. aureus.* The
199 GraRS, VraRS and WalKR operons are represented. The black-colored SNPs displayed on the
200 upper side of the operon scheme are those reported in the literature to confer a VISA phenotype
201 to *S. aureus*. (Hafer et al., 2012). The SNPs that were detected in our study are represented on
202 the bottom side of the operon scheme. They are colored in red if they were detected in the
203 diversification experiment *in vitro*. They are colored in blue if they were detected *in vivo.* They are
204 colored in green if they were identified in the *in vitro* experiments of artificial mixtures or

205 exogenous addition of Bsa. The SNPs that were identified in this work and already reported to
206 confer a VISA-phenotype are highlighted with a colored frame.
207
208 **Supplemental Figure S7 (Related to main figure 5 and 7): Mechanism for acquisition of**
209 **intermediate resistance to vancomycin of VISA strains. (A)** Vancomycin inhibits bacterial cell
210 wall synthesis by forming stable complexes with the C-terminal D-Ala-D-Ala residues of the Lipid
211 II-linked pentapeptide cell wall precursors in the nascent peptidoglycan (henceforth, simply
212 referred to as Lipid-II-AA). This blocks the access of the penicillin-binding proteins to their
213 substrates, which causes an inhibition of peptidoglycan crosslinking and thus, cell wall
214 biosynthesis (detail 1). Hence, Lipid-II-AA is the lethal target of vancomycin, which is positioned
215 at the tip of the division septum. Thus, cell wall synthesis in *S. aureus* exclusively occurs at the
216 division septum and not throughout the whole-cell wall (detail 2). This means that vancomycin
217 has to diffuse to the tip of the division septum to bind to Lipid-II-AA and inhibit cell wall synthesis
218 (detail 2). However, VISA strains show a thicker cell wall, which prevents the diffusion of
219 vancomycin to its site of action at the division septum and therefore, decreases the potency of
220 the drug. The thicker cell wall of VISA strains provides a barrier against vancomycin diffusion
221 using several blocking mechanisms (detail 3). First, the increase in cell wall thickness physically
222 hinders the penetration of vancomycin (Hiramatsu, 2001; Touhami et al., 2004). Second, the
223 thickened cell wall of VISA strains contains reduced crosslinking of peptidoglycan strands. This
224 increases the number of free D-Ala-D-Ala residues in the cell wall (Hiramatsu, 2001; Sieradzki et
225 al., 1999; Sieradzki and Tomasz, 2003), which are nonlethal binding sites for vancomycin, since
226 they do not participate in cell wall synthesis. These are able to retain vancomycin in the cell wall
227 and impede the penetration of the antibiotic to the division septum (Pereira et al., 2007). Third,
228 VISA strains showed reduced rates of autolysis and cell wall turnover, which reduces the number
229 of lethal targets of vancomycin (Cafiso et al., 2012; Cui et al., 2010). Fourth, VISA strains
230 contained an increased number of positive charges in the cell wall (Cui et al., 2010; Mishra et al.,

231 2009). This is due to the overexpression of the *dlt* operon, which is responsible for the D-alanil^{+}
232 esterification of the teichoic acids at the cell wall (Cao and Helmann, 2004; Perego et al., 1995).
233 Positive charges in the cell wall repel the binding of positively charged antimicrobial peptides,
234 such as vancomycin. Altogether, a model of intermediate resistance to vancomycin, commonly
235 referred to as “drug capture” (Sieradzki et al., 1999) or “clogging phenomenon” (Krismer et al.,
236 2014), has been proposed for VISA strains. The barrier to the diffusion of vancomycin provided
237 by a thickened cell wall prevents the binding of the antibiotic to its lethal target Lipid-II-AA at the
238 division septum, leading to the accumulation of vancomycin at the cell wall, where its action does
239 not affect cell viability **(B)** Fluorescence microscopy analysis showing the accumulation of
240 fluorescein-labeled vancomycin after diffusing through the cell wall of *S. aureus*. Fluorescein-
241 labeled vancomycin is referred to as Van-FL. The fluorescence signal is false-colored in green.
242 Left column shows the specific binding of Van-FL to the lethal target Lipid-II-AA. We have used
243 the method described by (Pereira et al., 2007) in which cells are grown to mid-exponential phase
244 in the presence of D-serine, leading to incorporation of this amino acid as the C-terminal residue
245 of the peptidoglycan muropeptides (Grohs et al., 2000; van Wageningen A, 1998). This leads to
246 a cell wall that is crosslinked with peptides containing D-Ala-D-Ser residues instead of D-Ala-D-
247 Ala, for which vancomycin has very low affinity. Subsequent growth in the presence of D-Ala for
248 15 min leads to the incorporation of this amino acid primarily into Lipid-II-AA and newly
249 synthesized and uncrosslinked chains of the peptidoglycan. Cells were then labeled with Van-FL,
250 which binds only the D-Ala-D-Ala-containing peptidoglycan, allowing the visualization of the new
251 cell wall synthesis sites. The fluorescence signal exclusively localizes at the division septum. Cell
252 membranes have been stained with Nile red for better visualization (false colored in red). Scale
253 bar is 1 μm. Right column shows the phenomenon of “drug capture” or “clogging phenomenon”
254 of vancomycin in *S. aureus* cells. Exponentially growing cells were treated with non-fluorescent
255 vancomycin (3 μg/ml) for 1 h before a quick exposure to an excess of Van-FL. Quick staining
256 with Van-FL enables the visualization of vancomycin accumulation at the cell wall and not at the

257 division septum. Cell membranes have been stained with Nile red for better visualization (false
258 colored in red). Scale bar is 1 µm **(C)** Quantitative analysis of the diffusion of fluorescein-labeled
259 vancomycin through the cell wall of O, W and Y cells. This is a version of the protocol published
260 by (Pereira et al., 2007). A total of 100 cells from each strain were monitored and classified into 3
261 categories according to their rate of Van-FL incorporation. Category 1 (C1): Cells showed higher
262 fluorescent signal associated with the cell wall than with the septum. Category 2 (C2): Cells
263 showed a uniform distribution of the fluorescent signal in the cell wall and in the septum.
264 Category 3 (C3): Cells showed higher fluorescent signal associated with the septum than with
265 the cell wall. Control experiment compares the diffusion of Van-FL in two isogenic VSSA/VISA
266 strains (N315/Mu50). As expected, the VISA strain showed lower fluorescence signal associated
267 with the division septum and higher fluorescence signal retained in the cell wall. A similar effect
268 is observed in Y cells in comparison to O and W cells. Comparison analysis has been performed
269 using Leica Application Software Analysis and FiJi. **(D)** Electronic micrographs of O, W and Y
270 cells, providing evidence that Y cells contained a thicker cell wall. Media and mean deviation of
271 the cell wall thickness is shown in the top of each panel (in nanometers). Scale bars are 0.3 µM.
272 **(D)** Cell wall thickness of several VISA strain in relation to their parental VSSA strains (Cui et al.,
273 2006; Howden et al., 2011; Reipert et al., 2003). Increase in cell wall thickness is approximately
274 1.4 fold.
275

276 **Supplementary Tables**

277 **Supplementary Table S1:** Strains and plasmids used in this study. Related to main figures 1-7.

