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Supplementary information (SI) for:

Adaptive laboratory evolution and independent component analysis disentangle complex vancomycin adaptation trajectories

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SI Materials and Methods:

Serial transfer experiments. The MRSA strain JE2, a plasmid-cured derivative of USA300 LAC, was grown on increasing concentrations of vancomycin in tryptic soy broth medium (TSB) using a two-step protocol derived from Rodriguez de Evgrafov *et al.* 2015 (1) (**Figure S6**). In the first step, the bacteria were challenged with increasing concentrations of vancomycin. Overnight cultures were diluted to 10^5CFU/ml and used to inoculate a microtiter plate containing 0.25 to 0.5µg/ml increments in vancomycin concentration. After 18h at 37°C in a fixed incubator, the plates were visually inspected for growth. The highest concentration to which JE2 grew (subMIC) was recorded, and this culture was used to inoculate fresh TSB media supplemented with vancomycin at the strains subMIC and allowed to grow overnight in 3mL cultures. This second step was used to stabilize any adaptive event occurring from the previous challenge. This two-step evolution procedure is referred to as an Exposure cycle (E). The culture was used to start a new cycle, and an aliquot was saved in glycerol at -80°C. 10 independent cultures were grown in parallel resulting in the lineages L1 to L10. This process was repeated for 30 exposure cycles (60 days) and thorough characterization was conducted every 10 cycles at timepoints E10, E20 and E30. L5_E20 and L9_E20 were re-adapted to vancomycin for 10 cycles resulting in the 2 additional clones L5_E30bis and L9_E30bis. L3_E20 contaminated a parallel culture during the laboratory evolution resulting in two E30 strains for the same lineage, L3_E30 and L3_E30bis. The cultures were regularly plated on TSA to visually check for contaminants.

Genome Sequencing and Mutation Analysis. DNA sequencing was performed on the JE2 strain, and the JE2-derived vancomycin adapted clones. Chromosomal DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen) and concentrations were normalized using a Qubit (Invitrogen). Sequencing libraries were prepared using the Nextera XT DNA sample preparation kit (Illumina). Paired-end sequencing was performed at Statens Serum Institut in Copenhagen using the NextSeq 550 system (Illumina). Data analysis was performed in CLC Genomics Workbench version 8.0. Reads were mapped against the *S. aureus* USA300_FPR3757 reference genome

(Genbank accession no. NC_07793). The frequency cut-off for variant calling was set at 30% and all the detected mutations were verified manually to ensure homogeneity of the variants across reads and avoid false detection due to imperfect mapping. The variants identified during alignment of JE2 to the *S. aureus* USA300_FPR3757 reference genome were excluded in the variant call for vancomycin-adapted clones. Detailed information on variant calling is presented in **Dataset S1**. We verified that the passaged populations were homogeneous by sequencing the whole population for L9 at E20 in addition to sequencing the single clone L9_E20. There were no additional mutations during sequencing of the L9 population compared to the single clone, suggesting that the populations are homogeneous (**Table S9**). However, we detected an additional mutation in the single clone L9_E20 that was not present in sequencing of the whole population, or in sequencing of the subsequent isolate L9_E30, which suggests that the additional mutation in L9_E20 was acquired during isolation of the single clone from the population prior to sequencing. The mutation is a synonymous mutation in the gene *relA*.

Construction of plasmids and strains. All plasmid constructs were cloned in *E. coli* IM08B (2) then transformed into *S. aureus* strain JE2. For expression of *vraX*, SAUSA300_RS02980, SAUSA300_RS08750, *sceD*, *vraR*, *vraS*, *vraT* and *vraU*, the genes including their predicted ribosomal binding site were cloned into the SalI and EcoRI sites of pSK9067 behind the IPTG-inducible P_{spac} promoter (3) using primer pairs listed in **Table S10**, concurrently removing the *gfp* reporter gene. The *vraT* and *walK* mutants were obtained by genetic recombination using the protocol by Monk and Stinear, 2021 (4). The *vraT* mutations present in clones L7_E30, L8_E20 and L10_E20 were amplified with 700bp flanking sequences. Due to gene essentiality, the entire sequence of *walK* was amplified with equal-sized flanking regions upstream and downstream of the mutations present in clones L1_E20 and L7_E20. The inserts were cloned into pIMAY-Z backbone using the SLiCE (Seamless Ligation Cloning Extract) ligation extract. After pIMAY-Z integration and plasmid excision, putative mutants were screened by sequencing.

Mutation co-occurrence analysis. To assess the co-occurrence of mutations across strains, we built a weighted graph in Networkx in which mutations were represented as nodes and co-occurrence of two mutations in strains were represented by edges. To inspect the chronology of mutation emergence, we constructed two separate weighted undirected graphs for each exposure level. The first graph accounted for mutations arising at E20 across 10 clones (L1 through L10), while the second graph accounted for mutations arising at E30 across 12 clones (L1 through L10, L3bis, L5bis and L9bis). Edges were weighted according to the reciprocal of the number of times two mutations co-occurred in the same strain. The Girvan-Newman method was then used to find communities in the graph. Communities are defined as a subset of nodes that share a significantly larger number of connections than with the rest of the network (5). In this specific case, communities represent sets of mutated loci that have a higher co-occurrence frequency. Thus, communities capture potential mutational trajectories at E20 and E30. Next, to represent the importance of a mutated locus within its community we calculated the harmonic centrality of each node. The harmonic centrality is described as the sum of the reciprocal of the shortest path distances $d(v,u)$ from all other nodes to node u.

