Comparative Tn-Seq reveals common daptomycin resistance determinants in *Staphylococcus aureus* despite strain-dependent differences in essentiality of shared cell envelope genes

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Abstract

Antibiotic-resistant *Staphylococcus aureus* remains a leading cause of antibiotic resistance-associated mortality in the United States. Given the reality of multi-drug resistant infections, it is imperative that we establish and maintain a pipeline of new compounds to replace or supplement our current antibiotics. A first step towards this goal is to prioritize targets by identifying the genes most consistently required for survival across the *S. aureus* phylogeny. Here we report the first direct comparison of gene essentiality across multiple strains of *S. aureus* via transposon sequencing. We show that mutant fitness varies by strain in key pathways, underscoring the importance of using more than one strain to differentiate between core and strain-dependent essential genes. Despite baseline differences in gene importance, several pathways, including the lipoteichoic acid pathway, become consistently essential under daptomycin exposure, suggesting core vulnerabilities that can be exploited to resensitize daptomycin-nonsusceptible isolates. We also demonstrate the merit of using transposons with outward-facing promoters capable of overexpressing nearby genes for identifying clinically-relevant gain-of-function resistance mechanisms. Together, the daptomycin vulnerabilities and resistance mechanisms support a mode of action with wide-ranging effects on the cell envelope and cell division. This work adds to a growing body of literature demonstrating the nuanced insights gained by comparing Tn-Seq results across multiple bacterial strains.

Author summary

Antibiotic-resistant *Staphylococcus aureus* kills thousands of people every year in the United States alone. To stay ahead of the looming threat of multidrug-resistant infections, we must continue to develop new antibiotics and find ways of making our current repertoire of antibiotics more effective, including by finding pairs of compounds that perform best when administered together. In the age of next-generation sequencing, we can now use transposon sequencing to find potential targets for new antibiotics on a genome-wide scale, identified as either essential genes or genes that become essential in the presence of an antibiotic. In this work, we created a compendium of genes that are essential across a range of *S. aureus* strains, as well as those that are essential in the presence of the antibiotic daptomycin. The results will be a resource for researchers working to develop the next generation of antibiotic therapies.

Introduction

Every year in the United States over two million people acquire an antibiotic-resistant infection, and over 23,000 people die from those infections [1]. More people die of infections caused by antibiotic-resistant *Staphylococcus aureus* than any other resistant pathogen [2]. The primary antibiotic class used to treat *S. aureus* infections is the β-lactams, but over 50% of *S. aureus* infections in the United States are resistant to methicillin and other β-lactamase-impervious β-lactams [3, 4]. These β-lactam-resistant

81 strains are termed methicillin-resistant *S. aureus* (MRSA). Antibiotics such as
82 vancomycin, linezolid, and daptomycin are used to treat MRSA infections but are
83 ineffective in an increasing number of cases [5-7]. We need a pipeline of new
84 compounds that can either kill *S. aureus* outright or resensitize antibiotic-resistant
85 isolates, regardless of genetic background. Thousands of *S. aureus* isolates have been
86 sequenced, revealing substantial differences in gene content [8, 9], but the functional
87 variability across the phylogeny has yet to be systematically investigated. Transposon
88 sequencing (Tn-Seq) can be used to attribute phenotypes to genes on a genome-wide
89 scale, enabling researchers to characterize the full essential genome of a bacterial
90 strain in a single experiment [10, 11]. Here we describe the first multi-strain Tn-Seq
91 study in *S. aureus*, using comparisons of results from favorable growth conditions to
92 define baseline variation in genetic dependencies and then exposing the libraries to
93 daptomycin to probe the factors that modulate daptomycin susceptibility.
94 Several transposon mutagenesis studies have formerly analyzed the essential
95 genes in individual *S. aureus* strains [12-17]. Aside from one study conducted in a
96 livestock-associated strain, all the studies involved one clonal complex, CC8, and most
97 emphasized laboratory-adapted strains. Only two methicillin-resistant strains have been
98 examined. Moreover, the conditions for library creation and analysis differed
99 substantially. With these limitations, it is difficult to assess the congruence of gene
100 essentiality across *S. aureus* strains. A recent comparative Tn-Seq analysis of
101 *Mycobacterium tuberculosis* showed strain-to-strain differences in gene essentiality,
102 with implications for antibiotic targets and acquisition of resistance; this work highlighted

103 the importance of carrying out functional genomics studies of important pathogens in
104 multiple strains using the same methodology [18].