DL99	*Escherichia coli* DH5α	(Reusch et al., 1986)
DL2	*Bacillus subtilis* 168	(Burkholder and Giles, 1947)
DL1	*Bacillus subtilis* NCIB3610	(Branda et al., 2001)
GK36	*S. aureus* HG003 [Laboratory strain corrected for Δ11-bp-*rsbU* deletion and *tcaR* mutation. The parental strains is NCTC8325]	(Herbert et al., 2010)
DL1603	*S. aureus* N315 [MRSA isolate from an airways infection (Japan)]	(Ito et al., 1999)

DL1120	*S. aureus* Newman [MSSA isolate from an osteomyelitis patient (UK)]	(Lipinski et al., 1967)
DL1217	*S. aureus* RN4220 [Laboratory strain. The parental strains is NCTC8325]	(Kornblum, 1990)
GK141	*S. aureus* LAC (TCH) [CA-MRSA isolate from a wirst abscess (USA)]	(McDougal et al., 2003)
CW58	*S. aureus* LAC (JE2)) [CA-MRSA isolate from a wirst abscess (USA)] (This is a plasmid-cured strain)	(Bae et al., 2004)
DL1479	*S. aureus* NewHG [Laboratory strain corrected for saeRS hyperactivation. The parental strains is Newman]	(Mainiero et al., 2010)
DL1125	*S. aureus* COL) [MRSA isolate from the air of a surgery room (UK)]	(Dyke, 1969)
DL1122	*S. aureus* Reynolds [MSSA isolate from a patient with bacteremia (USA)], Protoype serotype 5.	(Albus et al., 1991)
DL1480	*S. aureus* RN1 (also known as NCTC8325). [MRSA isolate from a patient with bacteremia (USA)]	(Novick, 1967)
DL1121	*S. aureus* Becker [MSSA isolate from a patient with bacteremia (USA)]. Protoype serotype 8.	(Sau and Lee, 1996)
DL1123	*S. aureus* MN8 [MSSA isolate from a menstrual toxic-shock syndrome patient (USA)]	(Yarwood and Schlievert, 2000)
DL1626	*S. aureus* 2330 [MRSA isolate from a patient with bacteremia (Germany)]	(Sader et al., 2006)
DL1127	*S. aureus* UAMS-1 [MSSA isolate from an osteomyelitis patient (USA)]	(Blevins et al., 2002)
DL1353	*S. aureus* SH1000 [Laboratory strain corrected for Δ11-bp-rsbU deletion. Its parental strains is NCTC8325]	(Horsburgh et al., 2002)
DL1607	*S. aureus* MT01 [MSSA isolate from the nose of a healthy host (JAPAN)]	(Tsai et al., 2011)
DL1627	*S. aureus* Mu50 [VISA strain isolated from a wound infection (Japan). Its parental strain is N315]	(Hiramatsu et al., 1997)
DL1129	*S. aureus* Sc-01) [CA-MRSA isolate from a hip wound infection (USA)]	(Beenken et al., 2003)
GK142	*S. aureus* Sc-01 derivative (Y strain)	This study
GK143	*S. aureus* Sc-01 derivative (O strain)	This study
GK144	*S. aureus* Sc-01 derivative (W strain)	This study
GK473	*S. aureus* Sc-01 derivative (YA strain)	This study
GK472	*S. aureus* Sc-01 derivative (YB strain)	This study
GK455	*S. aureus* Sc-01 derivative (Om1 strain)	This study
GK456	*S. aureus* Sc-01 derivative (Om2 strain)	This study
GK457	*S. aureus* Sc-01 derivative (Om3 strain)	This study
GK463	*S. aureus* Sc-01 derivative (Wm1 strain)	This study

GK464	*S. aureus* Sc-01 derivative (Wm2 strain)	This study
GK465	*S. aureus* Sc-01 derivative (Wm3 strain)	This study
GK469	*S. aureus* Sc-01 derivative (Ym1 strain)	This study
GK470	*S. aureus* Sc-01 derivative (Ym2 strain)	This study
GK471	*S. aureus* Sc-01 derivative (Ym3 strain)	This study
CW42	*E. coli* DH5α pMAD-Δ*bsa*::*cm*^{R}	This study
GK300	*S. aureus* LAC Δ*sigB*:: *erm*^{R}	This study
GK540	*S. aureus Sc-01* Δ*bsa*:: *cm*^{R}	This study
DL1646	*S. aureus* LAC Δ*sigB*:: *erm*^{R} Δ*bsa*::*cm*^{R}	This study
JM36	*E. coli* DH5α pDR111-*agrC-comP*	This study
JM37	*E. coli* DH5α pDG1661-P_{HP}*agrC-comP*	This study
JM39	*B. subtilis* pDG1661-P_{HP}*agrC-comP*	This study
DL720	*B. subtilis lacA*::P*srf*-YFP	(Lopez et al., 2009)
JM8	*B. subtilis* Δ*comX*::*spc*^{R}; *lacA*::P*srf*-YFP	This study
JM42	*B. subtilis* Δ*comX*::*spc*^{R} *lacA*::P*srf*-YFP *amyE*::P_{HP}*agrC-comP*	This study
DL1495	*S. aureus* Newman Δ*sigB*:: *erm*^{R}	This study
DL1501	*S. aureus* Newman Δ*agr*:: *tet*^{R}	This study
DL1699	*S. aureus* Newman Δ*sigB*:: *erm*^{R} Δ*agr*:: *tet*^{R}	This study
DL 1480	*S. aureus 4220 (graRS O)*	This study
GK560	*S. aureus 4220 (graRS Y)*	This study
GK562	*S. aureus 4220 (graRS Ym)*	This study
GK562	*S. aureus 4220 (graRS Ym)*	This study
GK569	*S. aureus 4220 (vraRS O)*	This study
GK570	*S. aureus 4220 (vraRS Y)*	This study
GK571	*S. aureus 4220 (vraRS Ym)*	This study
GK572	*S. aureus 4220 (vraRS Ym)*	This study
GK573	*S. aureus 4220 (rsbU-sigB O)*	This study
GK574	*S. aureus 4220 (rsbU-sigB W)*	This study
GK575	*S. aureus 4220 (rsbU-sigB Wm)*	This study
GK576	*S. aureus 4220 (walKR O)*	This study
GK577	*S. aureus 4220 (walKR Y)*	This study
GK580	*S. aureus 4220 (walKR Ym)*	This study
GK581	*S. aureus 4220 (walKR YA)*	This study
GK582	*S. aureus 4220 (walKR YB)*	This study
GK583	*S. aureus 4220 Δpsmα Δpsmβ*	(Geiger et al., 2012)

278

279 **Supplementary Table S2:** List of primers used in this study. Related to main figures 1, 3, 4, 5

280 and 7.

