Evolution under vancomycin selection drives divergent collateral sensitivity patterns in *Staphylococcus aureus*

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Abstract

Staphylococcus aureus bacteremia is typically treated empirically with vancomycin, with therapy later tailored based on susceptibility results. However, these tests occur before vancomycin exposure and do not account for adaptation during empiric treatment that can alter *S. aureus*' susceptibility to first-line drugs. To investigate these collateral drug responses, we experimentally evolved 18 methicillin-susceptible *S. aureus* (MSSA) populations under increasing vancomycin concentrations until they achieved intermediate resistance. Genomic sequencing revealed two distinct adaptive pathways characterized by mutations in the WalKR regulon, affecting cell wall metabolism, or *rpsU*, impacting translational stress responses. These pathways correlated with divergent collateral sensitivity profiles to first-line antibiotics. By developing a Collateral Response Score (CRS), we quantified the probability and magnitude of these responses, demonstrating that evolutionary dynamics critically influence resistance outcomes. Our findings suggest a probabilistic approach to antimicrobial therapy, advocating for rapid genomic diagnostics alongside susceptibility testing to better anticipate and respond to evolutionary changes.

Significance

Antibiotic treatment can influence bacterial evolution, altering the effectiveness of subsequent therapies by inducing collateral resistance or sensitivity. This study reveals that evolution toward vancomycin-intermediate resistance in the pathogen *Staphylococcus aureus* proceeds through at least two distinct evolutionary pathways: one characterized by alterations in cell wall metabolism and another by changes in global stress response. These adaptive trajectories result in contrasting collateral sensitivities to first-line antibiotics. By introducing the Collateral Response Score, we assess the uncertainty in these outcomes, providing a probabilistic framework to evaluate how past antibiotic exposure shapes future treatment responses. Further validation studies are needed; however, we believe that improved forecasting of pathogen evolution can enhance antibiotic stewardship, inform therapeutic decisions, and ultimately improve patient outcomes.

Introduction

Staphylococcus aureus is a Gram-positive pathogen often responsible for severe infections, including endocarditis, osteomyelitis, soft tissue infections, and device-associated infections (1–4). It typically enters the bloodstream through various routes, often via a wound, surgical site, or catheter, frequently leading to bacteremia (1, 3). When clinicians suspect a severe Staphylococcal infection, they commonly initiate treatment with broad-spectrum antibiotics, such as vancomycin, to address the possibility of methicillin-resistant *S. aureus* (MRSA). Blood culture results can take several days to return, at which time therapy may be adjusted based on the detected organism and its susceptibility profile. Currently, antistaphylococcal penicillins, including nafcillin and oxacillin, are recognized treatments for methicillin-susceptible *S. aureus* (MSSA) infections, and clinicians may administer these drugs if cultures exclude MRSA. However, we ask whether favorable susceptibility results alone provide a complete picture. This question rests on two key considerations.

First, the evolution of vancomycin-intermediate resistance during empiric treatment may affect *S. aureus*' susceptibility to other drugs (i.e., collateral drug responses). Intermediate resistance evolves via mutations in a diverse set of genes (5–8) and is associated with persistent infections and reduced treatment success (8). These mutations may alter MSSA's sensitivity to subsequent first-line therapies, even in the absence of prior exposure. For example, a large cohort study showed that the in-hospital mortality rate of cloxacillin-treated patients with MSSA correlated with vancomycin minimum inhibitory concentration (MIC) (9). MSSA should be susceptible to both cloxacillin and methicillin because these drugs have the same cellular target. Prior vancomycin adaptation is one possible contributing factor to these poor clinical outcomes.

Second, antibiotic susceptibility test results reflect a single snapshot in time, often before empiric antibiotic therapy, and are thus a lagging indicator of phenotypic state and agnostic to evolution. In current practice, clinicians sample a patient's blood and then administer broad-spectrum antibiotics. They isolate the causative agent of the infection from the blood cultures and rely on susceptibility tests to inform subsequent therapy. However, these tests provide information about the pathogen's susceptibilities before treatment and cannot account for any potential changes in its antibiotic response arising from intervening evolution under empiric therapy. Moreover, susceptibility results only reflect a subset of the bacterial population existing in a patient. Initial culture results may indicate that the infection is susceptible to an antistaphylococcal penicillin; however, this recommendation may be inaccurate, given that the infection has had the opportunity to evolve for several days (several hundred bacterial generations) in this new environment, potentially altering antibiotic susceptibility profiles in various ways (10, 11).

To address these issues, we investigated how empiric therapy impacts collateral drug sensitivities by experimentally evolving replicate MSSA populations under increasing vancomycin concentrations until they reached intermediate resistance levels. The populations exhibited diverse collateral responses to several first-line antibiotics. However, the lines broadly followed two adaptive routes under vancomycin selection, rendering some drug tradeoffs explicable by genetic background. Ideally, a treatment regimen for MSSA would include drugs that the infection has a high chance of being susceptible to, given its prior vancomycin exposure. Thus, background-specific interactions complicate therapy yet underscore the need for rapid and accurate genomic sequencing, alongside standard antimicrobial susceptibility tests, to inform therapeutic decisions. In that spirit, we propose the Collateral Response Score (CRS), a standardized metric that provides information about the direction and magnitude of changes in MIC.

Taken together, our results underscore the uncertainty and risk of not accounting for evolution when making therapeutic decisions. Instead, clinicians should anticipate that infections will evolve under empiric therapy, which might affect their susceptibility to first-line drug treatment. Our study, therefore, highlights the complexities of bacterial evolution and emphasizes that we should consider susceptibilities in a probabilistic light, just as we do with other inherently stochastic systems.

Results and Discussion

Evolution of vancomycin-intermediate resistance in MSSA

To model Staphylococcal evolution under empiric vancomycin therapy, we established 18 replicate MSSA populations from an ancestral clone of *S. aureus* subsp. *aureus* Rosenbach, a clinical isolate that is an international quality control standard. We transferred each population daily into a growth medium containing vancomycin. We gradually increased the concentration until the lines reached intermediate resistance levels (**Fig. 1*A***). Additionally, 87 control lines were established from the same ancestral strain and propagated under identical conditions except in the absence of vancomycin. It is important to note that controls in experimental evolution differ from those used in other fields of biology. When the null hypothesis is that an expected effect will or will not occur based on the presence of an experimental perturbation, single or paired controls make sense. Here, however, our null hypothesis is not that the control lines will have a specific, single outcome different from the experimental conditions, but rather that they will display an ensemble of outcomes that differs from those of the experimental group. In this study, we therefore examined the differences in the distributions of these mutational outcomes.

The 18 vancomycin-exposed lines had an initial average MIC of 1.3 µg/mL (standard deviation, *SD* = 0.3) and subsequently evolved to an average MIC of 5.4 µg/mL (*SD* = 0.5) (**Fig. 1*B***). The time required for the lines to increase their MIC varied significantly. It took an average of 9 days (*SD* = 2), with population 14 achieving vancomycin-intermediate resistance in just six days, while population 13 required 13 days to reach the same resistance level.