105 Here we have used our previously reported platform for creating transposon
106 libraries in *S. aureus* to make high-density transposon libraries in five strains [17].
107 These libraries comprise six sub-libraries, each made using a different transposon
108 construct. Several constructs contain outward-facing promoters, which minimize polar
109 effects within polycistronic operons. In addition, under some conditions, insertions of
110 outward-facing promoter constructs can upregulate nearby genes to provide a distinct
111 fitness advantage that is mechanistically informative [19]. The five *S. aureus* strains we
112 chose represent three clonal complexes (Fig 1B), including two MRSA/methicillin-
113 sensitive (MSSA) pairs from the same sequence type. MW2, from sequence type 1
114 (ST1), was the first lineage of community-acquired MRSA infections reported in the
115 United States [20-22]. MSSA476, a representative of a United Kingdom community-
116 acquired MSSA lineage, is likewise from ST1 [23]. We have chosen USA300-TCH1516
117 to represent the hypervirulent USA300 lineage from ST8, which has become the most
118 common community-acquired MRSA lineage in the United States [21, 24, 25]. The
119 commonly used MSSA lab strain HG003 is also ST8. To capture a larger scope of the
120 *S. aureus* phylogeny, we also included MRSA252, an ST36 hospital-acquired MRSA
121 strain predominantly found in Europe that has attracted attention for its unusually large
122 accessory genome [23].

123 We found 200 genes that are essential in all five strains and thus represent the
124 core essential genome. We also identified genes that were uniquely essential in a given
125 strain, as well as genes and pathways present in all five strains but essential in a

126 subset. These cases imply that strain-specific distal genetic determinants, whether
127 allelic in nature or horizontally acquired as with β-lactam resistance cassettes, can
128 profoundly reshape the role of even core pathways. The lipoteichoic acid (LTA) pathway
129 is one example of a conserved pathway that is differentially important across *S. aureus*
130 strains.

131 We probed to what extent the variable genetic dependencies evident in the
132 essential gene analysis shaped mutant fitness upon exposure to daptomycin, an
133 antibiotic used for *S. aureus* infections. Patients who cannot tolerate β-lactam or
134 vancomycin treatment, or who have resistant infections, are commonly treated with
135 daptomycin. Although still rare, daptomycin nonsusceptibility is on the rise [6]. It might
136 be possible to overcome infections that are resistant to daptomycin by co-administering
137 a compound that targets an intrinsic resistance factor found to be universally important
138 in withstanding daptomycin stress. A recent study comparing two *Streptococcus*
139 *pneumoniae* strains found major differences in factors that influenced daptomycin
140 susceptibility, raising questions about the prevalence of universally conserved
141 daptomycin intrinsic resistance factors in *S. aureus* [26]. Nevertheless, we identified
142 daptomycin intrinsic resistance factors conserved in all strains and demonstrated that
143 even pathways that appear variably essential among strains under normal growth
144 conditions can become essential in all upon exposure to daptomycin, illuminating a path
145 forward for combatting daptomycin resistance in *S. aureus*.

146

147 **Results**

148

149 **Creation of high-density *S. aureus* transposon libraries with both positive and**
150 **negative modulation of gene expression**
151
152 To characterize the functional diversity of *S. aureus*, we generated transposon
153 libraries with similar coverage in five strains (HG003, USA300-TCH1516, MSSA476,
154 MW2, and MRSA252). For each strain, we used our phage-based transposition method
155 to make six sub-libraries using different transposon constructs (Fig 1A) [17]. One
156 transposon construct contained an erythromycin resistance gene driven by its own
157 promoter (P_{erm}) and followed by a transcriptional terminator; another lacked the
158 transcriptional terminator to allow readthrough from the P_{erm} promoter; three constructs
159 contained the transcriptional terminator, but included an additional, outward-facing
160 promoter that varied in strength; and the sixth construct lacked the transcriptional
161 terminator and had an added outward-facing promoter, allowing for bidirectional
162 transcription. The sub-libraries were made separately and then pooled, with unique
163 barcodes in each transposon construct to enable disambiguation. These high-density
164 composite libraries often contained transposon insertions from multiple constructs at a
165 given TA insertion site. The constructs containing outward-facing promoters minimized
166 polar effects by allowing expression of downstream genes when an operon was
167 disrupted. Under some conditions, they also provided supplementary fitness
168 information. For example, under antibiotic exposure, an upregulated gene conferring a
169 growth advantage presented in the data as a strand-biased enrichment of promoter-
170 containing transposon insertions upstream of the gene. These “upregulation signatures”