RT-*crtM*F	5’-TGATGACAGTATAGATGTTTATGG-3’
RT-*crtM*R	5’-ACATGCTGAAGCGCCATCATG-3’
RT-*icaB*F	5’-TTATCACAGGTCATGTTGGG-3’
RT-*icaB*R	5’-ATCGTCATTCATCAAGCCAT-3’
RT-*spa*F	5’-TGAATCTCAAGCACCGAAAG-3’
RT-*spa*R	5’-TTGCTCACTGAAGGATCGTC-3’
RT-*epiB*F	5’-ACTGAAATTGGATTTGGCGAGG-3’
RT-*epiB*R	5’-AGCCACAATAAGTACGCTGCT-3’
RT-*hla*F2	5’-CAACAACACTATTGCTAGGTTCCATATT-3’
RT-*hla*R2	5’-CCTGTTTTTACTGTAGTATTGCTTCCA-3’
RT-*hlb*F2	5’-TGAAGATGGTGGCGTAGCGATTGT-3’
RT-*hlb*R2	5’-TCATGTCCAGCACCACAACGAGAA-3’
RT-*psmα*F	5’-TATCAAAAGCTTAATCGAACAATTC-3’
RT-*psmα*R	5’-CCCCTTCAAATAAGATGTTCATATC-3’
RT-*hld*F	5’-CCTAGATCACAGAGATGTGATGG-3’
RT-*hld*R	5’-AATACATAGCACTGAGTCCAAGG-3’
RT-*mprF*F	5’-GAACCACCGTTTTCAACTGAA-3’
RT-*mprF*R	5’-GTAAATCTAACTCTGGCAACCATC-3’
RT-*dltA*F	5’-ATGTTTAGCATCAGGCGGTAC-3’
RT-*dltA*R	5’-ACTTGGGAAACGGCTCACTAA-3’
RT-*cls2*F	5’-ACGCCCGTTTCGCAATAAAG-3’
RT-*cls2*R	5’-TGCGTATCTCGCCAATAGCC-3’
RT-*sgtB*F	5’-AATATACGCTTGAGGGCGCA-3’
RT-*sgtB*R	5’-TCGTGCTTACACGTTGCGTA-3’
RT-*murZ*F	5’-TGTTGCACCTACGCTAACCAT-3’
RT-*murZ*R	5’-TTACCGGGAGGATGTCCACT-3’
RT-*epiA*F	5’-TTCAGCAGGTGACGAACGTA-3’
RT-*epiAR*	5’-GCCCTTTCAGGCTCTATTTGC-3’
RT-*sle1*F	5’-AAGTATCTGGCTCAAGTAATTCTAC-3’
RT-*sle1R*	5’-ACTGGTACGTTCAGAGGTCG-3’
RT-*ssaAF*	5’-GGCGGAGAAATCGGTTCTACT-3’
RT-*ssaAR*	5’-TACTGAGAAAGGTCCGCCAC-3’
RT-*RNAIIF*	5’-TATGAATAAATGCGCTGATGATATACCACG-3’
RT-*RNAIIR*	5’-TTTTAAAGTTGATAGACCTAAACCACGACC-3’
RT-*RNAIIIF*	5’-CTAAGTCACCGATTGTTGAA-3’
RT-*RNAIIIR*	5’-ATGGAAAATAGTTGATGAGTTG-3’
GK118	5’-CGTTTTAACCGAAATTTAAAATCTTCAATTTCTTCTTGATTACG-3’
GK119	5’-CGTAATCAAGAAGAAATTGAAGATTTTAAATTTCGGTTAAAACG-3’
GK120	5’-AAAAGCATGCTTACAATTCGATTTCAATATCAGCCT-3’

GK127	5’-AAAAGCTAGCTAAGGAGGAACTACTGTGGAATTATTAAATAG-3’
GK157	5’-AAAAGGATCCTACATATAAGTAATGACAAGCTTTGG-3’
GK158	5’-GGGTAACTATTGCCGAGATAAAACCTCCTA-3’
GK159	5’-TAGGAGGTTTTATCTCGGCAATAGTTACCC-3’
GK160	5’-CCGTTAGAGCCCCAATCTGATCTGGAGCTGTAAT-3’
GK161	5’-ATTACAGCTCCAGATCAGATTGGGGCTCTAACGG-3’
GK162	5’-AAAAGTCGACTTCATCAACAAAATCACAATTGAT-3’
SEQ *rsbU* F	5’-GCGGCAATAACTGGTAGGATTAA-3’
SEQ *sigB* R	5’-AAAAAAAGCTAGCCTATTTATGTGCTGCTTC-3’
SEQ *rsbU* int F	5’-GAATATTAATCAAAATATGTTCGTCAC-3’
SEQ *rsbW* int R	5’-CCTAGGCCACCTTCGCGTAAAAAGTC-3’
SEQ *vraS* F	5’-GGATAACTATACAGTTAATATCTACG-3’
SEQ *vraS* R	5’-GCGTTGATAGATAACTTGAAATTCC-3’
SEQ *graS* F	5’-GGATAGTGCAATCGAAACAAAAGTAGG-3’
SEQ *graS* R	5’-CTGCATTGATGTATCAATTCTAAGTAAC-3’
SEQ *agrA* F	5’-GTGCAGATGATATACCACGTA-3’
SEQ *agrA* R	5’-AAAAGCCAGCTATACAGTGCATTT-3’
SEQ *walK* F	5’-AAAACTCGAGACGCGTAGAGGCGTTGG-3’
SEQ *walK* R	5’-GCAGTCTTTGTCCATTTCATCCCAATCACC-3’
SEQ *walK* int F	5’-GGATATTACATGTTAAGTGTATTAAG-3’
SEQ *fmtC* F	5’-TAAGAAGCACTCATAATCGGCTGT-3’
SEQ *fmtC* R	5’-ACTTGATGAAACTGATTGGACTGA-3’
SEQ *fmtC* int F	5’-CTGCATTATCAGGTTTAGTCAGCT-3’
SEQ *fmtC* int R	5’-ATTTTCATCACCTAACGGATCTCC-3’
SEQ *cls*2 F	5’-AGTGTGTTTTTCAAACTAAATTGTC-3’
SEQ *cls*2 R	5’-GGCCTGTATAATCATTTTGATGAATAGATTCC-3’
graRS F	5’-AAAAGCTAGCAGGAGGATGCAAATACTAC-3’
graRS R	5’-TTTTCTCGAGTTAAAATGACAAATTTGTC-3’
pvraSR F	5’-AAAAGGATCCTAACTATACAGTTAATATCTACG-3’
pvraSR R	5’-AAAAGAGCTCCTATTGAATTAAATTATG-3’
walKR F	5’-AAAAGGATCCTAATATATAAGTTTATATTGG-3’
walKR R	5’-TTTTGAATTCTTATTCATCCCAATCACCGTC-3’
rsbUsigB F	5’-AAAAGGATCCTTTTATTATAAAAGGATGTCTTAAG-3’
rsbUsigB R	5’-TTTTGGATTCCTATTGATGTGCTGCTTCTTGTAA-3’
rpoZ F	5’-TATCCGCTTGTTGATGGCCAAGG-3’
rpoZ R	5’-CGCGCTTCAGTATAACGCATTGC-3’

281

282 **Supplementary table S3:** RNA-Seq dataset. Read analysis statistics. Related to main figure 2.

LIBRARY READ FILE	O	W	Y
No. of input reads	4353946	5078847	7088389
No. of reads – PolyA detected and removed	1569588	1754263	2504657
No. of reads - Single 3' A removed	957264	943712	1891419
No. of reads - Unmodified	1827094	2380872	2692313

No. of reads - Removed as too short	39350	39306	31970
No. of reads - Long enough for alignment	4314596	5039541	7056419
Total no. of aligned reads	4193429	4834286	6881138
Total no. of unaligned reads	121167	205255	175281
Total no. of uniquely aligned reads	1670096	1807375	2185476
Total no. of alignments	12682210	15931291	22630589
Percentage of aligned reads (compared to total input reads)	96.31	95.18	97.08
Percentage of uniquely aligned reads (in relation to all aligned reads)	39.83	37.39	31.76
NC_010063.1 - No. of aligned reads	4230.852381	7224.842857	5718.030952
NC_010063.1 - No. of uniquely aligned reads	4210	7183	5692
NC_010063.1 - No. of alignments	4262	7279	5766
NC_010079.1 - No. of aligned reads	4189192.648	4827049.958	6875409.345
NC_010079.1 - No. of uniquely aligned reads	1665886	1800187	2179781
NC_010079.1 - No. of alignments	12677936	15923992	22624802
NC_012417.1 - No. of aligned reads	5.5	11.2	10.625
NC_012417.1 - No. of uniquely aligned reads	0	5	3
NC_012417.1 - No. of alignments	12	20	21

283

284 **Supplementary table S4:** Genes differentially expressed in W strain compared to the O strain.
285 Genes with ≥4-fold difference in the expression level are listed. Genes that belong to the SigB
286 regulon are marked in yellow. For instance, production of Bsa antibiotic, which are induced in W
287 strain more than 318-fold compared to the O strain. Related to main figures 2 and 4.