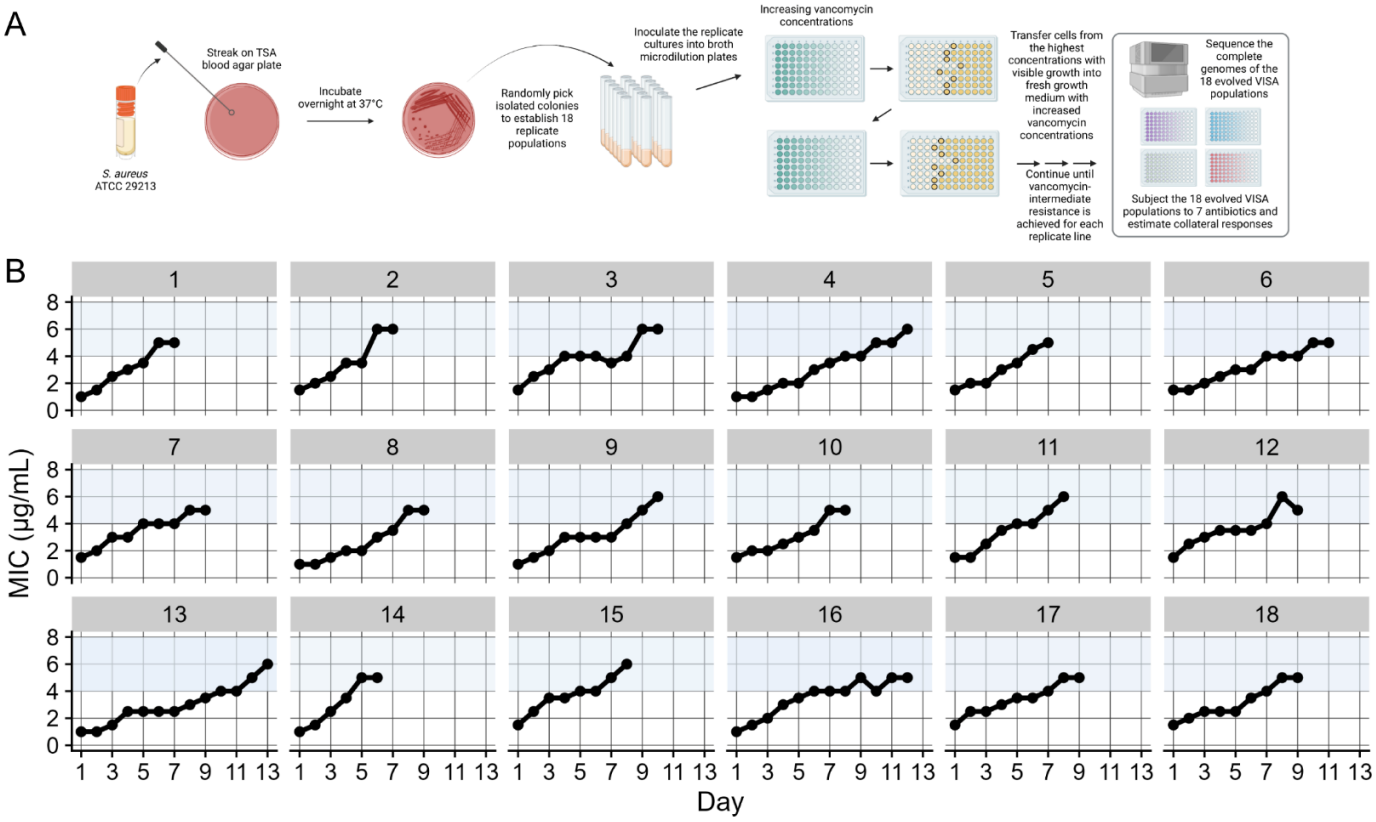


Fig. 1. Schematic illustration of our study design and evolution of intermediate vancomycin resistance. (*A*) We established 18 independent populations from a methicillin-susceptible *S. aureus* (MSSA) strain and experimentally evolved them under increasing vancomycin concentrations until they reached intermediate resistance levels. Additionally, we established 87 replicate control lines from the same ancestral strain and transferred them under identical conditions except in the absence of vancomycin (not shown). We then performed whole-genome sequencing on all evolved vancomycin-adapted and control populations, and examined the susceptibilities of the vancomycin-adapted lines to seven drugs used in the treatment of MSSA. **(*B*)** By day 13 of the evolution experiment, all vancomycin-adapted populations exhibited intermediate resistance, as defined by the Clinical and Laboratory Standards Institute (CLSI), characterized by a minimum inhibitory concentration (MIC) between 4 and 8 µg/mL (blue-shaded regions).

139 **Populations follow distinct adaptive pathways under vancomycin selection**

140 Next, we sequenced the ancestor and experimentally evolved populations to investigate the
141 genetic changes associated with vancomycin-intermediate resistance. The resistant and control
142 lines had 4,921 mutations at a frequency of ≥5% (**Fig. 2*A***): 81.9% were single-base substitutions,
143 and 14% were intergenic mutations located within 150 bp upstream of a gene, suggesting that
144 they affect regulation. Structural variants were rare, and gene amplification was almost non-
145 existent, likely due to the low abundance of repeat regions that mediate tandem amplifications
146 (12).

147 To assess the impact of vancomycin exposure on genomic parallelism, we compared the
148 gene-level similarity of mutations among independent lineages that evolved under vancomycin
149 selection with those that evolved under permissive, drug-free conditions. As described in the
150 *Materials and Methods*, we computed Dice's similarity coefficient for each pair of populations
151 using only the 3,812 qualifying mutations that unambiguously affected a single gene. The
152 vancomycin-adapted (VISA) lines exhibited a higher average pairwise similarity (S_v = 0.063)
153 compared to the control lines (S_c = 0.029); that is, on average, two independently evolved
154 populations under vancomycin selection shared 6.3% of their mutated genes, whereas lines that
155 evolved without drug exposure shared only 2.9%. A permutation test confirmed that this difference
156 was significant (P < 0.0001), demonstrating that vancomycin selection decisively promotes
157 greater genomic parallelism relative to permissive conditions.

158 Multivariate logistic regression identified several genes associated with vancomycin
159 exposure. First, *vraS* and *liaF* genes were the most frequently mutated in our lines (**Fig. 2*B***). They
160 occurred in seven out of 18 VISA populations but were absent in all 87 control lines (**Fig. 2*C***).
161 LiaF and VraS regulate the cell membrane stress response (13, 14) and are prevalent in clinical
162 VISA isolates (15). Second, *rpsU* mutations targeting the 30S ribosomal subunit occurred in six
163 VISA lines, and they were also absent in any control population. Ribosomal perturbation can
164 activate stress response in some bacteria. For example, *Listeria monocytogenes rpsU* mutants
165 exhibit upregulated σ^B-mediated stress genes and slower growth, trading fitness for broad stress
166 resistance (16). In our lines, *rpsU* mutations may similarly impact global regulation, resulting in an
167 elevated cell membrane stress response and reduced cell wall turnover, which can increase
168 vancomycin and daptomycin resistance (12, 17). Indeed, *rpsU* deletion mutants often require
169 compensatory mutations to restore fitness, consistent with this gene's role in core cellular
170 processes (16). Third, *yycH*, *yycI*, *walK*, and *atl* mutations repeatedly occurred in our evolved
171 VISA lines. These genes comprise the WalKR regulon, an essential cell-wall metabolic pathway
172 in *S. aureus* (**Fig. 2*D***) (18, 19). Loss-of-function mutations in any of these regulon genes cause

173 increased cell wall teichoic acid content, resulting in a thicker and more resistant cell wall against
174 vancomycin and daptomycin (19, 20). This finding is consistent with our results and an earlier
175 study, which showed that mutations in *walKR* cause increased resistance to these antibiotics in
176 clinical VISA strains (5). Together, these results illustrate that vancomycin selection promoted
177 strong genomic parallelism by driving repeated mutations in a limited set of genes primarily
178 involved in cell wall metabolism and general stress response, raising the question of whether
179 evolutionary constraints influenced these adaptive trajectories.

180 Given these observations, we next investigated whether selection for mutations in specific
181 pathways constrained the evolution of mutations in others, specifically, whether interactions
182 between mutations shaped distinct adaptive trajectories. Bayesian latent class analysis (BLCA)
183 — which considered all vancomycin-associated genes that occurred at least three times across
184 lines and without bias toward predefined functional pathways (e.g., the WalKR regulon) —
185 identified two distinct clusters among the 18 VISA populations, both defined by shared gene-level
186 mutations (**Fig. S2**). Notably, all populations in Cluster 2 evolved mutations in *yycH* and uniformly
187 lacked *rpsU* mutations, whereas six of the twelve populations in Cluster 1 harbored *rpsU*
188 mutations without co-occurring *yycH* alterations. Although the mutual exclusivity between *yycH*
189 and *rpsU* mutations was marginally nonsignificant by Fisher's exact test (P = 0.0537), these
190 findings suggest genomic divergence.

191 Two distinct adaptive strategies became apparent, however, when we extended our
192 analysis to the functional level, grouping genes by shared biological role. One adaptive strategy
193 modulated cell wall architecture via WalKR regulon mutations, while the other involved a broader
194 reprogramming of translational stress-response pathways mediated by *rpsU* mutations (**Fig. 2*D***).
195 In fact, near complete mutual exclusivity was observed between populations with WalKR regulon
196 mutations and those with *rpsU* mutations: 14 and six lines harbored WalKR regulon and *rpsU*
197 mutations, respectively, with an overlap in only two lines. A Fisher's exact test confirmed this
198 negative association (P = 0.0049). Moreover, 13 of the 14 regulon-mutated lines had a single
199 mutation in this pathway, while the remaining population had three mutations. The probability of
200 this distribution occurring by chance is < 0.0001. WalKR is the only essential two-component
201 system in *S. aureus*, as it controls the expression of enzymes that maintain cell wall architecture
202 during growth and division (18, 20–22). Thus, the partial disruption of this pathway likely imposes
203 a cost on *S. aureus* growth, possibly explaining the rare co-occurrence of WalKR regulon
204 mutations; their combined effect on fitness may be overly deleterious.