171 can identify molecular targets or mechanisms of resistance (Fig 1A, yellow gene;
172 Methods).
173 Tn-Seq analysis showed that pooling the six transposon sub-libraries afforded
174 libraries with high coverage in every genome (Fig 1B). Although there was a three-fold
175 difference in unique insertions, counting insertions from each sub-library as distinct,
176 between the library with the fewest insertions (MW2) and the library with the most
177 insertions (USA300-TCH1516), approximately 90% of the genes in each genome had
178 high coverage of TA dinucleotide insertion sites. This gave us confidence that we could
179 obtain a reliable estimate of gene essentiality differences across the strains.
180
181 **Essential gene analysis reveals unexpected differences between strains**
182
183 We performed Tn-Seq analysis on the five transposon libraries to identify
184 essential genes in each strain. For the purposes of this study, we defined an essential
185 gene as one whose loss precluded survival in competition with a heterogeneous
186 bacterial population. We categorized genes as essential, non-essential, or
187 indeterminate based on TA insertion site coverage and sequencing reads (S1 Table).
188 Most essential genes were essential in all five strains. These 200 genes represent the
189 core essential genome of *S. aureus* (S1 Fig, S2 Table), with an additional 11 that were
190 required for at least the three MRSA strains (Fig 2A). Gene ontology overrepresentation
191 analysis showed that the ubiquitously essential genes were enriched in DNA, RNA, and
192 protein metabolic processes (S3 Table). Phospholipid and monosaccharide synthesis
193 were also enriched pathways, reflecting the importance of fatty acids and other cell

194 envelope precursors. Hypothetical genes were significantly underrepresented among
195 the ubiquitously essential genes but dominated the group of essential genes that were
196 only present in a subset of the genomes. Only two such genes were annotated: the
197 antitioxin gene *pezA* found only in MW2 and a phage repressor found in HG003, MW2,
198 and MSSA476.

199 Among annotated genes present in all five strains, there were notable
200 differences in essentiality. Strains of the same sequence type displayed as much
201 variability in gene essentiality as strains from different sequence types (S1 Fig),
202 suggesting it may not be possible to draw general conclusions about a clonal complex
203 from a functional genomics analysis of one member. Variably essential genes were
204 found in multiple pathways. For example, the uniquely essential genes in MRSA252
205 included *sbcD,* which is homologous to an *E. coli* gene encoding a DNA hairpin
206 cleavage enzyme [27, 28]; *sagB*, which encodes a β-N-acetylglucosaminidase [29]*;* and
207 *gdpP*, which hydrolyzes the bacterial second messenger cyclic-di-AMP [30]. It is
208 important to emphasize that essentiality in a Tn-Seq analysis does not imply that
209 individual knockouts are not viable; however, it does suggest that there are substantial
210 differences in the fitness of mutants across strains and that it is worth considering the
211 broader genomic context in evaluating the cellular roles of genes.

212 We expected pathways involved in constructing the cell envelope to be equally
213 essential for all strains, given the importance of the cell envelope in protecting the cell
214 from outside stressors. We were therefore surprised to find essentiality differences
215 within the LTA biosynthesis pathway. LTAs are long glycerol or ribitol phosphate
216 polymers anchored in the membrane of Gram-positive bacteria. In *S. aureus*, LTAs are

important in cell division, autolysin regulation, and virulence, among other processes [31]. The LTA pathway consists of five genes: *pgcA, gtaB, ugtP, ltaA,* and *ltaS* (Fig 2B). The first three genes encode proteins involved in the biosynthesis of diglucosyl-diacylglycerol (Glc_2DAG), the membrane anchor on which LTAs are assembled. Glc_2DAG is made inside the cell and flipped to the cell surface by LtaA. The LTA synthase, LtaS, then assembles the polymer by sequential transfer of phosphoglycerol units from phosphatidylglycerol to Glc_2DAG. LTAs can then be further modified on the extracellular surface by D-alanine substitutions and N-acetyl glucosamine residues [32, 33]. If Glc_2DAG is not available due to deletion of an upstream gene, LtaS can synthesize LTAs on the alternative membrane anchor phosphatidylglycerol. The four genes upstream of *ltaS* were previously found to be nonessential for *S. aureus* viability [34-36], but *ltaS* mutants were reported to be nonviable except at low temperature and under osmotically-stabilizing conditions unless suppressors were acquired [37-40]. Our Tn-Seq analysis showed, however, that mutant fitness within the LTA pathway varied considerably by strain (Fig 2C). The *ltaA* gene was expendable in all strains, but the three genes responsible for synthesis of the Glc_2DAG anchor, *pgcA, gtaB,* and *ugtP* (*ypfP*), were uniquely essential by Tn-Seq analysis for USA300-TCH1516 (Fig 3A, S2 Fig). Conversely, *ltaS* was unexpectedly dispensable in MW2 and MSSA476 (Fig 3C, S2 Fig).