$$C(u) = \sum_{\mathrm{v} \neq \mathrm{u}} \frac{1}{d(v,u)} \qquad \text{Eq (1)}$$

In other words, a mutated locus is more central when it occurs across a larger number of clones and co-occurs with a larger number of nodes and is therefore more closely connected to all other nodes. We proceeded to compute the edge betweenness centrality, which is a measure of the importance of an edge in a graph. Mathematically, it is described as the fraction of shortest paths $\sigma(s,t)$ in a graph that include edge e and is calculated for all edges.

$$C_B(\mathrm{e}) = \sum_{\mathrm{s},t \in \mathrm{V}} \frac{\sigma(s,t|e)}{\sigma(s,t)} \qquad \text{Eq (2)}$$

To highlight nodes and edges that significantly skew the distribution of computed centralities, we iteratively removed nodes and edges and re-computed centrality values for each sub-network. We

then ran a normality test for each z-scored distribution and highlighted the nodes/edges for which the p-value dropped below 0.05.

Independent Component Analysis of transcriptomic profile. RNA-sequencing and ICA pipeline used to calculate iModulon structure and activities have been in described in full detail in previous publications (6, 7). Here, normalized transcripts per million (TPM) from 33 new RNA-seq profiles were calculated concatenated to the previously published 108 profiles (7). The combined dataset was centered to strain-specific reference conditions. These conditions are labeled "USA300_TCH1516_U01-Set000_CAMHB_Control_1", "USA300_TCH1516_U01-Set000_CAMHB_Control_2" for TCH1516; and "USA300_LAC_U01-Set001_CAMHB_Control_1", "USA300_LAC_U01-Set001_CAMHB_Control_2" for LAC. The combined centered dataset was used for ICA decomposition with 100 iterations, and 10e-8 tolerance and 46 components.

ICA outputs an $\mathbf{S}_{(\text{genes x IC})}$ and an $\mathbf{A}_{(\text{components x samples})}$ matrix, corresponding to structure and activity of iModulons, respectively. The $\mathbf{S}_{(\text{genes x IC})}$ matrix lists the weights of each gene (rows) in each extracted component (columns). Most gene weightings in each of the independent components of S are normally distributed. However, weightings of genes in an iModulon, representing the signal for each component fall outside this normal distribution, i.e. the absolute value of their weights are higher than what would be expected of a normally distributed variable (6). To enrich these genes, we used Scikit-learn's implementation of the D'Agostino K2 test, which measures the 'normality' of sample distribution based on skew and kurtosis (8, 9). For each component in **S**, weights were converted to their absolute value, and genes were sorted by the absolute weightings in that component. Genes with the highest weighting were then removed iteratively until the D'Agostino K2 test for the remaining distribution fell below the previously tested cutoff value of 280. The outlier genes which were removed constitute the gene set of an iModulon.

The analysis yielded 40 robust independent components (IC, **Dataset S2**). We proceeded to annotate the 40 iModulons with biological annotations. As a first step we compared each iModulon

to assembled and curated annotations containing features such as known regulons, genomic islands, prophages etc (7). Next, we mapped each iModulon to a regulon by either comparing iModulon gene sets to differentially expressed genes in transcription factor knockout experiments or by implementing the RegPrecise algorithm (10–14). The iModulon genes for each component were compared to this database for significant overlap using Fisher's exact test with FDR of 0.05. If iModulon genes had any significant overlap with the features in the dataset with precision ≥ 0.5 and recall ≥ 0.2, the iModulon was annotated with the feature name e.g. VraR, φSa3, CcpA etc.

SI Figures:

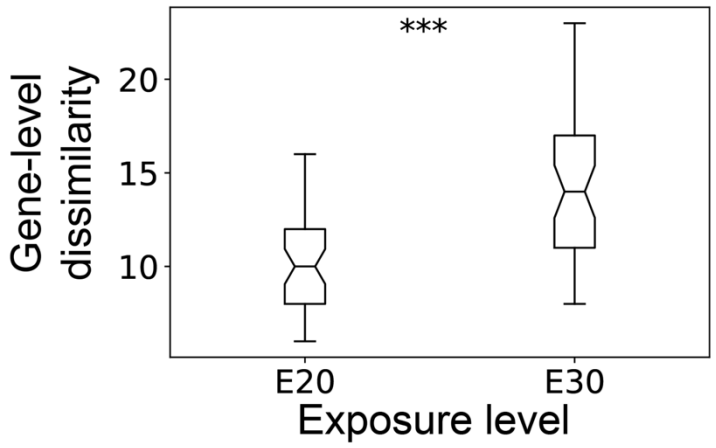
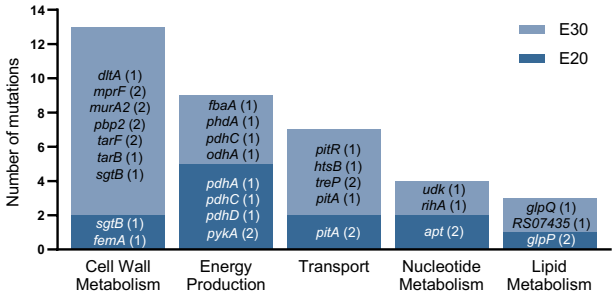


Figure S1: Mutational divergence indicated by increasing gene-level mutation dissimilarity across exposure levels. We compared the pairwise dissimilarity in the identity of mutated genes across exposure levels. On average, two clones differ by 9.7 +/-2.7 mutated genes at E20, and by 14.2 +/- 3.5 mutated genes at E30. A Student's t-test underscores a significance increase in dissimilarity in mutational profiles across evolutionary time ($p << 0.01$, statistic = -6.8). N.b., we excluded L3_E30, L5_E30 and L9_E30 profiles from our analysis, for the benefit of correctly accounting for mutations accumulated across L3_E30bis, L5_E30bis, and L9_E30bis, respectively, and to avoid the bias resulting from high similarity between replicate pairs.