205 In summary, our findings demonstrate that MSSA employed at least two adaptive
206 strategies, balancing the evolution of vancomycin resistance with maintaining core cellular

207 functions. The distinct genomic routes — centered on cell wall remodeling via WalKR regulon
208 mutations and translational reprogramming through *rpsU* alterations — underscore possible sign
209 epistasis that limits the co-occurrence of these mutations. Future work might focus on genomic
210 reconstructions of these evolved mutations into the ancestral background to better examine their
211 epistatic relationships. This divergence is particularly striking, considering that the replicate
212 populations descended from a single ancestor. This result complements previous studies, in
213 which *Escherichia coli* lineages from different genotypes followed divergent paths to increased
214 resistance (23, 24). Finally, our results are consistent with a recent study by Fait and colleagues
215 (25), who evolved 10 independent MRSA populations under vancomycin selection to intermediate
216 resistance and observed notable divergence in mutational profiles; some populations acquired
217 walK mutations, while others evolved mutations in *vraF*.

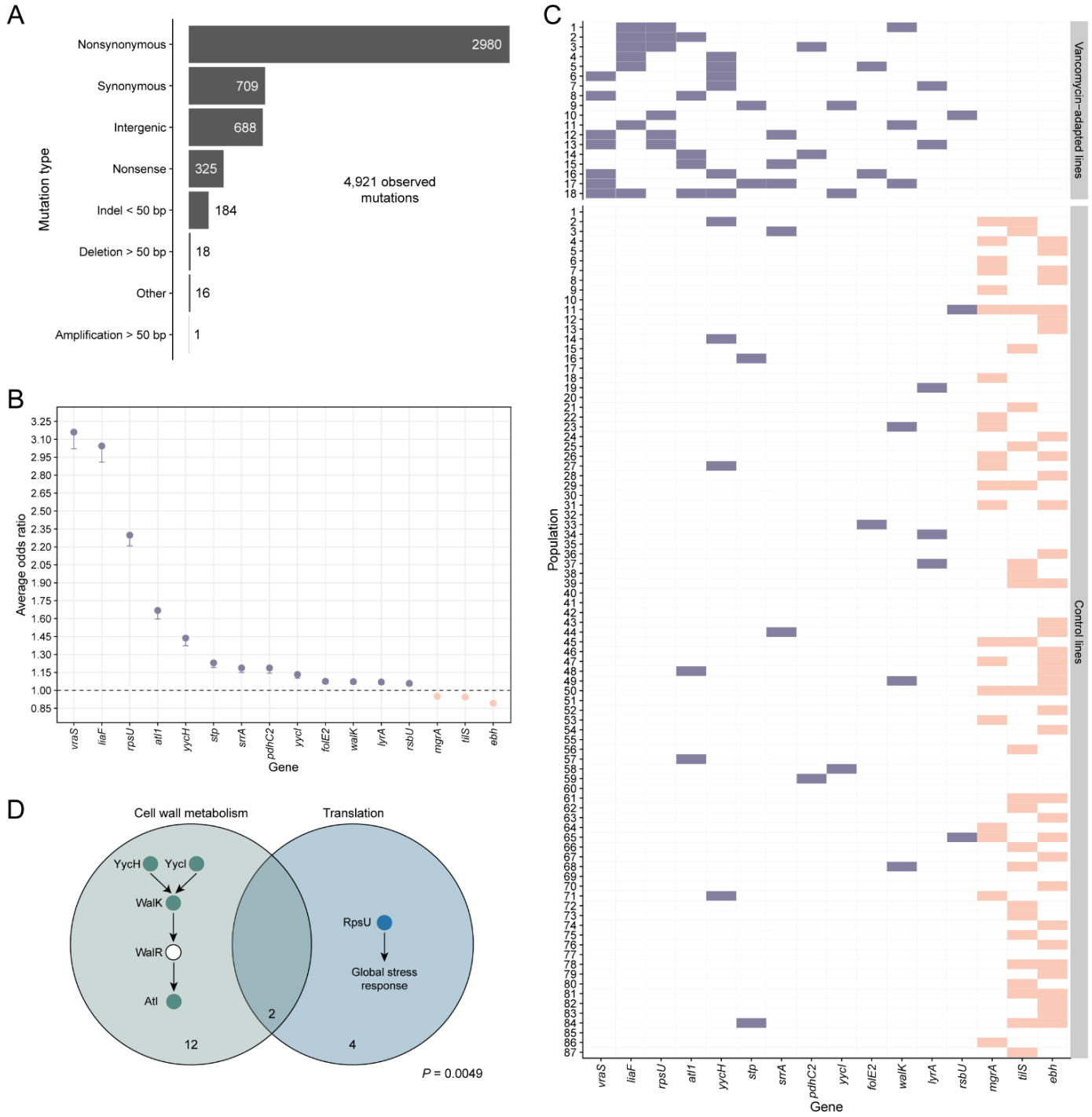


Fig. 2. Identification of mutated genes and pathways in experimentally evolved vancomycin-intermediate *S. aureus* (VISA) populations. (***A***) Classification of mutations by type. Numbers indicate the mutation counts for each category. (***B***) The average odds ratios for genes significantly associated with vancomycin exposure (violet-filled points) and control conditions (salmon-filled points) were evaluated across 250 iterations of a multivariate logistic

223 regression procedure (*Materials and Methods*). Error bars illustrate 95% confidence intervals,
224 calculated from the model coefficients and subsequently exponentiated. The horizontal dashed
225 line indicates an odds ratio of one. Point estimates are arranged by mean odds ratio. (***C***)
226 Identification of genes containing qualifying mutations, arranged by their mean odds ratio and
227 colored in the same manner as panel B. (***D***) A Venn diagram illustrating the extent of overlap
228 between the WalKR regulon and *rpsU* mutations in the evolved vancomycin-intermediate *S.*
229 *aureus* (VISA) populations. Filled circles in each pathway denote mutations impacting those
230 proteins, while the numbers indicate the total number of lines affected.

231 **Divergent genetic pathways under vancomycin exposure led to varied collateral responses**
232 We then examined how evolution under vancomycin selection affected the susceptibility of VISA
233 populations to commonly used first-line antibiotics. These so-called collateral drug responses are
234 widely observed in bacteria (2, 10, 11, 26–32) and cancer (33–36), and may impact therapeutic
235 outcomes (26). We compared the MICs of the ancestral clone against the 18 evolved populations
236 in cefazolin, clindamycin, daptomycin, gentamicin, meropenem, nafcillin, and trimethoprim-
237 sulfamethoxazole. These antibiotics are suggested treatments for MSSA bacteremia (27) and
238 endocarditis (2, 28). They also have diverse mechanisms of action (**Fig. 3*A***). For each antibiotic,
239 we quantified the collateral response of an evolved population as the difference in its $\log_2$-
240 transformed MIC relative to the ancestral clone. A population is collaterally resistant when its MIC
241 is higher than the ancestral MIC and collaterally sensitive when it is lower.
242 The VISA populations exhibited repeated and significant collateral responses to two
243 antibiotics (**Fig. 3*B***). First, 17 out of 18 lines had significantly increased daptomycin resistance
244 relative to the ancestor, as determined by a two-tailed Mann-Whitney test with *P*-values adjusted
245 using the Benjamini-Hochberg method (29) (adjusted $P = 0.0005$). Notably, nine of these lines had
246 MICs above the clinical resistance breakpoint for this phenotype (30) despite no prior exposure to
247 this drug. In contrast, no MIC met or exceeded the clinical resistance threshold to any other drug
248 in our study. Bacteria evolve thicker cell walls under vancomycin selection that hinder daptomycin
249 entry (31–34), likely explaining this finding as well as clinical accounts of collateral resistance (35).
250 Second, the VISA lines were consistently collaterally sensitive to clindamycin (adjusted $P =$
251 0.0468). Despite these encouraging results, clindamycin monotherapy is associated with higher
252 rates of recurrent infection in MSSA endocarditis because this drug is bacteriostatic (36).
253 Nonetheless, clindamycin is effective when given with trimethoprim-sulfamethoxazole (37).