We tested individual deletion mutants in the LTA pathway to confirm the Tn-Seq results. We were able to delete *ugtP* from USA300-TCH1516, consistent with results in other USA300 strain backgrounds, but a spot dilution assay showed that the mutant was less fit than the corresponding mutant in MW2 (Fig 3B). We also found that *ltaS* is

240 dispensable in MW2, but essential in other strains (Fig 4C). Although the MW2Δ*ltaS*
241 strain was more temperature-sensitive than MW2 wildtype, it grew on plates even at
242 37°C (S3 Fig). Because lethality due to *ltaS* deletion can be suppressed by deletion of
243 *gdpP*, *clpX*, and *sgtB* [37-39], we sequenced the transposon library parent strain but
244 found no nonsynonymous SNPs in *sgtB* or *gdpP* and only one nonsynonymous SNP in
245 *clpX*. The amino acid difference in ClpX is unlikely to explain the observed phenotype,
246 as it is located in an unstructured region of the protein and the amino acid is not strictly
247 conserved in the *S. aureus* phylogeny. Moreover, we did not find any evidence for a
248 duplication of *ltaS* in MW2 and confirmed that the MW2Δ*ltaS* mutant does not produce
249 LTAs (Fig 3D). The explanation for why LtaS is nonessential in MW2 must lie elsewhere
250 in the genome and will need to be investigated further.
251
252 ***S. aureus* strains share key daptomycin resistance factors**
253
254 While antibiotics targeting essential genes are invaluable, it may also be
255 advantageous to have compounds that resensitize bacteria to existing antibiotics,
256 though they may not be antimicrobial *in vitro* on their own. To find targets for such
257 compounds, we chose daptomycin as a case study. Daptomycin is a calcium-dependent
258 lipopeptide antibiotic frequently used to treat *S. aureus* infections. Although its
259 mechanism of action is not completely clear, it has been shown to insert into Gram-
260 positive bacterial cell membranes and change membrane curvature, mislocalize cell
261 division and cell wall synthesis proteins, depolarize the membrane, and ultimately kill
262 the cell [41-46]. The majority of *S. aureus* infections remain susceptible to daptomycin,

263 but there have been steady reports of daptomycin nonsusceptibility, making it
264 imperative to identify intrinsic resistance factors that can be targeted to restore
265 susceptibility [47].

266 We grew the five *S. aureus* transposon libraries in the presence and absence of
267 daptomycin in both cation-adjusted Mueller Hinton broth (MHBII) and Roswell Park
268 Memorial Institute supplemented with 10% lysogeny broth (RPMI+LB), as we wanted to
269 ensure the results were not dependent on a particular medium. We found substantial
270 overlap in the significantly depleted and upregulated genes in the two media, so the
271 results were considered as a union of the two conditions. For every strain, there were
272 more hits shared with at least one other strain than there were hits unique to that strain
273 (Fig 4A, S4 and S5 Table). Genes that were substantially depleted (>10-fold) in three or
274 more strains are shown in Fig 4B. Several vulnerabilities were above or very close to
275 the 10-fold cutoff in all five strains.

276 Many of the shared vulnerabilities to daptomycin are in genes related to the cell
277 envelope. These include *graRS/vraFG*, encoding the multi-component signaling system
278 that regulates cell envelope processes [48-50]; *arlR*, encoding a regulator of virulence
279 genes [51, 52]*; murA1*, encoding an enzyme that catalyzes the first step in
280 peptidoglycan biosynthesis [53, 54]; *alr1*, one of two genes encoding an alanine
281 racemase that supplies D-alanine for cell wall synthesis [55]; *mprF*, encoding a
282 phosphatidylglycerol lysyltransferase that modifies membrane charge [56]; and *ltaA*,
283 encoding the Glc_2DAG flippase gene in the LTA pathway [34]. The earlier LTA pathway
284 genes, *gtaB, pgcA,* and *ugtP,* were also significantly depleted in MW2, with the latter
285 two also depleted in HG003 (Fig 5A). In addition, we identified three depleted cell

286 division regulators, *gpsB*, *ezrA*, and *noc* (Figs 4B and 4C) [57-59]. A number of these
287 depleted genes have been previously associated with daptomycin susceptibility,
288 including *mprF* [60, 61], *graRS*/*vraFG* [49, 62], and *ezrA* [63], but some that were
289 substantially depleted in all five strains have not been connected to daptomycin,
290 including *gpsB* and *ltaA*. We confirmed that deletion of *ltaA* sensitizes HG003, MW2,
291 and USA300-TCH1516 to daptomycin and that the sensitivity can be reversed by *ltaA*
292 complementation (Fig 5B, S4 Fig). Therefore, Glc_2DAG-LTA in the cell membrane
293 contributes to *S. aureus* survival in the presence of daptomycin, and it may be possible
294 to exploit this observation to overcome daptomycin nonsusceptibility or limit its
295 development.

296 Just as there were more shared than unique depleted genes for each strain,
297 there were more shared than unique upregulated genes (Fig 4A). The outward-facing
298 promoters in several of our transposon constructs can upregulate downstream genes,
299 allowing us to identify genes that confer a fitness advantage when upregulated. They
300 are detected by strand-biased read enrichments in promoter-containing constructs, and
301 we have previously confirmed that these strand-biased read enrichments, or
302 upregulation signatures, do reflect increased expression of proximal genes [19]. Often
303 the genes identified this way are the targets of the antibiotic itself or reveal gain-of-
304 function resistance mechanisms. Standard transposon libraries cannot identify gain-of-
305 function resistance mechanisms, although loss-of-function resistance mechanisms can
306 be identified through intragenic read enrichments. Here we found that the genes
307 enriched in reads upon daptomycin exposure belonged to a wide assortment of cellular
308 processes and we could not discern a pattern amongst them (S6 Table).