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155 **Figure S2. Mutations in metabolism COG subsystems at E20 and E30.** The mutated genes in

156 each COG subsystem are presented in each graph bar. The number of times the gene was

157 mutated across lineages is shown in parentheses.

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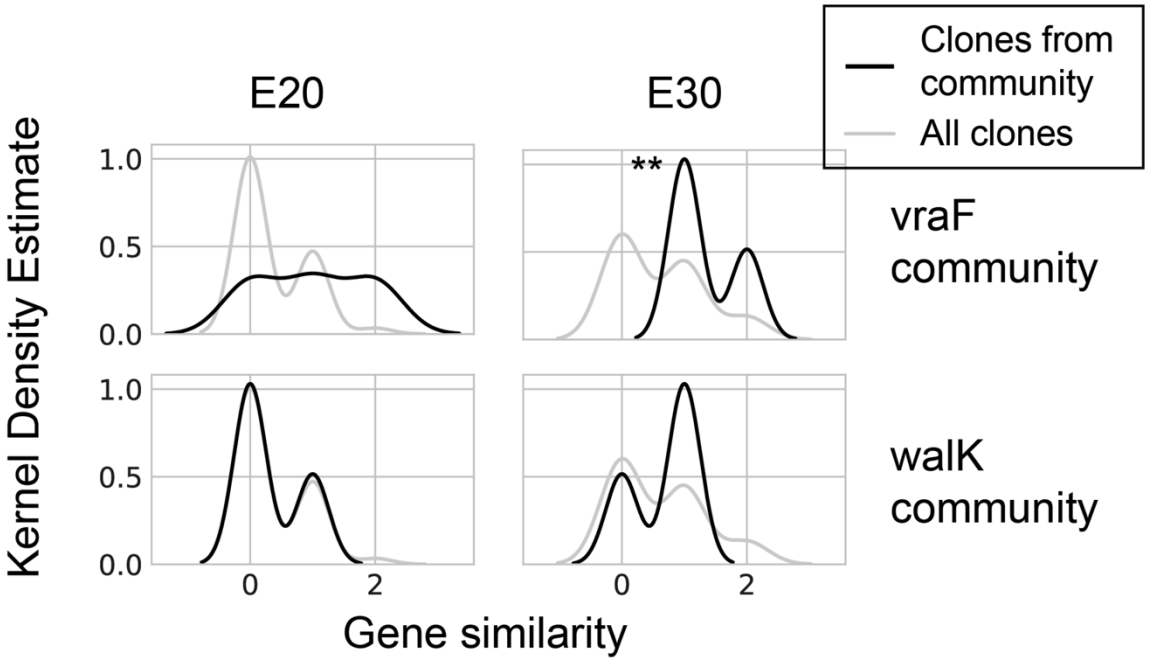


Figure S3: Gene level similarity across sub-communities of clones identified through network analysis of mutational patterns. We compare the gene level similarity (pairwise number of shared mutated genes) across the two sub-communities (in black, *vraF*: L2, L5 and L6, and *walK*: L4, L7 and L8) in comparison to the background (in grey, all strains) across exposure levels. Interestingly, we notice that while at E20, gene level similarities within the two sub-communities are comparable to background, the distribution is right shifted at E30, with a larger number of common genes being target by mutations within the respective sub-communities than in the background population. On average, two clones of the *walK* sub-community share 1.3 mutated genes (in contrast to only 0.6 for two random clones), while two clones from the *vraF* community share 0.66 mutated genes. We observe statistical significance for the *walK* community.