254 The evolved populations displayed a range of collateral responses to the five remaining
255 antibiotics, with no significant changes in either direction. We hypothesized that this variation was
256 explained by differences in the genomic profiles that evolved during vancomycin selection. To
257 investigate this possibility, we used point biserial tests to evaluate the associations between
258 collateral response estimates and the presence or absence of mutations. On balance, WalKR
259 regulon mutations were significantly correlated with nafcillin collateral sensitivity (Benjamini-
260 Hochberg adjusted P = 0.0178), and *yycHI* mutations alone were sufficient to explain this
261 phenotype (P = 0.0372) (**Fig. 3*D***). In contrast, *rpsU* mutations were significantly correlated with
262 cefazolin and nafcillin cross-resistance (adjusted P = 0.0379) (**Fig. 3*D***).
263 Our findings, however, may depend on the specific environmental conditions under which
264 antibiotic susceptibilities were assessed. For instance, Machado and colleagues (38) evolved
265 MRSA populations under different media conditions — cation-adjusted Mueller-Hinton broth (CA-
266 MHB) and Roswell Park Memorial Institute (RPMI) medium — to simulate bacteriological and
267 physiological conditions, respectively. Their results indicated trade-offs between environmental
268 conditions, with lines evolved in CA-MHB showing increased vancomycin resistance in RPMI, but
269 not vice versa. This result suggests that the susceptibility profiles observed in our VISA
270 populations, measured in CA-MHB, may also differ under physiologically relevant conditions.
271 Additionally, Machado and colleagues identified mutations in *walK*, *yycH*, and *vraS*, which are
272 consistent with our study, though *liaF* mutations were notably absent in their lines. This
273 discrepancy could reflect differences in genetic background, as we evolved MSSA populations,
274 whereas their study used MRSA. Thus, genetic background and environmental context together
275 shape collateral responses, reinforcing the clinical relevance of considering both factors when
276 interpreting experimental evolution studies.

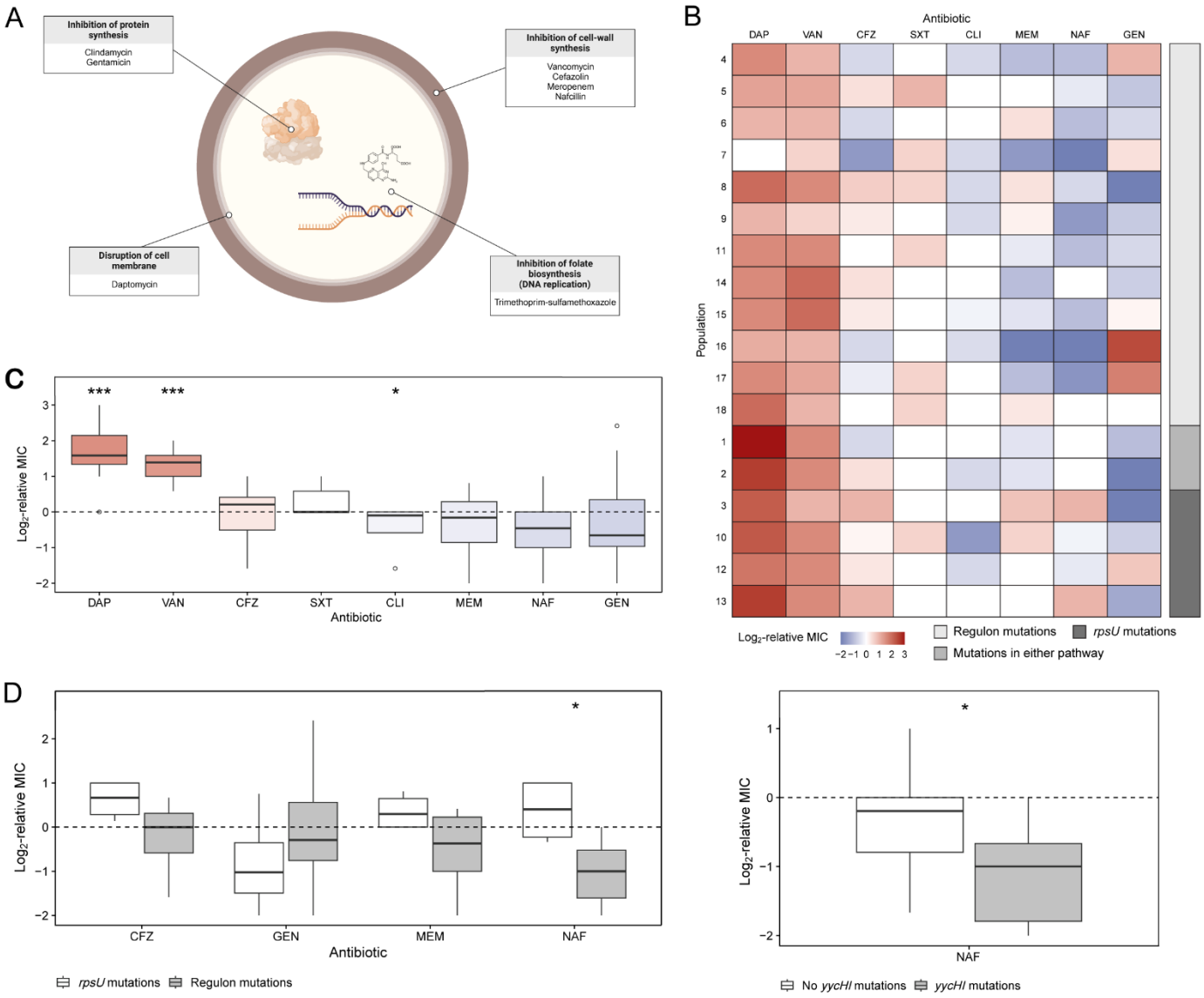


Fig. 3. Evolved vancomycin-intermediate *S. aureus* (VISA) lines exhibit diverging collateral sensitivity to cefazolin and nafcillin. (*A*) Bacterial targets and mechanisms of action of each drug. **(*B*)** Collateral resistance (red) or sensitivity (blue) of the 18 evolved VISA lines to each drug expressed as the $\log_2$-transformed minimum inhibitory concentration (MIC) relative to the ancestor. The lines are ordered by targeted pathway. **(*C*)** Boxplot representing the median and interquartile range of collateral responses in daptomycin (DAP), vancomycin (VAN) (the selecting drug), cefazolin (CFZ), trimethoprim-sulfamethoxazole (SXT), clindamycin (CLI), meropenem (MEM), nafcillin (NAF), and gentamicin (GEN). We ordered the eight antibiotics in decreasing order of median collateral resistance. **(*D*)** Variation in collateral responses between lines with WalKR regulon or *rpsU* mutations and with and without *yycHI* mutations.

287 Nafcillin and other antistaphylococcal penicillins are the current recommended treatments
288 for MSSA bacteremia. Nonetheless, many clinicians support first-line cefazolin therapy instead
289 because patients can better tolerate its side effects (39–42). Other clinicians prefer nafcillin
290 because cefazolin is susceptible to cleavage by *S. aureus*-produced penicillinases and, therefore,
291 may be less effective when there is a high bacterial titer (43, 44). We offer an evolutionary
292 perspective on this debate: specific mutations that arise during empiric vancomycin therapy may
293 negatively impact treatment effectiveness. On the one hand, *rpsU* mutations may predispose
294 MSSA infections to suboptimal treatment with cefazolin or nafcillin, as even marginal resistance
295 increases — such as those we observed experimentally — could become clinically significant if
296 initial susceptibility is already close to clinical resistance thresholds. On the other hand, mutations
297 in the WalKR regulon might promote favorable treatment outcomes. Although our results raise
298 concerns about the reliability of these drugs as first-line agents in specific contexts, further
299 research is needed to support these findings.
300 Our study, alongside corroborating evidence (25, 45), suggests that the evolution of
301 vancomycin resistance may predictably impact collateral drug sensitivities, highlighting the key
302 role that treatment history plays in shaping resistance outcomes. In this context, several studies
303 have shown that appropriately designed sequential therapies hold promise for steering
304 evolutionary trajectories to mitigate resistance (10, 26, 46, 47). Moreover, recent work that
305 accounts for fluctuating environmental pressures (48) further illustrates how a nuanced
306 understanding of evolution can inform treatment design. Together, these studies provide a
307 conceptual framework that not only aligns with our study but also suggests promising strategies
308 for integrating evolutionary insights into antimicrobial therapy.
309
310 **Using Collateral Response Scores to inform antibiotic treatment**
311 When clinicians use antimicrobial susceptibility test results to guide treatment decisions, they
312 participate in a decision-making process that involves uncertainty and risk. Results reflect the
313 pathogen's phenotypic state before empiric therapy and cannot account for adaptation during
314 treatment, potentially leading to shifts in susceptibility. Our findings highlight this possibility.
315 Confidence in the accuracy and relevance of initial susceptibility test results may, therefore, lead
316 to ineffective treatment choices. It is paramount that clinicians have a well-informed, probabilistic
317 understanding of how infections respond to treatment, informed by their prior evolutionary history.
318 We propose the Collateral Response Score (CRS) to address this issue. This composite
319 metric accounts for the stochastic nature of evolution by quantifying the net collateral effect of
320 antibiotic exposure. We defined it as,