288

289 **Supplementary table S5:** Genes differentially expressed in Y strain compared to the O strain.
290 Genes with at least ≥2-fold difference in the expression level are listed. Genes that belong to the
291 cell-wall stimulon, typically activated in VISA strains are marked in yellow. Related to main figure
292 2 and 5.

293

294 **Supplementary table S6:** List of the genomes of the different Staphylococcus aureus strains
295 that have been used in the MAUVE alignment of the region that contains the *bsa* gene cluster in
296 VISA and VSSA *Staphylococcus aureus* isolates. Related to main figure 5.

Number	Bacteria species name	Strain	Accession Number
1	*Staphylococcus aureus*	04-02981	NC_017340.1
2	*Staphylococcus aureus*	08BA02176	NC_018608.1
3	*Staphylococcus aureus*	subsp. *aureus* strain 11819-97	NC_017351.1
4	*Staphylococcus aureus*	subsp. *aureus* strain 71193	NC_017673.1
5	*Staphylococcus aureus*	subsp. *aureus* strain COL	NC_002951.2
6	*Staphylococcus aureus*	subsp. *aureus* strain ECT-R_2	NC_017343.1
7	*Staphylococcus aureus*	subsp. *aureus* strain ED133	NC_017337.1
8	*Staphylococcus aureus*	subsp. *aureus* strain ED98	NC_013450.1
9	*Staphylococcus aureus*	subsp. *aureus* strain HO5096_0412	NC_017763.1
10	*Staphylococcus aureus*	subsp. *aureus* strain JKD6008	NC_017341.1
11	*Staphylococcus aureus*	subsp. *aureus* strain JKD6159	NC_017338.1
12	*Staphylococcus aureus*	subsp. *aureus* strain LGA251	NC_017349.1
13	*Staphylococcus aureus*	subsp. *aureus* strain M013	NC_016928.1
14	*Staphylococcus aureus*	subsp. *aureus* strain MRSA252	NC_002952.2
15	*Staphylococcus aureus*	subsp. *aureus* strain MSHR1132	NC_016941.1
16	*Staphylococcus aureus*	subsp. *aureus* strain MSSA476	NC_002953.3
17	*Staphylococcus aureus*	subsp. *aureus* strain MW2	NC_003923.1
18	*Staphylococcus aureus*	subsp. *aureus* strain N315	NC_002745.2
19	*Staphylococcus aureus*	subsp. *aureus* strain NCTC_8325	NC_007795.1
20	*Staphylococcus aureus*	subsp. *aureus* strain Newman	NC_009641.1
21	*Staphylococcus aureus*	RF122	NC_007622.1
22	*Staphylococcus aureus*	ST228_10388	NC_020529.1
23	*Staphylococcus aureus*	ST228_15532	NC_020532.1
24	*Staphylococcus aureus*	ST228_16035	NC_020533.1
25	*Staphylococcus aureus*	ST228_18341	NC_020536.1
26	*Staphylococcus aureus*	ST228_18412	NC_020537.1
27	*Staphylococcus aureus*	subsp. *aureus* strain ST398	NC_017333.1
28	*Staphylococcus aureus*	subsp. *aureus* strain T0131	NC_017347.1
29	*Staphylococcus aureus*	subsp. *aureus* strain TCH60	NC_017342.1
30	*Staphylococcus aureus*	subsp. *aureus* strain TW20	NC_017331.1
31	*Staphylococcus aureus*	subsp. *aureus* strain USA300_FPR3757	NC_007793.1
32	*Staphylococcus aureus*	subsp. *aureus* strain USA300_TCH1516	NC_010079.1
33	*Staphylococcus aureus*	subsp. *aureus* strain VC40	NC_016912.1
34	*Staphylococcus aureus*	subsp. *aureus* strain JH1	NC_009632.1
35	*Staphylococcus aureus*	subsp. *aureus* strain JH9	NC_009487.1
36	*Staphylococcus aureus*	subsp. *aureus* strain Mu3	NC_009782.1
37	*Staphylococcus aureus*	subsp. *aureus* strain Mu50	NC_002758.2

297

298 **Supplementary table S7:** Definition of a cut-off number to define high and low bacterial
299 load in the organs of infected mice. We considered mice infection models that have been
300 previously established to assay staphylococcal infections (Intravenous, intranasal, intraperitoneal
301 and subcutaneous murine infection models). The bacterial loads of target organs (CFU/g of
302 organ) were compiled from published experiments that distinguish between superficial bacterial
303 burden and deep tissue infections. We classified these experiments into three categories: Group
304 1 contains the CFU/g values of infections from published experiments that compare virulent
305 strains (+) and attenuated (-) strains of *S. aureus*. Group 2 contains CFU/g values of
306 staphylococcal infections from published experiments that compared antibiotic treated (+) and
307 non-treated (-) mice. Group 3 contains CFU/g values of staphylococcal infections from published
308 experiments that compare immunized (+) and non-immunized (-) mice. All groups of experiments
309 compare staphylococcal infections that likely develop into deep tissue infections and infections
310 with attenuated strains (Group 1) or antibiotic-treated mice (Group 2 and 3), in which the
311 development of severe infections is unlikely (i.e. superficial bacterial burden). The bacterial load
312 of superficial bacterial burden from groups 1-3 occurred in a range of 10^3-10^5 CFU/g in target
313 organs. In contrast, deep tissue infections contained a bacterial load of 10^6-10^{10} CFU/g of organ.
314 Thus, deep tissue infections show a bacterial load of $>10^5$ CFU/g of organ and superficial
315 bacterial burden show a bacterial load of $\leq 10^5$ CFU/g of organ. To ascertain whether similar
316 values occur in our *in vivo* experiment, we assayed an intravenous infection model and the
317 intranasal infection model to study kidney and lung infections, respectively. We used these two
318 infection models to determine the bacterial loads of superficial bacterial burden and deep tissue
319 infections. To do this, we used 2 cohorts of 5 mice to test infections of a virulent strain (LAC) and
320 an attenuated strain (LAC Δ*agr* mutant). Likewise, 2 additional cohorts of 5 mice were used to
321 assay LAC infections in the presence and absence of vancomycin treatment (total number of
322 mice n = 40). Organs were aseptically harvested and CFU/g were determined. Superficial
323 bacterial burden was associated with a bacterial load of 10^3-10^5 CFU/g. Deep tissues infections

324 displayed a bacterial load of 10^6-10^{10} CFU/g. Based on this analysis, organs with bacterial loads
325 >10^5 CFU/g are considered deep tissue infections (i.e. high bacterial load) and organs with
326 bacterial load ≤ 10^5 CFU/g are considered as a superficial bacterial burden (i.e. low bacterial
327 load). Therefore, 10^5 CFU/g is the empirically derived cut-off number to distinguish between
328 superficial bacterial burden (i.e. low bacterial load) and deep tissue infections (i.e. high bacterial
329 load) in our infection experiments. Related to figure 6 and 7.