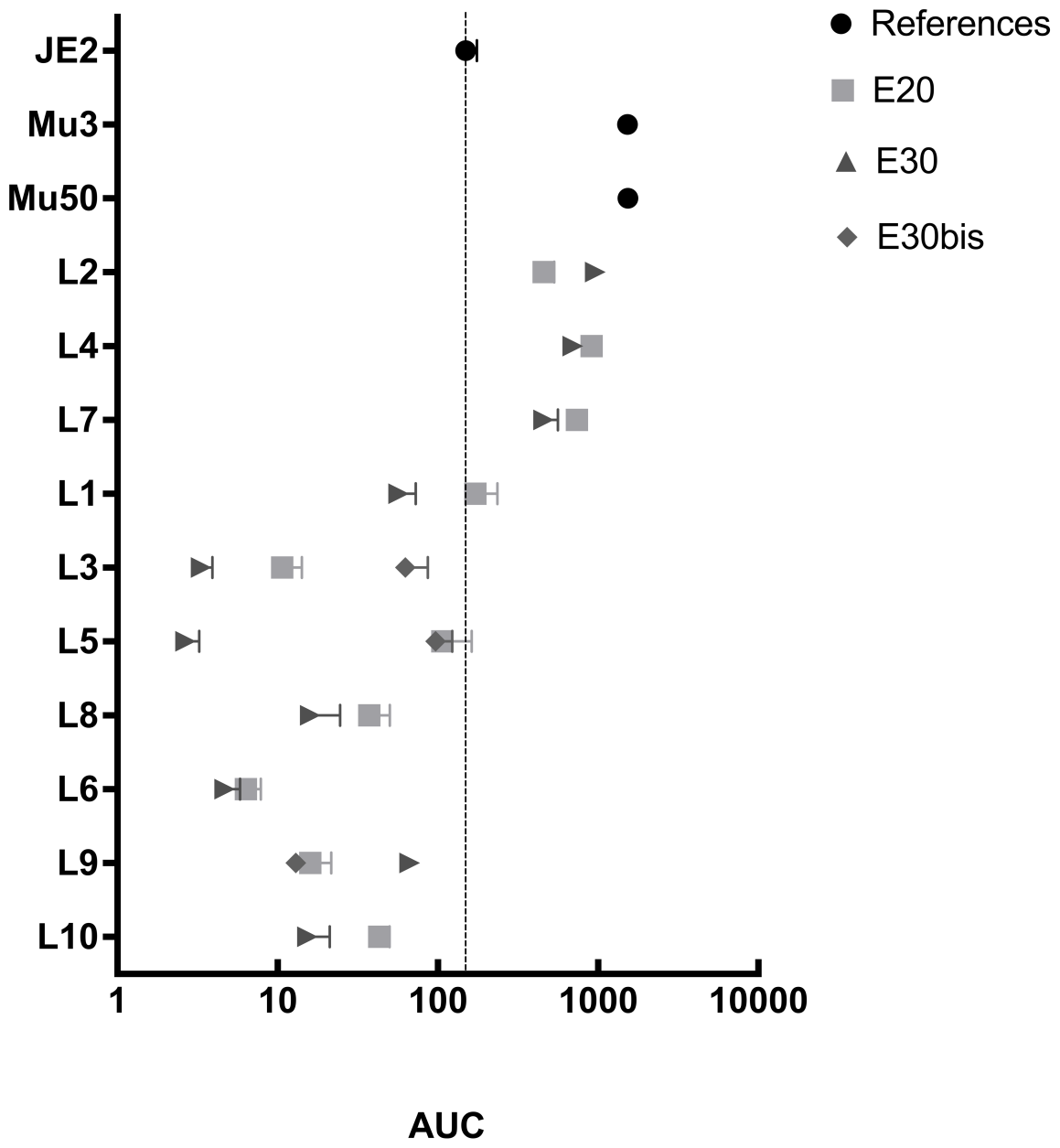
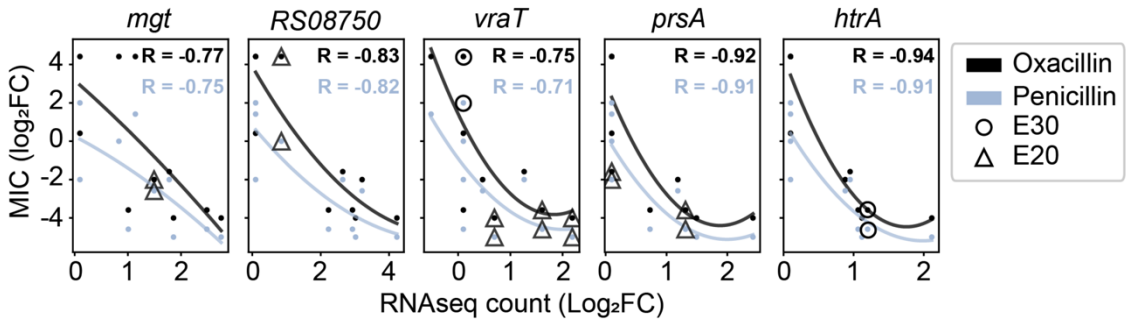


Figure S4. Oxacillin susceptibility of JE2-derived VISA strains by PAP-AUC. Each datapoint shows the mean AUC for 3 to 6 biological replicates. The error bars represent standard deviations.



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179 **Figure S5. Correlation between transcriptional profile of vancomycin-adapted strains and**

180 **antibiotic susceptibility.** Correlation between gene expression and antibiotic susceptibility (each

181 dot represents a clone, triangles are traced around the dots if the clone is mutated for that gene,

182 blue: penicillin, black: oxacillin.