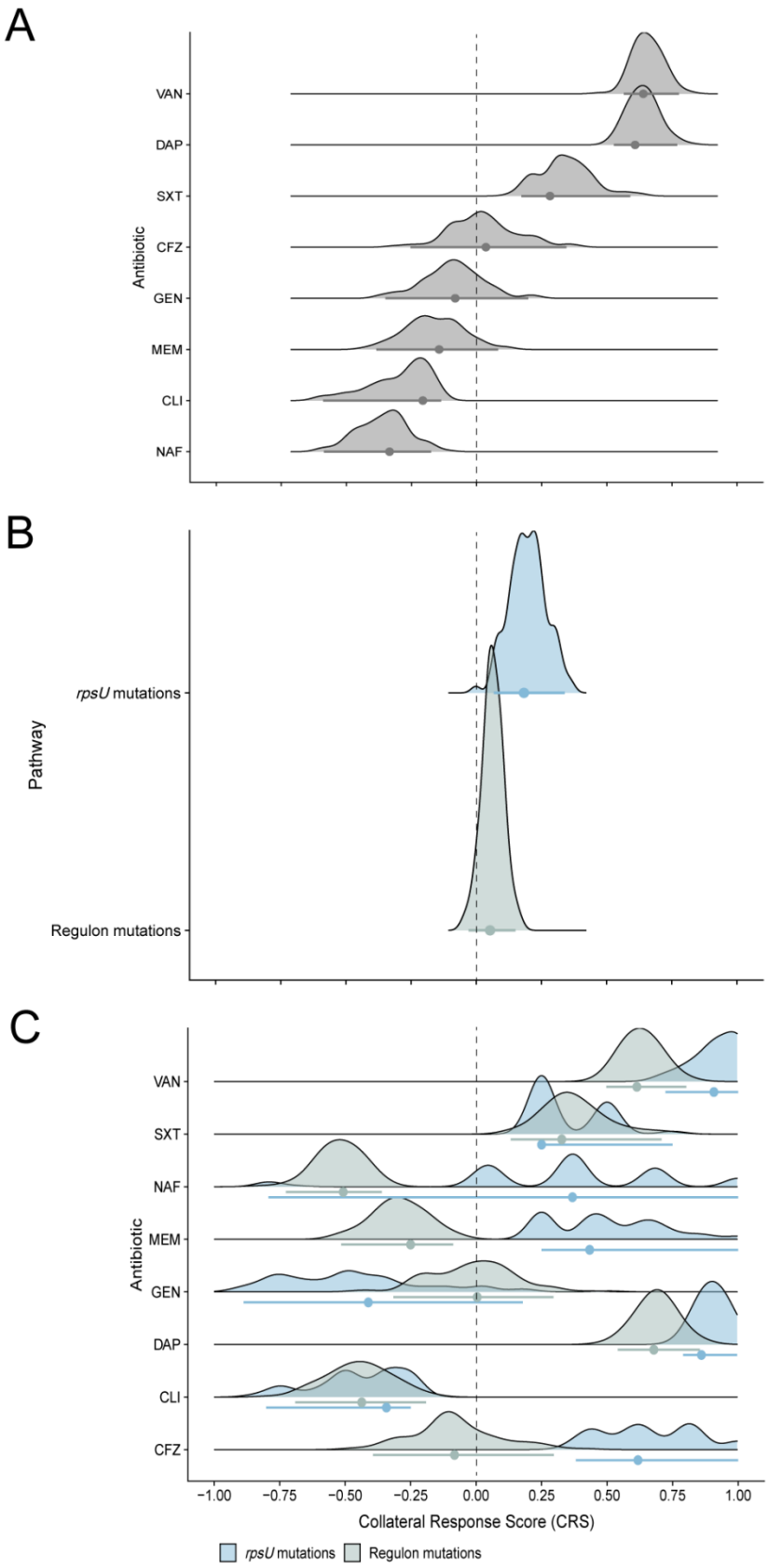
321 $$C = \frac{\frac{1}{N}\sum_{i=1}^{N} R_i}{\max_{1 \le i \le N} |R_i|}$$

322 where R_i denotes the $\log_2$-fold change in MIC between replicate i and the ancestor, $R_i =$
323 $\log_2\left(\mathrm{MIC}_{i,\mathrm{evolved}}/\mathrm{MIC}_{\mathrm{ancestor}}\right)$, and N indicates the number of replicate populations.

324 The CRS is standardized and easily interpretable, yielding values between −1 and 1 that
325 reflect the overall direction and magnitude of changes in antibiotic susceptibility relative to a prior
326 genotypic state. Negative values indicate that, on average, evolved lines exhibit collateral
327 sensitivity, and positive values indicate collateral resistance. A CRS close to −1 or 1 signifies
328 substantial and probable changes in either direction, whereas values near zero denote only
329 modest changes (or, importantly, balanced divergent changes).

330 This score is also amenable to statistical investigation. The tests discussed in the previous
331 sections evaluated whether the distributions of MICs differed significantly between the ancestral
332 clone and its derived VISA populations, or whether there was a correlation between specific
333 genomic profiles and collateral response estimates. Nevertheless, these tests alone cannot
334 provide information about the likelihood of a particular collateral response, nor its strength and
335 direction. These attributes are essential information for clinicians making therapeutic decisions.
336 Consider, for example, a situation where a susceptibility test indicates that an MSSA infection,
337 previously treated with vancomycin, is sensitive to both cefazolin and nafcillin. How should a
338 clinician tailor therapy, given that sensitivity might change with the evolution of *rpsU* mutations?
339 In that spirit, we calculated bootstrap distributions of CRS, categorized by drug, pathway, and
340 their interaction. The net collateral effect of vancomycin exposure on first-line drug susceptibility
341 is significant if the bootstrap 95% confidence interval of a CRS estimate does not include zero.

342 Overall, the CRS distributions support our earlier findings while revealing important
343 nuances in collateral responses. For example, although VISA populations exhibited the highest
344 overall collateral sensitivity to gentamicin (**Fig. 3*C***), the considerable variability among replicates
345 meant that individual populations could be nearly equally likely to evolve sensitivity or resistance
346 (**Fig. 4*A***). The CRS effectively captures this variability, emphasizing its ability to represent both
347 the magnitude and uncertainty inherent in evolutionary responses. Furthermore, mutations in
348 *rpsU* modestly increased collateral resistance across drugs (**Fig. 4*B***), while mutations in the
349 WalKR regulon and *rpsU* were associated with a higher likelihood of nafcillin collateral sensitivity
350 and cross-resistance, respectively.


A
VAN
DAP
SXT
CFZ
GEN
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CLI
NAF
Antibiotic
B
rpsU mutations
Regulon mutations
Pathway
C
VAN
SXT
NAF
MEM
GEN
DAP
CLI
CFZ
Antibiotic
−1.00
−0.75
−0.50
−0.25
0.00
0.25
0.50
0.75
1.00
Collateral Response Score (CRS)
rpsU mutations
Regulon mutations

351

352 **Fig. 4. Bootstrap distributions of Collateral Response Scores (CRS)** across (***A***) antibiotics,
353 (***B***) genomic pathways, and their (***C***) interaction. The CRS is the mean $\log_2$-fold change in MIC
354 between the evolved VISA populations and the ancestor, normalized by the maximum absolute
355 change in MIC. Values range from –1 (uniform collateral sensitivity) to 1 (uniform collateral
356 resistance). Each distribution represents 100 bootstrap replicates, with point estimates
357 representing the mean CRS and error bars indicating the percentile-based 95% confidence
358 interval. The dashed vertical line at zero denotes the threshold between collateral sensitivity and
359 resistance.

360 Importantly, however, drug response outcomes might change in different experimental and
361 clinical contexts. Here, we outline three possible future studies that address these contexts,
362 generalize our findings, and possibly validate the CRS. First, one could examine whether *S.*
363 *aureus* clinical isolates with different genetic backgrounds exhibit similar patterns of collateral
364 sensitivity or resistance. A bacterium's background may direct evolution toward some pathways
365 while constraining others (49, 50), similar to how the evolution of WalKR regulon or *rpsU*
366 mutations precluded the other in our MSSA lines. This process, known as historical contingency,
367 may also influence how effectively a population compensates for increased susceptibility to first-
368 line drugs through subsequent evolution (23, 51–53). Second, one might investigate how biofilm
369 formation impacts collateral drug responses in MSSA and how different antibiotic delivery systems
370 alter these patterns. These studies would bridge the gap between laboratory findings and clinical
371 applicability, especially considering the relevance to implant-associated infections caused by
372 biofilms and heart valve vegetations (54, 55). Third, we acknowledge that our experimental
373 design, which involves progressively increasing vancomycin concentrations, differs from the
374 dosing regimens typically used in clinical settings. This approach may have influenced the
375 evolutionary trajectories observed in our study, potentially leading to the accumulation of
376 mutations distinct from those that would occur under constant therapeutic doses. For example,
377 Oz and colleagues showed that when *E. coli* lines evolve antibiotic resistance under strong
378 selection, they exhibit more collateral resistance and have more mutations in drug pathway-
379 specific genes than populations evolved under weak selection (56). While our method allowed us
380 to explore a range of evolutionary responses, we acknowledge that our approach may limit the
381 clinical relevance of our findings. Further studies under higher, clinically relevant doses (and over
382 shorter durations) are warranted to understand the dynamics of resistance evolution in therapeutic
383 contexts.