330

1) CFU/g organ from mice infected with virulent (+) and attenuated (-) *S. aureus* strains				
Infection model	**Organ**	**Strain virulence**	**CFU/g organ**	**Reference**
Intravenous infection model	Kidneys	+	10^7	(Bubeck Wardenburg et al., 2006; Cheng et al., 2010)
		-	10^5	
	Livers	+	10^7	(Bae et al., 2006; Marincola et al., 2012)
		-	10^5	
	Hearts	+	10^8	(McAdow et al., 2012; Panizzi et al., 2011)
		-	10^5	
Intranasal infection model	Lungs	+	10^7	(Chen et al., 2013; Mitchell et al., 2013)
		-	10^4	
Intraperitoneal infection model	Kidneys	+	10^8	(Cohn, 1962; Kapral, 1966)
		-	10^5	
	IP lesions	+	10^7	(Dye and Kapral, 1981; Kapral et al., 1980)
		-	10^5	
Subcutaneous infection model	Skin abscess	+	10^7	(Bunce et al., 1992; Ford et al., 1989)
		-	10^5	
2) CFU/g organ from infected mice treated (+) and non-treated (-) with antibiotics				
Infection model	**Organ**	**Antibiotic treatment**	**CFU/g organ**	**Reference**
Intravenous infection model	Kidneys	-	10^{10}	(Georgopoulos et al., 1992; Oh et al., 2010)
		+	10^5	
	Livers	-	10^7	(Piper et al., 2012; Tsao et al., 2003)
		+	10^5	
	Hearts	-	10^8	(Gibson et al., 2007; Jabes et al., 2011)
		+	10^3	
Intranasal infection model	Lungs	-	10^8	(Harada et al., 2013; Jacqueline et al., 2014)
		+	10^5	
Intraperitoneal infection model	Kidneys	-	10^8	(Lobo and Mandell, 1972; Sande et al., 2012)
		+	10^4	
	IP lesions	-	10^7	(Kokai-Kun et al., 2007; Sandberg et al., 2011)
		+	10^4	
Subcutaneous infection model	Skin abscess	-	10^7	(Foletti et al., 2013; van Sorge et al., 2013)
		+	10^5	
3) CFU/g organ from mice immunized (+) and non-immunized (-) against *S. aureus* infections				

Infection model	Organ	Immunization	CFU/g organ	Reference
Intravenous infection model	Kidneys	-	10^7	(Kim et al., 2012; McKenney et al., 1999)
		+	10^4	
	Livers	-	10^7	(Hu et al., 2009; Rozalska and Wadstrom, 1993)
		+	10^5	
	Hearts	-	10^8	(Ekstedt and Yoshida, 1969; McAdow et al., 2011)
		+	10^5	
Intranasal infection model	Lungs	-	10^7	(Bubeck Wardenburg and Schneewind, 2008)
		+	10^4	
Intraperitoneal infection model	Kidneys	-	10^6	(Kim et al., 2011; Mariotti et al., 2013)
		+	10^3	
	IP lesions	-	10^8	(Easmon and Glynn, 1975; Rauch et al., 2012)
		+	10^5	
Subcutaneous infection model	Skin abscess	-	10^8	(Tkaczyk et al., 2013; Weinandy et al., 2014)
		+	10^5	

4) Experimental quantification of CFU/g organ from mice infected with virulent (+) and attenuated (-) *S. aureus* strains

Infection model	Organ	Cohort	Strain virulence	CFU/g organ	Range	Reference
Intravenous infection model	Kidneys	n=5	-	10^5	10^3-10^5	This study
				10^5		
				10^4		
				10^5		
				10^3		
		n=5	+	10^9	10^7-10^9	This study
				10^8		
				10^8		
				10^7		
				10^8		
Intranasal infection model	Lungs	n=5	-	10^4	10^3-10^4	This study
				10^3		
				10^4		
				10^4		
				10^3		
		n=5	+	10^6	10^6-10^8	This study
				10^7		
				10^7		
				10^6		
				10^8		

5) Experimental quantification of CFU/g organ from infected mice that were treated (+) and non-treated (-) with vancomycin (200mg/Kg)

Infection model	Organ		Antibiotic treatment	CFU/g organ	Range	Reference
Intravenous infection model	Kidneys	n=5	-	10^8	10^8-10^{10}	This study
				10^9		
				10^{10}		
				10^8		
				10^8		
		n=5	+	10^3	10^3-10^5	This study

				10^5		
				10^5		
				10^4		
				10^5		
Intranasal infection model	Lungs	n=5	-	10^6	10^6-10^7	This study
				10^7		
				10^7		
				10^7		
				10^6		
		n=5	+	10^3	10^3-10^4	This study
				10^4		
				10^4		
				10^4		
				10^4		

6) Range of CFU/g or organ of infected mice and definition of cut-off		
Organ colonization	**Progression of infection**	**CFU/g organ (range)**
Low bacterial burden (low bacterial load)	-	10^3 - 10^5
Deep tissue infection (high bacterial load)	+	10^6 – 10^{10}
Cut-off number of bacterial load		10^5

331

332 **Supplemental Experimental Procedures**

333 **Strains, Media and Culture Conditions.**

334 Strains used in this study were *S. aureus* derivative of Sc01 (Beenken et al., 2003),
335 Newman (Lipinski et al., 1967), N315 (Ito et al., 1999), HG003 (Herbert et al., 2010) and LAC
336 (Kazakova et al., 2005) isolates. The laboratory *S. aureus* strain RN4220 (Kornblum, 1990) was
337 used for cloning purposes. *Bacillus subtilis* strain 168 was occasionally used as genetic
338 background to generate a transducer strain that senses the presence of AIP and to assay for the
339 activity of Bsa in antibiogram experiments. The strain *Escherichia coli* DH5α was used for
340 propagating plasmids and genetic constructs in laboratory conditions. A complete strain list is
341 shown in table S1.

342

343 *B. subtilis* and *E. coli* strains were regularly grown in LB medium. When required,
344 selective media were prepared in LB agar using antibiotics at the following final concentrations:
345 ampicillin 100 μg/ml, kanamycin 50 μg/ml, chloramphenicol 5 μg/ml, tetracycline 5 μg/ml, and

346 erythromycin 2 μg/ml. *S. aureus* strains were routinely propagated in liquid TSB medium
347 incubated with shaking (200 rpm) at 37°C for 24h. When required, selective media were
348 prepared in TSB agar using antibiotics at the following final concentrations: kanamycin 50 μg/ml,
349 chloramphenicol 10 μg/ml, tetracycline 5 μg/ml, and erythromycin 2-80 μg/ml. To generate
350 multicellular aggregates, a inoculum TSB plate was grown for 12h at 37°C. 2 μl of a cell
351 suspension from the inoculum were spotted on the surface of TSB agar plates supplemented
352 with $MgCl_2$ 100 mM and incubated at 37°C for 5 days. When specified, AIP was added at
353 concentrations of 1-5 μM and Bsa was added to different concentrations that are specified in the
354 body of the paper. Specific growth conditions are presented in the figure legends.
355
356 To grow *S. aureus* biofilms, a preculture grown overnight in TSB liquid medium was
357 diluted 1:100 in TSB + Glucose 0.5% + NaCl 3% (Blevins et al., 2003), dispensed in polystyrene
358 well plates and incubated overnight at 37° C. Biofilms formed by *S. aureus* were stained with
359 crystal violet (1%) for better visualization according to (O'Toole and Kolter, 1998). Secretion of
360 hemolytic toxins was monitored by spotting 3 μl of *S. aureus* cultures in TSB agar plates
361 containing 5% sheep blood and measuring the diameter size after 24h of incubation at 37° C.
362 Subsequent incubation at 4°C was used to asses β-hemolysis. Production of the pigment
363 staphyloxanthin was quantified by correlating production of staphyloxanthin with the optical
364 density of the culture. To purify staphyloxanthin, cell pellets were dissolved 1:1 (v/v) in ethanol
365 and incubated for 2 h at 45°C. After centrifugation, the pellet was discarded and the ethanol
366 containing the pigments was concentrated by evaporation (Pelz et al., 2005; Wieland et al.,
367 1994). Quantification of the concentration of the pigment was performed by spetrophotometry
368 analysis (λ=460mm) (Giachino et al., 2001). To assess the ability of the subpopulation to spread
369 on solid surfaces, 2 μl of an overnight culture was spotted on TSB 0.24% agar plates and
370 incubated at 37°C for 24 hours (Tsompanidou et al., 2011). To assess the resistance of *S.*