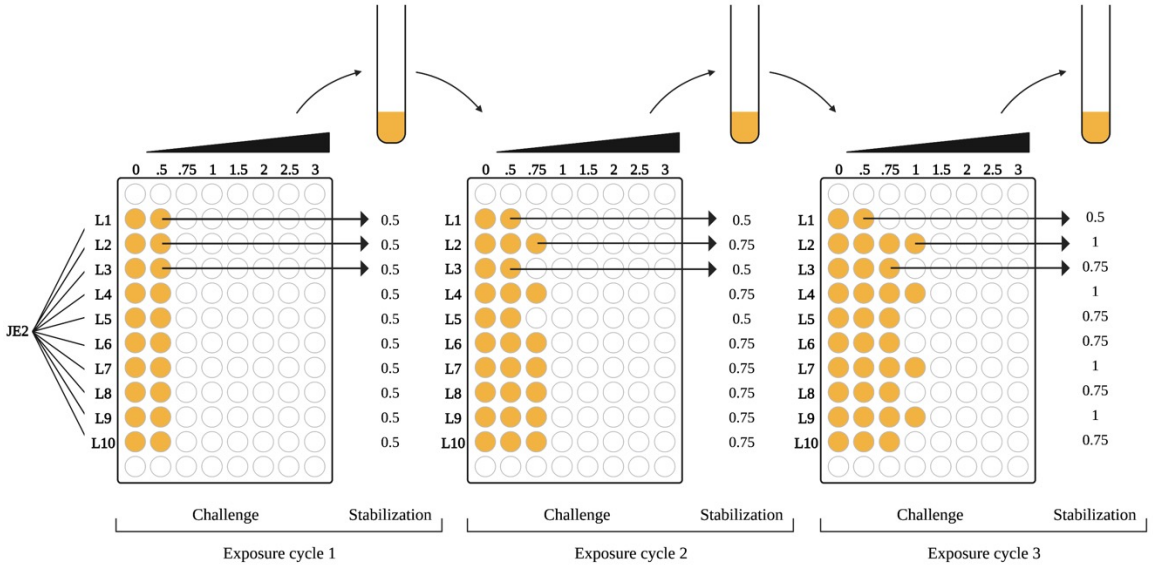

Figure S6. Illustration of vancomycin adaptive laboratory evolution. The MRSA strain JE2 was inoculated in 10 parallel cultures and adapted to vancomycin following a two-step protocol. In the first step, the cultures were challenged with increasing concentrations of vancomycin in 96-well plates. The plates were incubated at 37°C overnight. Aliquots from the highest concentration where growth was observed was used to inoculate tubes containing fresh media and sub-inhibitory concentrations of vancomycin. This second step was used to stabilize any possible adaptation from the vancomycin challenge. The cultures were incubated at 37°C overnight and used to start a new exposure cycle. Yellow coloring represents bacterial growth.

SI Tables:

Table S1. Phenotypic characterization of vancomycin adapted JE2 clones at timepoints E10, E20 and E30. Doubling time expressed in minutes. Vancomycin and oxacillin susceptibility tested using population analysis profile – area under the curve (PAP-AUC).

	Doubling time (min)				PAP-AUC Vancomycin (ratio to Mu3)				PAP-AUC Oxacillin (AUC)			
JE2 (MRSA, ancestor)	31				0.6				149			
Mu3 (hVISA)	34				1				1528			
Mu50 (VISA)	45				3.04				1535			
	E10	E20	E30	E30bis	E10	E20	E30	E30bis	E10	E20	E30	E30bis
L1	35	40	41		0.89	1.07	1.77		177	171	57	
L2	39	43	46		1.42	1.86	2.45		559	457	962	
L3	35	38	45	54	1.02	1.19	2.15	2.42	17	11	3	63
L4	35	47	51		1.41	1.88	2.74		97	910	694	
L5	38	38	44	41	1.00	1.20	2.39	1.45	103	106	3	97
L6	37	48	51		0.90	1.65	1.69		111	6	5	
L7	32	37	40		0.87	1.22	2.04		263	738	457	
L8	37	44	48		1.20	2.42	3.16		50	37	16	
L9	34	41	44	47	1.22	2.47	2.88	3.07	160	16	67	13
L10	35	38	42		1.34	1.40	2.15		63	43	15	

Table S2. Type of mutations from sequencing timepoint E20 and E30 of *in vitro* vancomycin evolved strains. ORF: Open reading frame; SNP: Single nucleotide polymorphism.

	Mutations	Intergenic	ORFs	InDels	SNPs	Synonymous	Frameshift	Stop codon
E20	64	9	55	17	47	3	10	4
E30	109	11	98	25	84	3	13	5

Table S3. Most mutated genes in JE2-derived hVISA and VISA lineages. Genes/adjacent genes/operons mutated in more than one lineage. Bold: mutations in E30 not present in E20; underlined: mutations in E20 not present in E30.

Gene	Locus	Lineages mutated			
vraT	SAUSA300_RS10195	L8	L9	L10	**L7**
/	SAUSA300_RS04225	L1	L5	**L2**	**L10**
graS	SAUSA300_RS03465	**L1**	**L3bis**		
vraF	SAUSA300_RS03470	L9	**L3**	**L5**	**L10**
vraG	SAUSA300_RS03475	**L7**			
pitR	SAUSA300_RS03480	L4			
pitA	SAUSA300_RS03485	L4	L8	**L3**	
ssaA	SAUSA300_RS12415	L3	L5	L10	
autolysin	SAUSA300_RS09395	L3	L6	**L9**	
walKR	SAUSA300_RS00110	L4	L7	L1	
	SAUSA300_RS00105	L8			
	SAUSA300_RS00115	L8			
rpoB	SAUSA300_RS02820	L6			
rpoC	SAUSA300_RS02825	L2	L9		
rpoD	SAUSA300_RS08300	L2			
pdhC	SAUSA300_RS05360	L6	**L3**		
pdhA	SAUSA300_RS05350	L5			
pdhD	SAUSA300_RS05365	L8			
puuR	SAUSA300_RS05375	L5			
pykA	SAUSA300_RS08975	L4	L7		
apt	SAUSA300_RS08670	L1	L10		
mprF	SAUSA300_RS06820	**L4**	**L8**		
rseP	SAUSA300_RS06255	L2	L6		
pbp2	SAUSA300_RS07315	**L1**	**L9**		
prsA	SAUSA300_RS09800	L3	L9		
tarF	SAUSA300_RS01325	L8	L9		
murA	SAUSA300_RS11440	**L7**			
fbaA	SAUSA300_RS11445	**L6**			

206 **Table S4. TCS associated genes mutated in E20 and E30.** We investigated the mutations which
207 were classified under cellular processes and signaling. We focused on the set of core *S. aureus*
208 two-component systems (15), and collected recently determined regulons for each of eight two-
209 component systems.