309 Many of the genes for which we identified upregulation signatures in multiple
310 strains were previously implicated in daptomycin resistance in *S. aureus* (Table 1).
311 Daptomycin nonsusceptibility normally develops through the acquisition of multiple
312 independent mutations that result in changes to the cell envelope [47], and many of the
313 mutations are thought to be gain-of-function mutations that result in increased activity.
314 For example, mutations in *mprF* have been selected *in vitro* and in the clinic and are
315 thought to increase lysyl phosphatidylglycerol in the outer leaflet of the membrane [64].
316 Other gain-of-function resistance mechanisms identified in our Tn-Seq libraries include
317 *cls2*, one of two cardiolipin synthases in *S. aureus* [65]; the staphyloxanthin
318 biosynthesis gene *crtM* [66]; and two signaling systems, *graRS* and *walKR* (*yycFG*) [62,
319 67]. Some of these resistance mechanisms have been reported for daptomycin
320 resistance in other bacteria as well [68]. These hits affirm the utility of upregulation
321 signatures for identifying clinically-relevant mechanisms of resistance. The gene *murA2,*
322 whose function was identified as important for withstanding daptomycin exposure in the
323 depletion analysis, also had upregulation signatures in several strains. The importance
324 of MurA activity identified in this study – both through the depletion analysis and the
325 upregulation analysis – is consistent with recent studies showing that a combination of
326 daptomycin and fosfomycin is significantly more effective at curing MRSA bacteremia
327 than daptomycin alone, leading to an ongoing phase III clinical trial [69-71]. One caveat
328 of the upregulating transposons is that they may have distal effects, upregulating both
329 the most proximal gene and other downstream genes, especially within operons.
330 Individual mutants need to be tested to confirm that the proximal gene is most
331 responsible for the phenotype. For example, we identified an upregulation signature for

332 *ugtP* in USA300-TCH1516 and in MSSA476. The *ugtP* gene is upstream of *ltaA* in an
333 operon, so it is unclear whether upregulating *ugtP* on its own would recapitulate the
334 transposon-induced fitness advantage or whether *ltaA* upregulation is also necessary.
335 Regardless of whether upregulation of one or both genes in the operon drives the
336 fitness advantage, the result is consistent with findings from the depletion analysis that
337 Glc_2DAG-LTA is important for withstanding daptomycin stress.
338

339 **Table 1. Upregulation signatures identify genes previously linked to reduced**
340 **susceptibility to daptomycin.**

Gene	Function	HG003	USA300	MSSA476	MW2	MRSA252
cls2	Cardolipin synthesis	■		■	■	■
crtM	Staphyloxanthin synthesis	■	■	■		
graRS	Cell wall stress response	■	■	■	■	
mprF	Lysyl phosphatidylglycerol synthesis	■	■	■		
murA1	Rate-limiting step in peptidoglycan precursor synthesis			■	■	
murA2		■	■	■	■	
walKR	Cell wall stress response	■	■		■	■
00969	Unknown	■	■	■	■	■
02149	Unknown	■	■	■	■	■

341 Only showing genes with upregulation signatures in at least two strains. Strains sharing
342 upregulation signature indicated with blue shading. Upregulation and/or suspected gain-
343 of-function SNPs in all of these genes were previously found to confer a survival
344 advantage in the presence of daptomycin. Hypothetical genes are listed according to
345 their NCTC 8325 locus tag numbers.
346

347 Upregulation signatures were found upstream of the genes *SAOUHSC_02149*
348 and *SAOUHSC_00969* in all five strains. These genes each encode a small protein of
349 unknown function with a single transmembrane helix. We previously identified both as
350 daptomycin resistance factors in a Tn-Seq study of a single *S. aureus* strain and
351 confirmed that upregulation confers resistance while inactivation increases susceptibility
352 to daptomycin [19]. The observation that these genes are important for daptomycin
353 resistance in all five *S. aureus* strains suggests some effort should be devoted to
354 evaluating the physiological roles of their encoded products.
355

356 ## Discussion

357

358 Declining sequencing costs and efficient transposon mutagenesis methodologies
359 enable investigation of the genetic basis of phenotypes on a large scale. Tn-Seq
360 experiments are typically conducted in a single strain that is considered representative,
361 and it is assumed that results from that strain can be extrapolated to other members of
362 the species. Recent studies have challenged this notion, demonstrating clear strain-
363 dependent gene requirements in *Mycobacterium tuberculosis, Streptococcus*
364 *pneumoniae,* and *Pseudomonas aeruginosa* [18, 26, 72].
365 In the present study, we reveal how complicated the concept of gene essentiality
366 can be. We found that the LTA pathway, which has always been presumed to be
367 essential for all *S. aureus* strains, was variably essential under normal growth
368 conditions, ranging from required in USA300-TCH1516 to expendable in MW2. We
369 suspect that intracellular Glc_2DAG has an unidentified function that USA300-TCH1516