384 Nonetheless, our study reveals that vancomycin adaptation in MSSA proceeds through at
385 least two distinct evolutionary pathways — one characterized by alterations in cell wall
386 metabolism and the other by changes in global stress response. These adaptive trajectories result
387 in contrasting collateral responses, with WalKR regulon mutations conferring enhanced sensitivity
388 to nafcillin, while *rpsU* mutations tend to maintain or increase resistance to both nafcillin and
389 cefazolin. Contingency highlights the importance of accounting for stochasticity when designing
390 therapeutic strategies, underscoring that anticipating evolutionary variability may be crucial for
391 preserving antibiotic efficacy and enhancing clinical outcomes.
392

393 ## Materials and Methods

394 ### Ancestral strain and experimental evolution

395 We used *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC 29213) as the ancestor of our
396 evolution experiment. This methicillin-susceptible strain was originally isolated from human pleural
397 fluid in 1884 (57) and is an international quality control standard with defined susceptibilities to
398 many antibiotics. According to the CLSI (58), vancomycin's MIC on this strain is 0.5 – 2 μg/mL.
399 Clinical *S. aureus* isolates with MICs <2 μg/mL are therefore considered susceptible to this drug.
400 The clinical MIC breakpoints differentiating intermediate- and complete-resistant phenotypes are 4
401 – 8 and >16 μg/mL, respectively. We used these breakpoints to classify phenotypes in our study.
402 All experiments were performed at 37˚C, unless otherwise noted. We revived the ATCC
403 29213 strain from a frozen stock by streaking cells onto tryptic soy agar (TSA) plates
404 supplemented with 5% sheep blood (Remel, Lenexa, KS). We randomly chose isolated colonies
405 from these plates to establish 18 replicate populations in tryptic soy broth (TSB) (Cleveland Clinic,
406 Cleveland, OH). To start the evolution experiment, we prepared a linear series of vancomycin
407 dilutions in TSB, ranging in concentrations from 0.25 to 1.5 μg/mL. Each replicate was aliquoted
408 into equal volumes of the vancomycin-amended TSB in 96-well plates, resulting in a total dilution
409 of 1:400, and incubated without shaking for 22 hours. Next, we transferred 1:400 cells from the
410 highest concentration with visible growth for each replicate into fresh TSB with increased
411 vancomycin concentrations. We transferred the replicate populations every 18–24 hours in the
412 unshaken medium until they grew at concentrations between 4 and 8 μg/mL for two consecutive
413 transfers. Replicates that did not grow at any concentration after 24 hours were left to incubate
414 until they did so, but no longer than 96 hours. Additionally, we randomly chose 87 isolated colonies
415 of the ATCC ancestor to establish replicate control populations. We propagated them for seven
416 days under the same conditions, but in TSB without vancomycin. Undiluted cultures of the evolved

417 populations were frozen at –80°C in TSB supplemented with 15% glycerol as a cryoprotectant.
418 The Cleveland Clinic Institutional Biosafety Committee approved all methods.
419
420 **Library preparation and whole-genome sequencing**
421 Frozen glycerol stocks of ancestral and VISA population samples were cultured overnight in TSB
422 with 1.5 µg/mL vancomycin, while the control populations were grown in vancomycin-free TSB.
423 We centrifuged the overnight cultures at 8,000 rpm for 3 minutes and removed the supernatant
424 from the bacterial pellets. SeqCenter (Pittsburgh, PA) prepared sample libraries from the ancestor
425 and vancomycin-adapted lines using the Illumina DNA Prep kit and IDT 10bp UDI indices,
426 followed by sequencing on an Illumina NextSeq 2000. Demultiplexing, quality control, and adapter
427 trimming were performed using bcl-convert (v3.9.3).
428 SeqCoast Genomics (Portsmouth, NH) lysed the control samples using MagMAX
429 Microbiome bead-beating tubes and extracted DNA using the Qiagen DNeasy 96 PowerSoil Pro
430 QIAcube HT Kit. Samples were prepared for whole-genome sequencing using the Illumina DNA
431 Prep tagmentation kit with Illumina Unique Dual Indexes. Sequencing was performed on the
432 Illumina NextSeq 2000 platform using a 300-cycle flow cell kit to produce 2x150 bp paired-end
433 reads. Moreover, 1 – 2% PhiX control was spiked into the run to support optimal base calling.
434 Read demultiplexing and trimming, and run analytics were performed using DRAGEN v4.2.7, an
435 onboard analysis software on the NextSeq 2000. All resulting FASTQ files of paired-end reads
436 for the ancestor, control, and vancomycin-adapted populations were deposited in the NCBI
437 Sequence Read Archive (accession no PRJNA1075422) (59).
438
439 **Mutation identification and statistical methodology**
440 The sequencing reads were filtered to remove low-quality bases using Trimmomatic v0.39 (60).
441 We clipped the reads when the average quality score was <20 in a 4-bp window and to a minimum
442 length of 36 bp. Next, we used *breseq* v0.39.0 (61) to identify mutations in two steps. First, we
443 used this bioinformatic pipeline with default parameters to map the ancestral strain reads to the
444 ATCC 29213 reference genome. This step accounted for mutations present at the beginning of
445 our evolution experiment. Second, we applied these background mutations to the ATCC 29213
446 genome and reran *breseq* in polymorphism mode to map the evolved population reads to this
447 updated reference.
448 We manually curated the sequencing results by excluding mutations with frequencies below
449 5% and within multicopy elements, such as ribosomal RNA operons and synthetases responsible
450 for charging tRNA. Gene conversions may cause these mutations, but short-read sequencing

451 data cannot fully resolve them (24). We chose the 5% frequency threshold given the high genomic
452 coverage of our samples. Moreover, we included only those "qualifying" mutations that
453 unambiguously impact a single gene (24, 62). These include nonsynonymous point mutations,
454 small indels that occur within genes, mutations within 150 bp upstream of the start of a gene, and
455 large deletions if at least one affected gene was mutated in another population.

456 We calculated the similarity of gene-level mutations between independent lines to
457 evaluate genomic parallelism in the VISA populations relative to control lines. For each population
458 pair, we calculated Dice's similarity coefficient, S, as,

459 $$S = \frac{2|X \cap Y|}{|X| + |Y|}$$

460 where $|X|$ and $|Y|$ represent the sets of genes with qualifying mutations in each population and
461 $|X \cap Y|$ denotes the number of genes mutated in both populations. A value of zero indicates that
462 the pair shares no mutations in common, whereas a value of one indicates complete overlap (24,
463 62, 63). We calculated S for every pair of populations within the vancomycin and control
464 conditions, and then derived the average pairwise similarity for each group. The difference
465 between the average similarity in the vancomycin-adapted lines (S_v) and in the control lines (S_c)
466 served as the test statistic; a positive difference indicates that the VISA lines exhibit greater
467 genomic parallelism than the control lines.

468 To determine the statistical significance of this difference, we employed a permutation test.
469 In each of the 10,000 permutations, the populations were randomly reassigned to the vancomycin
470 or control groups, and the difference $S_v - S_c$ was recalculated, thereby generating an empirical
471 distribution under the null hypothesis of equal genomic parallelism. We then obtained an
472 approximate *P*-value from the proportion of permutations with a test statistic at least as extreme
473 as the observed value.