Regulator	Locus tag	Name	E20	E30
vraRST	SAUSA300_RS10135	*mgt*	1	2
	SAUSA300_RS08750	/	1	1
	SAUSA300_RS11440	*murA2*	0	1
	SAUSA300_RS09800	*prsA*	2	4
	SAUSA300_RS10195	*vraT*	4	7
saeRS	SAUSA300_RS12415	*ssaA*	4	7
walKR	SAUSA300_RS00105	*walR*	1	1
	SAUSA300_RS09395	autolysin	2	4
	SAUSA300_RS05375	*puuR*	1	2
	SAUSA300_RS09800	*prsA*	2	4
	SAUSA300_RS12415	*ssaA*	4	7
	SAUSA300_RS08750	/	1	1
	SAUSA300_RS00110	*walK*	3	3
	SAUSA300_RS10500	MAP domain	0	1
graSR	SAUSA300_RS03475	*vraG*	0	1
	SAUSA300_RS00105	*walR*	1	1
	SAUSA300_RS06255	*rseP*	2	2
	SAUSA300_RS10590	phiNM3(p)	0	1
	SAUSA300_RS04515	*dltA*	0	1
	SAUSA300_RS03470	*vraF*	1	5
	SAUSA300_RS02925	*sdrE*	0	1
	SAUSA300_RS02625	*ftsH*	0	1
	SAUSA300_RS03465	*graS*	0	2
	SAUSA300_RS01170	*glpQ*	0	1
	SAUSA300_RS06890	*femA*	1	1
nreABC	SAUSA300_RS03665	*fruR*	1	1
sarA	SAUSA300_RS03665	*fruR*	1	1
lytSR	SAUSA300_RS02825	*rpoC*	2	3
	SAUSA300_RS02820	*rpoB*	1	1
	SAUSA300_RS07105	*odhA*	0	1
	SAUSA300_RS06375	*rny*	1	2
	SAUSA300_RS05520	*ylbN*	0	1
arlRS	SAUSA300_RS06375	*rny*	1	2
	SAUSA300_RS12570	/	0	1
	SAUSA300_RS11315	*murA2*	0	1

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Table S5. Communities extracted from two co-occurrence graphs constructed at E20 and E30. Genes included are targeted by a mutation in at least one clone.

	E20	***E30***
WalK community	*pdhD*, *walK*, *pitR*, *pitA*, SAUSA300_RS13540, SAUSA300_RS10925, *walR*, *glpP*, *vraT*, SMSAP5(p1), SAUSA300_RS06395, SAUSA300_RS09680, *stp1*, *fruR*, *rsbW*, *dnaQ*, *pykA*	*pitA*, TarF_1, *walR*, *glpP*, SMSAP5(p1), PepSY domain p1, SAUSA300_RS09680, *dnaQ*, *vraG*, *vraT*, *stp1*, *fruR*, *walK*, *tcaR*, SAUSA300_RS10925, *ebh*, SAUSA300_RS06395, *pykA*, *pdhD*, SAUSA300_RS13540, *yqfL*, *mprF*, *murA2*, *pitR*, *rsbW*
VraF community	*vraF*, *rny*, *puuR*, SAUSA300_RS04225, *rpoC*, *gdpP*, *ysdC*, *relA*, *apt*, *prsA*, *ssaA*, *sgtB*, SAUSA300_RS11565, *sagB*, SAUSA300_RS08750, *pdhC*, *rpoD*, *pdhA*, *rpoB*, *mfd*, *rseP*, *femA*	SAUSA300_RS03100, *vraF*, *rny*, *puuR*, *rihA*, SAUSA300_RS04225, *ylbN*, SAUSA300_RS05040, *rpoC*, *gdpP*, SAUSA300_RS08675, *fbaA*, *ysdC*, *relA*, *apt*, *prsA*, SAUSA300_RS08095, *graS*, *ssaA*, *odhA*, *sgtB*, *sdrE*, *pbp2*, *sagB*, SAUSA300_RS11565, T2SS, SAUSA300_RS08750, *rpoD*, *pdhC*, *rlmH*, *pdhA*, *rpoB*, *mfd*, *rseP*, *femA*, TsaD, *dltA*
WalK community	L1_E20, L10_E20, L4_E20, L9_E20, L7_E20, L8_E20	L9_E30bis, L7_E30, L3_E30, L8_E30, L10_E30, L4_E30, L1_E30
VraF community	L2_E20, L3_E20, L1_E20, L10_E20, L6_E20, L9_E20, L5_E20	L9_E30bis, L2_E30, L6_E30, L3_E30, L5_E30, L5_E30bis, L10_E30, L3_E30bis, L1_E30

Table S6. Effect of vancomycin-adaptive *vraT* and *walK* mutations on vancomycin and oxacillin susceptibility. Mutations in *vraT* and *walK* were amplified from JE2-derived VISA isolates (L7_E30, L8_E20, L10_E20, L1_E20 and L7_E20) and inserted into the chromosome of WT strain JE2. Vancomycin and oxacillin MIC was determined using Etests.

	Vancomycin MIC (mg/L)	Oxacillin MIC (mg/L)
JE2	0.75	16
JE2*vraT*$_{L7\_E30}$	1	256
JE2*vraT*$_{L8\_E20}$	1.5	256
JE2*vraT*$_{L10\_E20}$	1	256
JE2*walK*$_{L1\_E20}$	1	1
JE2*walK*$_{L7\_E20}$	1	1.5

Table S7. Overexpression of selected genes in MRSA strain JE2. Genes were cloned in plasmid pSK9067 (3) behind the IPTG-inducible promoter P_{spac}. Vancomycin and oxacillin MIC was determined using Etests in presence or absence of IPTG.