370 depends upon for survival because the Glc_2DAG biosynthesis genes (*pgcA, gtaB,* and
371 *ugtP)* contained almost no transposon reads, whereas *ltaA,* which encodes the flippase
372 responsible for translocating Glc_2DAG to the cell surface, contained many insertions.
373 The central role of Glc_2DAG in the viability of USA300-TCH1516 may provide a tool to
374 interrogate what other roles the molecule has in the cell. Likewise, having a strain like
375 MW2 that can ostensibly survive without LTAs provides new opportunities for
376 characterizing the functions of LTAs, as future investigations can interrogate what
377 wildtype MW2 can do that an *ltaS* knockout cannot. Whether strains that can survive
378 without LTA *in vitro* – due to the presence of known suppressors or to other
379 uncharacterized genetic background differences – can survive in the more challenging
380 environment of an animal infection needs to be assessed. LTAs are associated with
381 virulence and it has been shown in the *S. aureus* Newman strain that *ltaA* is required for
382 fitness in a mouse abscess model [34]. The results here also show that the LTA
383 pathway becomes universally required in the presence of daptomycin; therefore, LTA
384 pathway inhibitors may resensitize nonsusceptible isolates to daptomycin even if they
385 cannot kill the cells on their own.

386 We have also demonstrated an improved method for identifying gain-of-function
387 resistance mechanisms via Tn-Seq. The upregulation signatures that we found under
388 daptomycin exposure using our transposons with outward-facing promoters largely
389 echoed the depletion hits. In other words, if removing a gene sensitizes a bacterium to
390 an antibiotic, upregulating that gene is often protective. For daptomycin, these intrinsic
391 resistance factors were predominantly cell envelope, cell division, and stress response
392 system genes. Although a standard Tn-Seq library can identify intrinsic resistance

393 factors through sensitization of knockouts, sensitization readouts are limited to only
394 looking at nonessential genes where a decline in insertions can be detected. The
395 upregulation signatures, on the other hand, are indifferent to the essentiality of the
396 gene. For example, *ugtP* was upregulated under daptomycin stress in USA300-
397 TCH1516 even though it was found to be essential in that strain, and *walR,* an essential
398 multicomponent signaling system gene, was upregulated in multiple strains. As
399 validation for the approach, most of the genes that had upregulation signatures have
400 been previously reported as suspected gain-of-function resistance mechanisms for
401 daptomycin.

402 In summary, our comparison of Tn-Seq data across multiple strains provided a
403 more nuanced illustration of *S. aureus* genetic dependencies than we could have
404 attained using a single strain. The comparative data provide the most comprehensive
405 list of core essential genes in *S. aureus* to date and have identified myriad strain-
406 dependent essential genes. The strain-to-strain gene essentiality differences under
407 normal growth conditions provide a basis to more deeply interrogate *S. aureus* biology.
408 Meanwhile, those factors that were universally essential may be useful targets for future
409 antibiotic development. Under daptomycin stress the commonalities between the strains
410 likewise proved valuable, as they give us a means to prioritize targets for the
411 development of synergistic compounds. Moreover, Tn-Seq results can be used to
412 complement findings from other systems biology approaches [9, 73]. As sequencing
413 continues to become more affordable, we envision performing Tn-Seq in multiple strains
414 will become routine and will lead to a more comprehensive understanding of the scope
415 of bacterial phenotypic diversity.

416

417 ## Materials and methods

418

419 ### Materials, bacterial strains, plasmids, and oligonucleotides

420

421 All reagents were purchased from Sigma-Aldrich unless otherwise indicated.
422 Bacterial culture media were purchased from BD Sciences. Restriction enzymes and
423 enzymes for Tn-seq preparation were purchased from New England Biolabs.
424 Oligonucleotides and primers were purchased from Integrated DNA Technologies. DNA
425 concentrations were measured using a NanoDrop One Microvolume UV-Vis
426 Spectrophotometer (Thermo Scientific). DNA sequencing was performed by Eton
427 Bioscience unless otherwise noted. KOD Hotstart DNA polymerase (Novagen) was
428 used for PCR amplification. *E. coli* strains were grown with shaking at 37°C in lysogeny
429 broth (LB) or on LB plates. *S. aureus* strains were grown at 30°C in tryptic soy broth
430 (TSB) shaking or on TSB agar plates unless otherwise noted. For *S. aureus* strains,
431 compounds for selection or gene induction were used at the following concentrations: 5
432 µg/mL chloramphenicol and erythromycin; 50 µg/mL kanamycin and neomycin; or 2.5
433 µg/mL tetracycline. For *E. coli* strains, 100 µg/mL carbenicillin was used for selection.
434 The bacterial strains, plasmids, and oligonucleotide primers used in this study are
435 summarized in S7 Table.