474 Next, we used penalized logistic regression with L1 (LASSO) and L2 (Ridge) regularization
475 to identify genes associated with vancomycin selection. Given the high dimensionality of the initial
476 mutation set, we performed feature selection in two steps. First, we assessed the convergence of
477 model coefficient estimates over 1,000 iterations. In each iteration, the optimal regularization
478 strength (λ) and mixing parameters (α) were determined via three-fold cross-validation to
479 minimize deviance, and a multivariate logistic regression model was fitted using these
480 parameters. We only included in our model qualifying mutations that occurred in at least three
481 populations, resulting in 446 gene-level mutations. For every qualifying mutation, we computed
482 the cumulative mean of its coefficient to assess whether the point estimate stabilized as the

483 number of iterations increased. In parallel, we calculated the cumulative standard error, reflecting
484 the estimate's precision across iterations. We considered convergence achieved at iteration 250,
485 as the cumulative mean (**Fig. S3*A***) and standard error (**Fig. S3*B***) exhibited a marginal change
486 with further iterations. Second, we performed the penalized logistic regression procedure as
487 before with 250 iterations. Given the sparsity-inducing property of L1 regularization, we tracked
488 the frequency of non-zero coefficients across iterations. Mutations exhibiting non-zero coefficients
489 in at least 20% of the iterations were retained, constituting the final set of informative features for
490 subsequent Bayesian latent class analysis (BLCA) (SI Methods). We computed the 95%
491 confidence intervals for each gene's coefficient and then exponentiated the endpoints to obtain
492 the confidence intervals for the odds ratio. We provide the datasets and details of our statistical
493 analyses in an R Notebook on GitHub (64).
494
495 **Estimating collateral drug responses**
496 We estimated the collateral responses of the 18 evolved VISA populations to first-line antibiotics
497 using the broth microdilution method outlined by the CLSI (58). We supplemented Mueller-Hinton
498 broth (MHB) (Fisher Bioreagents, Ottawa, CA) with 20–25 mg/L Ca^{2+} ($CaCl_2 \cdot 2H_2O$, Fisher
499 Bioreagents, Ottawa, CA) and 10–15 mg/L Mg^{2+} ($MgCl_2$, Invitrogen, Vilnius, LT). We used this
500 cation-adjusted MHB (CA-MHB) to test the susceptibilities of the evolved populations to cefazolin,
501 clindamycin, daptomycin, gentamycin, meropenem, nafcillin, and trimethoprim/sulfamethoxazole.
502 For daptomycin and nafcillin susceptibility tests, we used CA-MHB that was additionally
503 supplemented with 25 mg/L Ca^{2+} (for a total calcium concentration of 50 mg/L) and 2% (w/v) NaCl
504 (Fisher Bioreagents, Ottawa, CA), respectively (65). CA-MHB was stored at 4°C to prevent ion
505 precipitation.
506 We inoculated 100 µL of cells from frozen samples into CA-MHB with 1 µg/mL vancomycin
507 (i.e., the MIC of the ancestral strain) to maintain resistance. After overnight growth, the cultures
508 were diluted to a McFarland 0.5 standard and then again by 200-fold in MHB. We aliquoted an
509 equal volume of these cells across a series of linear concentrations of a given antibiotic in MHB.
510 These concentrations ranged from 0.125 to 3× the median MIC of the wild-type *S. aureus* ATCC
511 29213 clone. To meet the CLSI standard (58), we incubated the vancomycin and nafcillin cultures
512 for 24 hours at 35°C. The other cultures were incubated at 37°C for 18–20 hours. The MIC of each
513 sample was the lowest antibiotic concentration that inhibited visual growth.
514 We quantified the susceptibility of three technical replicates for each of the 18 evolved VISA
515 populations across the seven antibiotics, totaling 378 MIC measurements. In addition, we included
516 the ancestor in each broth microdilution plate as a control and reference. The susceptibility of this

strain was estimated using eight technical replicates per drug, resulting in 56 MIC measurements. We calculated the collateral drug response, R, for each technical replicate, i, from population, j, as:

$$R_{i,j} = log_2\left(\frac{MIC_{i,evolved}}{MIC_{ancestor}}\right)$$

We analyzed the average collateral response values among the three technical replicates for each population.

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Author Contributions

K.J.C., D.C., and J.G.S. conceived of and designed the study. K.J.C., D.C., J.C., and A.S. performed the experiments. K.J.C., D.C., A.D., and J.G. analyzed all data. K.J.C. and A.D. wrote the associated analysis script and prepared figures. K.J.C. and J.G.S. supervised the project. K.J.C., D.C., and A.D. wrote the original draft. K.J.C., D.C., A.D., R.A.B., Z.D.C.B., and J.G.S. reviewed and edited the paper. All authors approved the final version.

Competing Interests

The authors declare no competing interests.

Data Availability

We provide the datasets and analysis code on GitHub (https://github.com/KyleCard/S_aureus_evolution) (64). Sequence read data have been deposited in the National Center for Biotechnology Information Sequence Read Archive (accession no PRJNA1075422) (59).

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SI Methods

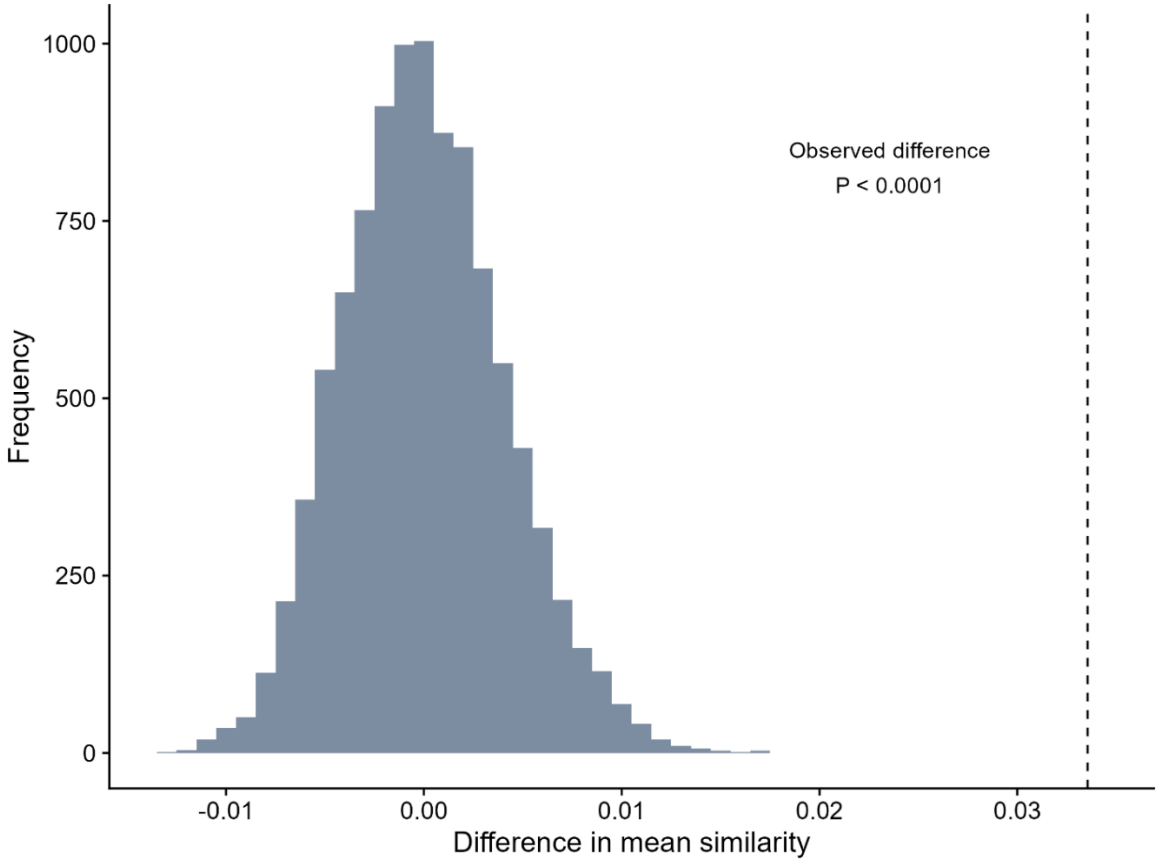
Following the feature selection by multivariate logistic regression, we performed Bayesian latent class analysis (BLCA) using the R package BayesLCA (1) with selected features (genes) mutated in at least three lines to identify distinct evolutionary trajectories. In this framework, the posterior distribution of parameters given the observations can be written as,

$$\prod^{N}\prod^{G}\tau^{z_{ig}+\delta_g-1}\prod^{M}\theta_{gm}^{x_{im}z_{ig}+\alpha_{gm}-1}(1-\theta_m)^{(1-x_{im})z_{ig}+\beta_{gm}-1}$$

where τ is the estimate for the population frequency of a particular group, θ is a GxM dimensional matrix of mutation probability given the group assignment, and δ, α, and β are parameters for the conjugate prior distributions for the Dirichlet and Beta distributions, respectively. We set the δ parameter to 1.0 as a uniform prior over class distributions and α and β to 0.5 to increase density around 0.0 and 1.0. Given the computational constraints of Markov chain Monte Carlo-based estimation, we used the expectation-maximization algorithm. To further improve the robustness of the clustering, we used the BLCA over 1000 iterations, where a random 90% subsampling was performed for both features and observations at each iteration. The subsampled data was clustered with k set from 1 to 5, and the best number of clusters was selected using AIC. At each clustering iteration, we tracked which observations were clustered together. Subsequently, we created a consensus matrix quantifying the frequency of pairwise observations, and a final clustering was performed using hierarchical clustering with Ward's criteria on the frequency matrix.