371 *aureus* cells to lysostaphin treatment, Staphylococcal strains were resuspended in 1 ml PBS.
372 100 μl of the suspension was then incubated for 15 minutes at 37°C in the presence of
373 lysostaphin (10 μg/ml) (Cui et al., 2006; Daum et al., 1992). Serial dilutions were plated on LB
374 and TSB plates to determine CFU and compared to untreated cell suspensions.

375

376 **Strain generation**

377 A LACΔ*sigB* and Sc01Δ*bsa* mutants were generated by phage transduction. Briefly, φ11
378 phage lysates were generated from *S. aureus* Newman Δ*sigB*::*erm*^{R} to infect LAC. Clones
379 resistant to erythromycin were further verified using PCR. To generate the *S. aureus* strain with a
380 deletion of the *bsa* operon, 500 bp flanking the operon, as well as the chloramphenicol cassette
381 were amplified using primers GK157/GK158, GK161/GK162, and GK159/GK160 respectively.
382 The fragments were subsequently joined together using a long-flanking homology PCR (LFH-
383 PCR) (Wach, 1996). This fragment was then cloned into pMAD (Arnaud et al., 2004) using
384 BamHI/SalI and transformed into *S. aureus* RN4220. φ11 phage lysates were used to infect
385 USAΔ*sigB* and double homologous recombination was carried out as previously described
386 (Arnaud et al., 2004). Clones were verified using PCR.

387

388 To measure AIP production, a *Bacillus subtilis* reporter strain was created in which a
389 chimeric version of the membrane-bound sensor kinase ComP replaces the native kinase
390 (Magnuson et al., 1994; Solomon et al., 1995). The chimera contains the sensing domain of the
391 staphylococcal AgrC sensor kinase that recognizes the AIP pheromone (Novick and Geisinger,
392 2008). To generate the chimera, the DNA fragments corresponding to the specific protein
393 regions of AgrC and ComP kinases were fused by LFH-PCR, using the primers GK127/GK118
394 (for AgrC) and GK119/GK120 (for ComP). The resultant DNA fragment was cloned into pDR111
395 vector and subcloned into pDG1663 vector. The final construct was linearized and integrated into
396 the *amyE* neutral locus of *B. subtilis* chromosome (strain 168). Additional genetic modifications to

397 this strain were as follows: Δ*comQXP*::*spc* mutation was included to fully inactivate the native
398 quorum sensing signaling pathway of *B. subtilis*. Furthermore, a transcriptional reporter P_{srf}-*yfp*
399 was inserted in the *lacA* neutral loci of the bacterial chromosome. The expression of this reporter
400 is strongly regulated by the activation of the AgrC-ComP chimera and its activation results in the
401 expression of a yellow fluorescent protein YFP (Nakano et al., 1991). Thus, the genetic
402 description of the reporter strain is *B. subtilis* 168 Δ*comQXP*::spc; *lacA*::P_{srf}-*yfp*; *amyE::*P_{hp}*agrC-*
403 *comP.* To monitor AIP levels, O, W and Y strains were grown overnight in TSB medium. Cells
404 were pelleted and supernatants filter-sterilized (pore size: 0.2 μM). An overnight culture of the
405 reporter strain was diluted 1:100 in LB medium and subsequently diluted in passages to fresh
406 medium every two hours, to maintain the reporter strain exponentially growing for a long time
407 period. Then, 180 μl were mixed together with 20 μl of each AIP containing supernatant.
408 Fluorescence was monitored using a Tecan plate reader (excitation: 485 nm, emission: 535 nm)
409 at 30°C. Data points prior to maximum fluorescence were used for comparison (about 2 h after
410 initiation of the assay).

411

412 **RNA extraction**

413 O, W and Y strains went through several isolation processes on TSB plates to verify the
414 purity of the cultures prior RNA extraction. Cells were grown overnight on a TSB plate before
415 resuspended in RNA Protect (Qiagen®, USA) according to the manufacturer's instructions. Cell
416 pellets were resuspended in 1ml RLT buffer and mechanically lysed using glass beads in a Fast
417 Prep Shaker (2 times, 45 s, speed: 6.5). The cell lysate was centrifuged for 10 min, 11, 000 x g,
418 4°C. The supernatant was used for RNA isolation using the RNeasy mini kit (Qiagen®, USA)
419 according to the protocol. The isolated RNA was treated with RNase-free DNase I (New England
420 Biolabs®, USA) to remove any DNA traces.

421

422 **RNA- Seq library construction, sequencing and qRT-PCR analysis**

423 The cDNA libraries were generated as described previously for eukaryotic microRNAs but
424 omitting the RNA size-fractionation step prior to cDNA synthesis (Dugar et al., 2013) (Vertis
425 Biotechnologie AG, Germany). For this the RNA samples were poly(A)-tailed by using poly(A)
426 polymerase. The 5'-PPP were removed using tobacco acid pyrophosphatase (TAP) followed by
427 the ligation of the RNA adapter to the 5'-monophosphate of the RNA. First-strand cDNA
428 synthesis was performed with an oligo(dT)-adapter primer and the M-MLV reverse transcriptase.
429 The resulting cDNA was PCR-amplified to reach a concentration of 20-30 ng/µl using a high
430 fidelity DNA polymerase. The cDNA was purified using the Agencourt AMPure XP kit (Beckman
431 Coulter Genomics) and was analyzed by capillary electrophoresis. The primers used for PCR
432 amplification were designed for TruSeq sequencing according to the instructions of Illumina. The
433 following adapter sequences flank the cDNA inserts: TrueSeq_Sense: 5'-
434 AATGATACGGCGACCACC-GAGATCTACACTCTTTCCCTACACGACGCTCTTCCGAT-T-3'
435 TrueSeq-AntisenseNNNNNN (NNNNNN = Barcode) 5'-CAAGCAGAAGACGGCATAC-
436 GAGATNNNNNNGTGACTGG-AGTTCAGACGTGTGCTCTTCC-GATC(dT25)-3'. The combined
437 length of the flanking sequences is 146 bases. The libraries were sequenced with an Illuminua
438 HiSeq machine with 100 cycles in single end mode. To perform qRT-PCR analysis, total RNA
439 was extracted using RNeasy Mini Kit (Quigen), according to the manufacturer's instructions. For
440 quantification of gene expression, total RNA was reverse transcribed using hexameric random
441 primers followed by qRT-PCR using SsoAdvanced SYBR Green Supermix (BioRad, USA),
442 according to the manufacture's instructions and using primers listed in table S2. qRT-CPR
443 experiments of this report follows the standard MIQE guidelines for publication of qRT-PCR
444 experiments (Bustin et al., 2009).