436

437 ### Transposon library construction

438

439 Construction of the transposon library in the laboratory *S. aureus* strain HG003
440 has been described [17]. A similar strategy was used to make libraries in other *S.*
441 *aureus* genetic backgrounds after deleting non-compatible DNA restriction systems and
442 endogenous antibiotic resistance genes that would interfere with transposon library
443 construction. In the community acquired *S. aureus* MW2 and MSSA476 strains, the
444 clonal complex CC1 *hsdR* Type I restriction system in each background was first
445 deleted using the temperature sensitive shuttle vector pKFC with ~1 kb DNA homology
446 flanking arms. A second restriction system unique to MSSA476 was deleted in a
447 subsequent round using pKFC to generate the transposon library host. Libraries were
448 constructed in the *S. aureus* community acquired strain USA300-TCH1516 by first
449 curing the endogenous plasmid pUSA300HOUMR through destabilizing replication via
450 integration of pTM283. Cointegrated pUSA300HOUMR-pTM283 was passaged at 30°C
451 for two rounds of outgrowth in 10 mL of TSB media before streaking to single colonies.
452 Colonies exhibiting kanamycin and erythromycin sensitivity (encoded on
453 pUSA300HOUMR) were further checked by PCR to confirm loss of plasmid. The
454 resulting strain, TM283, was used as host for transposon library construction. A
455 transposon library of the hospital-acquired, methicillin-resistant *S. aureus* MSRA252
456 strain was made by first converting the *hsdR* (SAR0196) Type I restriction gene deletion
457 shuttle vector pGKM305 into a high frequency transduction vector by adding a ϕ11 DNA
458 homology region (primers GKM422-423) into the SfoI site to make pGKM306. The
459 plasmid was electroporated into RN4220 and transduced into wildtype MRSA252 using
460 ϕ11-FRT with temperature permissive selection at 30°C [17]. The SAR0196 gene was
461 then deleted as described above. Both copies of the endogenous duplicated

462 erythromycin resistance gene (SAR0050 and SAR1735) were likewise deleted in two
463 rounds using pKFC_SAR0050, except plasmids were directly electroporated into the
464 restriction negative parent strain GKM361. Due to endogenous aminoglycoside
465 resistance, the transposase expressing plasmid pORF5 Tnp+ was converted into
466 chloramphenicol resistant plasmids to enable selection in strain TXM369 and library
467 construction.
468
469 **Transposon sequencing**
470
471 To identify the essential genes in each of the *S. aureus* strains (HG003, USA300-
472 TCH1516, MSSA476, MW2, and MRSA252), library aliquots were thawed and diluted to
473 an OD_{600} between 0.2 and 0.3 in 10 mL of MHBII in duplicate. The cultures were then
474 incubated shaking at 30°C until they reached an OD_{600} of approximately 0.4, roughly
475 1.5-1.75 hours. The cells were pelleted, and the DNA was extracted and prepared for
476 Tn-Seq as previously described [17]. Samples were then submitted to either the
477 Harvard Biopolymers Facility or the Tufts University Core Facility for sequencing on a
478 HiSeq 2500 instrument.
479 To identify genes affected by daptomycin exposure, transposon library aliquots
480 for the six strains were inoculated in either MHBII or RPMI+LB. The cultures were
481 incubated for 1.5 hours at 30°C in a shaking incubator as described above. The cultures
482 were diluted to OD_{600} 0.005 in either 2 mL MHBII or RPMI+LB containing a series of
483 daptomycin concentrations (µg/mL): 0, 0.12, 0.25, 0.5, and 1. The cultures were then
484 incubated at 37°C in a shaking incubator and their ODs were monitored using a

485 Genesys 20 spectrophotometer (Thermo Scientific). The 2 mL cultures were harvested
486 by centrifugation when the OD_{600} reached 1.5. The cell pellets were stored at -80°C until
487 processing for Tn-Seq. The genomic DNA was extracted and prepared as described
488 previously [17]. Samples were submitted to the Tufts University Core Facility for
489 sequencing on a HiSeq 2500 instrument.
490
491 **Transposon sequencing data analysis**
492
493 Transposon sequencing data was split by transposon and sample, trimmed,
494 filtered, and mapped using the Galaxy software suite as previously described [17, 19,
495 74, 75]. A workflow for the processing is provided on GitHub. The resulting SAM files
496 were converted into tab-delimited hop count files using Tufts Galaxy Tn-Seq software
497 (http://galaxy.med.tufts.edu/) or custom python scripts, likewise provided on GitHub, and
498 then converted further into IGV-formatted files, as previously described [17, 19].
499 Chromosome nucleotide FASTA files for NCTC 8325 (NC_007795.1 - HG003
500 parent strain, as the HG003 genome is not closed), USA300-TCH1516 (NC_010079.1),
501 MSSA476 (NC_002953.3), MW2 (NC_003929.1), and MRSA252 (NC_002952.2) were
502 downloaded from the NCBI genomes database. The genomes were reannotated via
503 Prokka and the pangenome was aligned with Roary, splitting by homolog and using a
504 90% ID cutoff [76, 77]. Roary group names were then adjusted based on common *S.*
505 *aureus* pangenome gene names found on AureoWiki [78].
506 Genes in each strain were labeled as essential, non-essential, or uncertain using
507 the TRANSIT software Gumbel method [79]. Only the transposon constructs with