	Vancomycin MIC		Oxacillin MIC	
	no IPTG	IPTG 800µM	no IPTG	IPTG 800µM
JE2p*gfp* (control)	0.75	0.75	16	16
JE2p*vraX*	0.75	0.75	12	16
JE2p*cwrA*	0.75	0.75	16	16
JE2pRS02980	0.75	0.75	16	24
JE2pRS08750	0.75	0.75	16	12
JE2p*sceD*	0.75	0.75	16	16
JE2p*vraR*	0.75	0.75	16	64
JE2p*vraS*	0.75	0.75	12	16
JE2p*vraT*	0.75	0.75	12	12
JE2p*vraU*	0.75	0.75	12	12

226 **Table S8. Strains used in the study.** (16–20)

Bacterial strains	Description		Source
S. aureus laboratory strains			
JE2	Plasmid-cured derivative of MRSA strain USA300 LAC		16
L1, L2, L3,…, L10	Vancomycin-adapted lineages independently evolved from JE2		This study
L1_E10 to L10_E10	Vancomycin-adapted JE2 clones from 10 rounds of *in vitro* passaging		This study
L1_E20 to L10_E20	Vancomycin-adapted JE2 clone from 20 rounds of *in vitro* passaging	Derived from E10 clones	This study
L1_E30 to L10_E30	Vancomycin-adapted JE2 clone from 30 rounds of *in vitro* passaging	Derived from E20 clones	This study
N315.1 to N315.5	Vancomycin-adapted N315 clone from 20 rounds of *in vitro* passaging		This study
110822.1 to 110822.5	Vancomycin-adapted 110822 clone from 20 rounds of *in vitro* passaging		This study
109384.1 to 109384.5	Vancomycin-adapted 109384 clone from 20 rounds of *in vitro* passaging		This study
109652.1 to 109652.5	Vancomycin-adapted 109652 clone from 20 rounds of *in vitro* passaging		This study
110788.1 to 110788.5	Vancomycin-adapted 110788 clone from 20 rounds of *in vitro* passaging		This study
19902.1 to 19902.5	Vancomycin-adapted 19902 clone from 20 rounds of *in vitro* passaging		This study
JE2*vraT*$_{L7\_E30}$	JE2 with *vraT* mutation Thr8Met from L7_E30		This study
JE2*vraT*$_{L8\_E20}$	JE2 with *vraT* mutation Ser7Leu from L8_E20		This study
JE2*vraT*$_{L10\_E20}$	JE2 with *vraT* mutation Glu375_Ala377del from L10_E20		This study
JE2*walK*$_{L1\_E20}$	JE2 with *walK* mutation Asn335_E336insAspSerPheLeuLeuAspLeuAsn from L1_E20		This study
JE2*walK*$_{L7\_E20}$	JE2 with *walK* mutation Ser273Asn from L7_E20		This study
JE2p*gfp*	JE2 pSK9067_*gfp* - original plasmid carrying the *gfp* gene behind IPTG-inducible Pspac promoter		This study
JE2p*vraX*	JE2 pSK9067_*vraX* - plasmid obtained by cloning the *vraX* gene behind IPTG-inducible P_{spac} promoter		This study
JE2pRS02980	JE2 pSK9067_RS02980 - plasmid obtained by cloning SAUSA300_RS02980 behind IPTG-inducible Pspac promoter		This study
JE2pRS08750	JE2 pSK9067_RS08750 - plasmid obtained by cloning SAUSA300_RS08750 behind IPTG-inducible Pspac promoter		This study
JE2p*sceD*	JE2 pSK9067_*sceD* - plasmid obtained by cloning the *sceD* gene behind IPTG-inducible Pspac promoter		This study
JE2p*vraR*	JE2 pSK9067_*vraR* - plasmid obtained by cloning the *vraR* gene behind IPTG-inducible Pspac promoter		This study
JE2p*vraS*	JE2 pSK9067_*vraS* - plasmid obtained by cloning the *vraS* gene behind IPTG-inducible Pspac promoter		This study
JE2p*vraT*	JE2 pSK9067_*vraT* - plasmid obtained by cloning the *vraT* gene behind IPTG-inducible Pspac promoter		This study
JE2p*vraU*	JE2 pSK9067_*vraU* - plasmid obtained by cloning the *vraU* gene behind IPTG-inducible Pspac promoter		This study
S. aureus clinical isolates			
Mu50	VISA clinical isolate from Japanese hospital in 1996		17
Mu3	hVISA clinical isolate from Japanese hospital in 1996		18
N315	MRSA clinical isolate from Japanese hospital in 1982		19
110822	Clinical isolate		20
109384	Clinical isolate		20
109652	Clinical isolate		20
110788	Clinical isolate		20

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Table S9. Assessment of population heterogeneity by sequencing L9 mixed population at E20 and single clone L9_E20. An aliquot sampled from population L9 at E20 during serial passaging, and a single clone isolated from this population, were used for genome sequencing.