732 **SI Figures**

733



734

735 **Fig. S1. The null distribution of the difference in mean gene-level similarity between**
736 **vancomycin-adapted and control populations.** We calculated Dice's similarity coefficients for
737 each population pair using only the qualifying mutations that unambiguously affected single
738 genes. We assessed the difference in average similarity between the vancomycin-adapted and
739 control lines. Then, we shuffled the population labels randomly between the two treatment groups
740 and recalculated the difference in mean similarity. This process was repeated 10,000 times. The
741 resulting distribution of differences is presented here. The observed difference of 0.034 surpassed
742 all permutations ($P < 0.0001$), indicating a significantly higher level of genomic parallelism under
743 vancomycin selection.

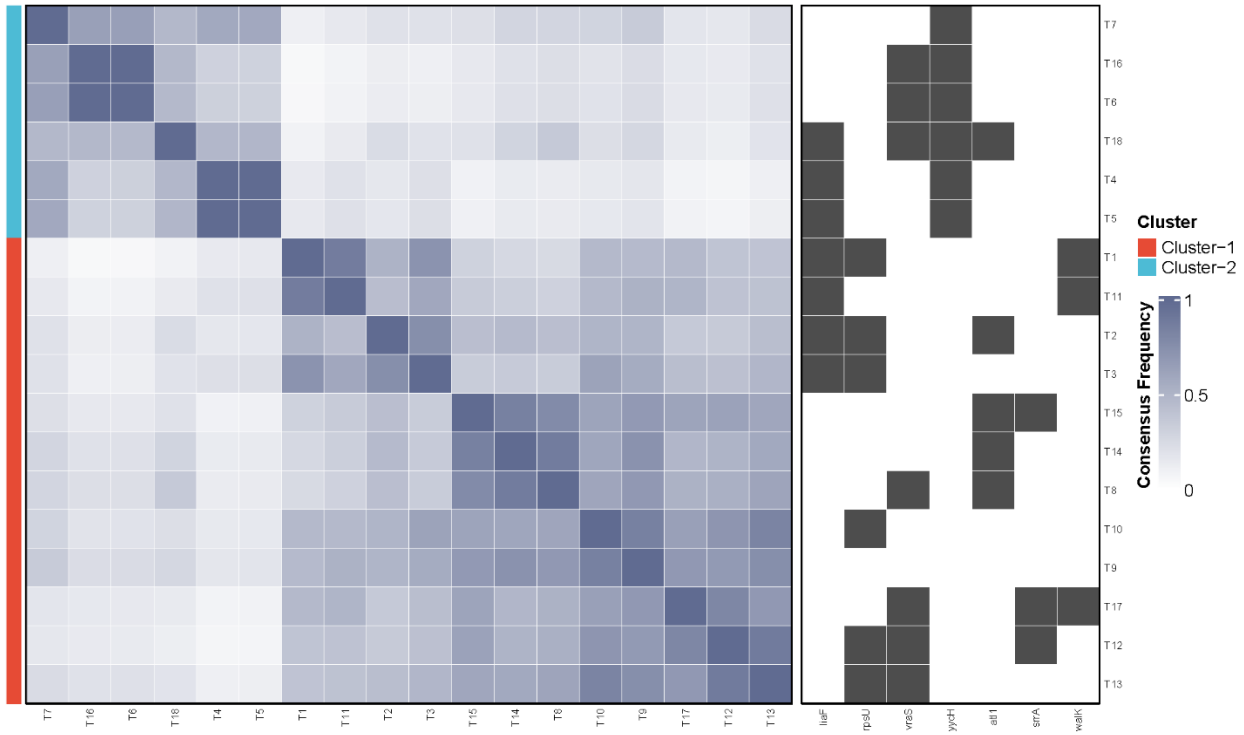
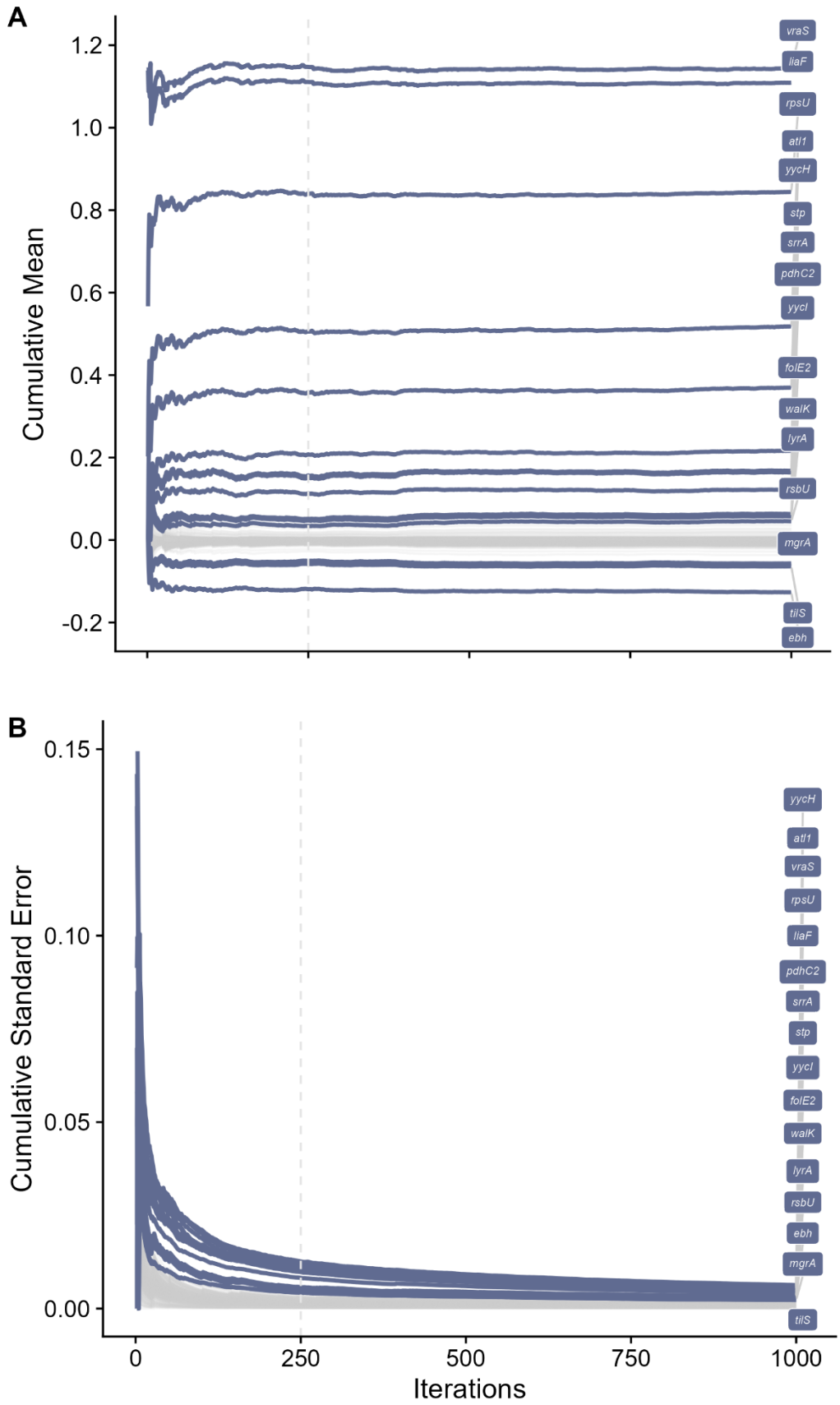


Fig. S2. Two distinct adaptive trajectories occurred under vancomycin selection. The heatmap represents the consensus matrix, generated through iterative Bayesian latent class analysis (BLCA) with random subsampling of observations and genomic features (Supplemental Methods), indicating the presence of two distinct clusters that correspond to unique genomic profiles.


A
Cumulative Mean
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
vraS
liaF
rpsU
atl1
yycH
stp
srrA
pdhC2
yycI
folE2
walK
lyrA
rsbU
mgrA
tilS
ebh
B
Cumulative Standard Error
0.15
0.10
0.05
0.00
yycH
atl1
vraS
rpsU
liaF
pdhC2
srrA
stp
yycI
folE2
walK
lyrA
rsbU
ebh
mgrA
tilS
0
250
500
750
1000
Iterations

750

751 **Fig. S3. Tests for convergence in logistic regression model coefficients.** Over 1,000
752 iterations of a penalized regression procedure (Materials and Methods), we estimated (***A***) the
753 cumulative mean and (***B***) the standard error for coefficients corresponding to qualifying gene-level
754 mutations. Blue highlighted lines denote genes with non-zero coefficients in at least 20% of the
755 iterations. Labels are ordered by decreasing cumulative mean and standard error. Grey
756 highlighted lines indicate all other genes. The dotted line indicates the iteration where
757 convergence is achieved.

758

SI Reference

1. A. White, T. B. Murphy, BayesLCA: An R package for Bayesian latent class analysis. *J. Stat. Softw.* **16**, 1–28 (2014).