445

446 **Bioinformatical analysis**

447 The pooled sequence reads were demultiplexed and the adapter sequences were
448 removed. After that the reads in Fastq format were quality trimmed using fastq_quality_trimmer

449 (from the FastX suite version 0.0.13 - http://hannonlab.cshl.edu/fastx_toolkit/) with a cut-off
450 Phred score of 20 and converted to Fasta format using Fastq_to_Fasta (also from the FastX
451 suite). The read processing (poly(A) removal, size filtering (min 12 nt length), statistics
452 generation, coverage calculation and normalization was performed with the RNA-analysis
453 pipeline RAPL version 0.1 with default parameters which used segemehl version 0.1.3
454 (Hoffmann et al., 2009) for the read alignment and DESeq 1.12.0 (Anders and Huber, 2010) for
455 differential gene expression analysis. As reference sequences and annotations the data of *S.*
456 *aureus* LAC FPR3757 (NCBI IDs: NC_010063.1, NC_010079.1, NC_012417.1) was used.
457 Scatter plots comparing the gene wise expression levels were normalized by the size estimation
458 factor calculated by DESeq of the different strain, which were generated using matplotlib
459 (http://matplotlib.sourceforge.net/). Genes with an up- or down-regulation factor of 1.5 and a p-
460 value of maximum 0.05 were allocated to functional groups. For this, the information about genes
461 and their functional group were downloaded from the JCVI Comprehensive Microbial Resource
462 ftp server (Peterson et al., 2001) which contains functional allocation for *S. aureus* LAC
463 FPR3757. All genes of the *S. aureus* LAC TCH1516 (NC_010063.1, NC_010079.1,
464 NC_012417.1) were aligned against all genes from *S. aureus* LAC FPR3757 (NC_007790.1,
465 NC_007791.1, NC_007792.1, NC_007793.1) and vice versa with a maximum e-value of 0.01
466 and a minimal bit score of 60 using makeblastdb and blastn from the BLAST+ suite version
467 2.2.28 (Camacho et al., 2009). If two genes had a best-reciprocal-hit-relationship they were
468 treated as orthologs. Via this ortholog allocation the function group associations were made for
469 the up- and down-regulated genes determined before. The number of such genes was summed
470 up for each group and visualized. The demultiplexed files and coverages files in wiggle format
471 have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are
472 accessible through GEO Series accession number GSE49636
473 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=49636).
474

475 ***in silico* genome comparison**

476 Genomics regions comprising 1.5 Kb upstream and 1.5 Kb downstream the location of
477 the *bsa* gene cluster were extracted from the *S. aureus* genomes subsp. *aureus* LAC_TCH1516
478 (Accession number NC_010079.1) as the reference sequence and, subsequently from subsp.
479 *aureus* JH1 (NC_009632.1), subsp. *aureus* JH9 (NC_009487.1), subsp. *aureus* Mu50
480 (NC_002758.2) and subsp. *aureus* Mu3 (NC_009782.1) using extractseq from EMBOSS Suite
481 (http://emboss.bioinformatics.nl/cgi-bin/emboss/extractseq) (Rice et al., 2000). These regions
482 were aligned using the open-source MAUVE aligner version 2.3.1 (Darling et al., 2004; Darling et
483 al., 2011). A non-progressive alignment was performed using a seed-weight of 19 while the
484 remaining parameters were run by default. Whole Genome Sequences (WGSs) alignment was
485 performed using the MAUVE version 2.3.1 with its progressive algorithm. The 37 complete
486 genomes were aligned using a seed-weight of 19 and assuming collinear genomes, while the
487 other mandatory parameters were run by default. The complete list of the genomes and their
488 accession numbers can be found in the supplementary table. After the initial set of multiple
489 alignments, MAUVE generates a distance matrix and constructs a Neighbor Joining guide tree
490 that clusters organisms with similar genomic content (Darling et al., 2004; Darling et al., 2011).
491 The graphical representation of this guide tree was obtained using the open-software FigTree
492 (http://tree.bio.ed.ac.uk/software/figtree/).

493

494 **Purification of small molecules**

495 We followed published to obtain an enriched fraction of AIP (P et al., 2001) and Bsa (Daly
496 et al., 2010) small molecules. Briefly, a 500 ml culture of the W strain was grown overnight in
497 TSB broth and, after the removal of bacterial cells by centrifugation, the supernatant was filtered
498 through a 0.22 mm membrane filter. The filtered supernatant was loaded into a C18 Sep-Pak
499 cartridge (Waters®, USA) previously stabilized with aqueous 20% CH_3CN and 0.1%
500 trifluoroacetic acid. Bsa was eluted from the cartridge with 10 mL of aqueous 60% CH_3CN and

501 0.1% trifluoroacetic acid and neutralized with ammonium. Elution of AIP was achieved with a 20-
502 45% concentration range of CH_3CN. Fresh AIP fractions were used in each experiment due to
503 the high instability of the preparation.
504

505 **Flow Cytometry**

506 For flow cytometric analysis, cells were dispersed from biofilms with 12 sonication pulses
507 (power output 0.7 and cycle 50%). After dispersion, cells were fixed with a treatment of 4%
508 paraformaldehyde, washed and resuspended in PBS buffer. Dilution of samples 1:100 was
509 necessary prior flow cytometry analyses. Further sonication treatment was required to separate
510 single cells in the sample. In this case, samples were subjected to three consecutive series of 12
511 pulses of (power output 50% and cycle 0.7 sec). Flow cytometry analysis was carried out in a BD
512 Fortessa flow cytometer (BD Biosciences, USA). For GFP fluorescence, we used a laser
513 excitation of 488 nm coupled with 530/30 and 505LP sequential filters. The photomultiplier
514 voltage was set between 400 and 500 V. No gates were required during the analysis of the
515 samples. Every sample was analyzed measuring 50,000 events using FACS Diva (BD
516 Biosciences, USA) software to capture the data. Further data analysis was performed in FlowJo
517 9.2 (http://www.flowjo.com).
518

519 **Western Blot**

520 Western blot analysis was performed to determine semi-quantitatively the levels of SigB
521 and RsbW in O, W and Y strains. The strains were grown for 6 h in TSB medium. Cultures were
522 pelleted and resuspended in SMM buffer (1M sucrose, 40 mM $MgCl_2$, 40 mM maleic acid; pH
523 6.5) containing 20 µg/ml lysostaphin. After 10 min incubation at 37 °C cells were lysed using a
524 French Press (SLM Aminco Instruments®, USA) at 20.000 psi for three times. Cell debris was
525 removed by centrifuging lysate at 15.000 rpm, 10 min at 4°C. Subsequently, supernatant was
526 centrifuged at 80.000 rpm for 1h at 4°C to remove membrane fractions. 20 µg of proteins of the