	Region	Gene	Locus tag	Old locus tag	Type	Reference	Allele	Length	Coverage	Frequency	Average quality	AA change
L9_E20 single colony	592140	*rpoC*	SAUSA300_RS02825	SAUSA300_0528	SNV	C	T	1	383	99	33	Arg958Cys
	610942	/	/	/	SNV	G	A	1	18	100	30	
	721032	*vraF*	SAUSA300_RS03470	SAUSA300_0647	SNV	C	T	1	89	99	34	Ser55Leu
	1131799	/	/	/	SNV	T	C	1	75	100	33	
	1959544	/	/	/	SNV	G	T	1	67	100	33	
	2027481	*vraT*	SAUSA300_RS10195	SAUSA300_1867	SNV	T	C	1	127	99	34	Glu156Gly
	2027645	*vraT*	SAUSA300_RS10195	SAUSA300_1867	Deletion	T	-	1	131	94	33	Val102fs
	2586148	/	SAUSA300_RS13290	SAUSA300_2400	SNV	C	T	1	195	100	34	
	1972154..1972225	*prsA*	SAUSA300_RS09800	SAUSA300_1790	Deletion			72				Leu204_Glu227del
	1740991	*relA*	SAUSA300_RS08665	SAUSA300_1590	SNV	G	A	1	128	100	34	*Synonymous*
L9_E20 population	592140	*rpoC*	SAUSA300_RS02825	SAUSA300_0528	SNV	C	T	1	345	99	32	Arg958Cys
	610942	/	/	/	SNV	G	A	1	24	100	36	
	721032	*vraF*	SAUSA300_RS03470	SAUSA300_0647	SNV	C	T	1	80	100	33	Ser55Leu
	1131799	/	/	/	SNV	T	C	1	72	99	32	
	1959544	/	/	/	SNV	G	T	1	70	100	32	
	2027481	*vraT*	SAUSA300_RS10195	SAUSA300_1867	SNV	T	C	1	134	100	34	Glu156Gly
	2027645	*vraT*	SAUSA300_RS10195	SAUSA300_1867	Deletion	T	-	1	138	96	34	Val102fs
	2586148	/	SAUSA300_RS13290	SAUSA300_2400	SNV	C	T	1	182	99	32	
	1972154..1972225	*prsA*	SAUSA300_RS09800	SAUSA300_1790	Deletion			72				Leu204_Glu227del

Table S10. Oligonucleotides used in the study.

Name	Sequence 5'-3'
vraX-FW_SalI	GATACAGTCGACGCAAAGGAGGTAATATAGGTTATG
vraX-RV_EcoRI	GATACAGAATTCTTAACTAACTTTCATATGATCTATATCGTC
cwrA-FW_SalI	GATACAGTCGACATATAAAGGAGTATGATAGCGATGAG
cwrA-RV_EcorI	GATACAGAATTCTTAAAAGAAATCAGATGGGTTAAATTC
RS02980-FW_SalI	GATACAGTCGACAATGTTAGGATGTAAATATGTCTTAGAG
RS02980-RV_EcorI	GATACAGAATTCTCAATATCCCTCACTCAATGTAAAC
RS08750-FW_SalI	GATACAGTCGACCATTGGAGGCGAACTATGAG
RS08750-RV_EcorI	GATACAGAATTCCTATTGATAAGCATTTTCAGATTTTAGTT
sceD-F_SalI	GATACAGTCGACGAGAAACAAATTACTTGTAGGAG
sceD-R_EcorI	GATACAGAATTCTTATGCAGTAACCCAATGTCC
vraR-FW_SalI	GATACAGTCGACAATAAGGAGGATTCGTATGACG
vraR-RV_EcorI	GATACAGAATTCCTATTGAATTAAATTATGTTGGAATGC
vraS-FW_SalI	GATACAGTCGACTATCGGAGACGTAGAGGTG
vraS-RV_EcorI	GATACAGAATTCTTAATCGTCATACGAATCCTC
vraT-FW_SalI	GATACAGTCGACATAGAAAGGCGGCGAAAC
vraT-RV_EcorI	GATACAGAATTCTCATCGATAAATCACCTCTACG
vraU-FW_SalI	GATACAGTCGACTAAAAGGTGATAGTTATGAACTATGTTG
vraU-RV_EcorI	GATACAGAATTCCTATGCCACAGCGTTCA
pSK9067_FW	ATGAACCAAGACAGCATCG
pSK9067_RV	ATGCGTAAGGAGAAAATACCG
walK_L1_L7_FW	CCTCACTAAAGGGAACAAAAGCTGGGTACCATGAAGTGGCTAAAACAACTAC
walK_L1_L7_RV	CGACTCACTATAGGGCGAATTGGAGCTCTTATTCATCCCAATCACCGTC
vraT_L7_L8_FW	CCTCACTAAAGGGAACAAAAGCTGGGTACCTGTTGCTCAAATTGAGCATAC
vraT_L7_L8_L10_RV	CGACTCACTATAGGGCGAATTGGAGCTCTCATCGATAAATCACCTCTACG
vraT_L10_FW	CCTCACTAAAGGGAACAAAAGCTGGGTACCGCACTACCTTTTAATGCTAGATG

SI Datasets:

Dataset S1. Mutations from sequencing timepoint E20 and E30 of *in vitro* vancomycin evolved strains. Mutations highlighted in blue were present in E20 and conserved in E30. Mutations highlighted in yellow were present in E20 and lost in E30. Mutations not highlighted are mutations acquired between E20 and E30. (Excel file)

Dataset S2. Feature table for the iModulon structure extracted from the transcriptomic data using independent component analysis. ICA yields an activity matrix, which represents the activity level of each independent component in each strain, and a weight matrix which contains the weight of each gene across each independent component. (Excel file)

Dataset S3. Expression in E20 strains for selected cell wall and VISA genes. (Excel file)

Dataset S4. Spearman correlation between gene expression levels and antibiotic MIC. Gene expression-antibiotic links are filtered for Spearman R > 0.7. (Excel file)

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