527 cytosolic fractions were loaded on a 12.5 % SDS-PAGE (for SigB detection) or 15 % SDS-PAGE
528 (for RsbW detection), respectively. Proteins were transferred to a PVDF membrane incubated
529 overnight in the presence of polyclonal antibodies against SigB or RsbW, kindly provided by Dr.
530 S. Engelmann (University of Greisfwald, Germany). Proteins were detected after incubation with
531 the secondary antibody (anti-rabbit IgG-HRP, BioRad®, USA) using the chemiluminescent
532 substrate Kit (Thermo Scientific®, USA). Chemiluminescence was recorded with the Illumination
533 System ImageQuant LAS4000 (General Electric®, USA).
534
535 **Fluorescence microscopy and transmission electron microscopy**
536 To assay fluorescence microscopy, cells were resuspended in 1 ml of 4%
537 paraformaldehyde solution and incubated at room temperature for seven minutes. After washing,
538 samples were resuspended in PBS buffer. Samples were repeatedly washed prior single-cell
539 analysis. Images were processed using Leica Application Suite V3.7 software. Microscopy
540 images were taken on a Leica DMI6000B microscope equipped with a Leica CRT6000
541 illumination system. The microscope was equipped with a HCX PL APO oil immersion objective
542 with 100x1.47 magnification that was used in this study. The microscope was also equipped with
543 a color camera Leica DFC630FX. Image processing was done using Leica Application Suite
544 Advance Fluorescence Software and Photoshop. YFP fluorescence signal was detected using
545 an excitation filter BP500/20 and an emission filter BP535/30. Excitation time was between 100
546 and 200 msec. Transmitted light images were taken with 36 msec of excitation time.
547
548 Subcellular localization of fluorescein-labeled vancomycin (Van-FL) was performed
549 following the protocols published in (Pereira et al., 2007). To specifically visualize new cell wall
550 synthesis, cells were grown in the presence of D-Serine 60 mM until mid-stationary phase. Cells
551 were harvested, washed and grown in regular medium for 15 min. Cells were harvested and
552 incubated at 4°C for 5 min before Van-FL labeling. For Van-FL labeling, cells were incubated

553 with Van-FL (1 μg/ml) for 5 min. Additional membrane staining was performed by incubating cells
554 with Nile Red (Sigma, USA) to a final concentration of 0.5 μg/ml for 10 min. After labeling, cells
555 were washed several times with PBS buffer and then analyzed by fluorescence microscopy. To
556 specifically visualize the diffusion of vancomycin into *S. aureus* cell wall, cells were grown until
557 mid-stationary phase. Non-fluorescent vancomycin (3 μg/ml) was added and cells were
558 incubated 1 hour at room temperature. After washing, cells were harvested and incubated at 4°C
559 for 5 min before Van-FL labeling (Van-FL 5 μg/ml for 1 min). We performed additional membrane
560 staining with Nile Red (0.5 μg/ml for 10 min) for better visualization of the cells. Subcellular
561 quantification of the fluorescent membrane signal was performed using Leica Application Suite
562 Advance Fluorescence Software and ImageJ. Quantitative analysis of the diffusion of
563 fluorescein-labeled vancomycin through the cell wall of O, W and Y cells was performed
564 according to the protocol published by (Pereira et al., 2007). Cells were harvested and incubated
565 at 4°C for 5 min before Van-FL labeling. For Van-FL labeling, cells were incubated with Van-FL
566 (1 μg/ml) for 5 min. 100 dividing cells were randomly selected from each strain and the Van-FL
567 signal measured using fluorescence microscopy and using Leica Application Software Analysis
568 and FiJi. Fluorescence of bound Van-FL signal was considered only in cells with complete
569 formed septa. The signal was measured at the peripherical cell wall and at the division septum.
570 Cells were classified into 3 categories attending to their Van-FL diffusion rate. Category 1 (C1):
571 Cells showed higher fluorescent signal associated with the cell wall than with the septum.
572 Category 2 (C2): Cells showed a uniform distribution of the fluorescent signal in the cell wall and
573 in the septum. Category 3 (C3): Cells showed higher fluorescent signal associated with the
574 septum than with the cell wall. Control experiment compares the diffusion of Van-FL in two
575 isogenic VSSA/VISA strains (N315/Mu50). To generate Van-FL, 500 μl of vancomycin 10 mg/ml
576 was mixed with 50 μl of 5(6)-carboxyfluorescenin-N-hydroxysuccinimideester 5 mg/ml (Roche,
577 USA) to generate a 5:1 mixture of vancomycin:fluorescein. The reaction was incubated overnight
578 at 4 °C and 1:1 diluted in Tris buffer and resulting solution was stored at - 20 °C in aliquots. The

579 final concentration of Van-FL of this stock was assumed to be 5 mg/ml with relatively little free
580 fluroescein. The minimal inhibitory concentration of Van-FL was similar to that of the original
581 vancomycin (~ 1µg/ml).
582
583 For transmission electron microscopy, cells were grown overnight on LB plates. Cells were
584 collected and washed with PBS. Subsequently cells were fixed for 2h in fixation buffer (2 %
585 paraformaldehyde, 2.5 % glutardialdehyde, 0.1 M sodium cacodylate; pH 7.2) at 4°C. After
586 washing the cells twice with 50 mM sodium cacodylate (pH 7.2), and afterwards with distilled
587 water, samples were stained with aqueous uranyl acetate (0.5 %) overnight at 4°C.
588 Subsequently samples were dehydrated and embedded in Epon812 before inspecting them
589 under an Zeiss EM900 electron microscope. Negatives were digitalized by scanning and
590 processed with Adobe Photoshop.
591
592 **Mouse infection studies**
593 All animal studies were approved by the local government of Lower Franconia, Germany
594 (license number 55.2-2531.01-06/12) and were performed in strict accordance with the
595 guidelines for animal care and animal experimentation. Female BALB/c mice (16–18 g) were
596 purchased from Charles River® (Germany), housed in polypropylene cages and supplied with
597 food and water ad libitum. The *S. aureus* derivative was cultured for 18 h at 37°C on a LB plate.
598 Subsequently, cells were collected and washed three times with PBS and diluted to the desired
599 concentration. Viable cell counts were determined by plating dilutions of the inoculum on
600 mannitol salt-phenol red agar plates. 100 µl *S. aureus* culture was injected into the tail vein of 5
601 mice. 6 days after bacterial challenge, organs were aseptically harvested and the CFU
602 determined. For this purpose, kidneys, livers, heart, spleen and lungs were homogenized in 2 ml
603 of sterile PBS using Dispomix (Bio-Budget Technologies GmbH, Germany) (Marincola et al.,
604 2012). Joints were ground in a mortar prior Dispomix homogenization. Serial dilutions of each

605 organ were plated on mannitol salt-phenol red agar plates, LB and TSB plates and incubated at
606 37°C for at least 48 h. CFUs were counted and the bacterial burden calculated as CFU/g of
607 organ. In addition, the occurrence of subpopulations was determined by selecting for color and
608 vancomycin resistance. To compare the infective potential of O, W and Y strains, 3 cohorts of 5
609 mice were infected 100 µl *S. aureus* culture containing 10^7 cells injected into the tail vein. Each
610 strain was used to infect one cohort of mice. The infections were allowed to progress until severe
611 infections symptoms occurred or to an endpoint of 5 days. Criteria for determining severe
612 infection included body weight, ruffled fur, hunched posture, decreased activity and labored
613 breathing. Animals were sacrificed when they meet the following criteria: 1) loss of at least 20%
614 of body weight; 2) loss of at least 15% of body weight and ruffled fur; 3) loss of at least 10% of
615 body weight and hunched posture 4) 5 days of infection. Organs were aseptically harvested and
616 the CFU determined. To determine cut-off number of bacterial load, 8 cohorts of 5 mice were
617 infected with 10^7 cells via tail vein injection (in the case of the intravenous infection mouse
618 model) or intranasal inoculation (in the case of the intranasal infection mouse model). 5 days
619 after bacterial challenge, target organs were aseptically harvested and the CFU determined. For
620 this purpose, kidneys were harvested from the intravenous infection mouse model and the lungs
621 were harvested from the intranasal infection mouse model.

622

623 **Supplementary References**

624

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