508 transcriptional terminators were included (four of six transposon constructs). For each
509 TRANSIT Gumbel run, data files for each transposon construct in both replicates (for a
510 total of eight files) were submitted and the mean replicates parameter was chosen.
511 Permutation tests with 20,000 permutations were then conducted between each pair of
512 strains for each gene to determine whether differences in essentiality were significant,
513 using the sum of the same data sets used in the Gumbel analysis. Data were
514 normalized by average reads per TA site with reads (non-zero means normalization)
515 and the p-values from each file pair were corrected for multiple hypothesis testing using
516 the Benjamini-Hochberg method. A gene was considered significantly different between
517 two strains if the q-value was less than 0.05. The list of genes essential for all five
518 strains was then analyzed using the online gene ontology tool PANTHER version 13.1,
519 comparing the NCTC 8325 locus tags for the essential genes to the *S. aureus* reference
520 gene list and using default settings for the overrepresentation test [80].
521 Genes depleted or enriched under daptomycin stress were identified by
522 normalizing the treated sample data by non-zero means and performing a Mann-
523 Whitney U test for each gene. The p-values were then corrected for multiple hypothesis
524 testing by the Benjamini-Hochberg method. A depleted gene was considered to be a hit
525 if there were at least 100 reads in the control file, the q-value was less than 0.05, and
526 the treated:control read ratio was less than 0.1. An enriched gene was considered to be
527 a hit if there were at least 100 normalized reads in the treated file, the q-value was less
528 than 0.05, and the read ratio was greater than ten. We then selected one representative
529 daptomycin concentration for each strain in each medium, chosen to have a similar

530 selective pressure (Table 2). Specifically, the highest concentration file with twenty-five
531 or fewer 10-fold depleted genes with a significant q-value was chosen.
532
533 **Table 2. Daptomycin samples included in the multi-strain comparison of depleted**
534 **and upregulated genes.**

Strain	Medium	Concentration (µg/mL)	Depleted Genes
USA300-TCH1516	RPMI+LB	0.12	8
USA300-TCH1516	MHBII	0.5	12
MW2	RPMI+LB	0.25	18
MW2	MHBII	0.5	8
MSSA476	RPMI+LB	0.25	7
MSSA476	MHBII	0.5	8
HG003	RPMI+LB	0.25	9
HG003	MHBII	1	25
MRSA252	RPMI+LB	0.5	19
MRSA252	MHBII	1	18

535
536 Genes upregulated under daptomycin stress were identified by a bootstrapping
537 approach. We defined the upstream region (UR) of a gene to be the 500 bp ahead of a
538 gene, based on the 99th percentile of 5' untranslated regions of transcripts in *E. coli* [81].
539 Only TA sites located in a UR that had at least one read in either the control or the
540 treated data were included in the analysis. Read differences for each site were then
541 calculated by subtracting the control data reads from the treated data reads. For each
542 strand and for each gene, a distribution for the expected difference in UR reads
543 between the treated sample and the control was created by counting the number of

544 qualifying TA sites in the UR (N) and generating 200,000 summed random samples of N
545 read differences from across the genome. A one-sided p-value for each strand was
546 calculated as the proportion of the null distribution values that was greater than the
547 actual value for that gene, and a q-value was obtained using the Benjamini-Hochberg
548 method. We defined an upregulation signature as any UR in which the q-value for the
549 DNA strand matching the gene direction was less than 0.05, there was more than one
550 TA site in the UR, and the opposing strand had a read difference less than the 90th
551 percentile for all URs. Again, only those samples shown in Table 2 were included.
552
553 **Whole genome sequence comparisons**
554
555 To obtain the genomic sequences of the transposon library parent strains, the
556 strains were cultured in MHBII at 37°C shaking overnight. The DNA was harvested
557 using a Promega Wizard Genomic DNA purification kit and cleaned using a Zymo DNA
558 Clean Up Kit. DNA was tagmented using the Illumina Nextera DNA Library Prep kit, but
559 using 1/20th of the volume recommended by the manufacturer and starting DNA
560 concentrations of 0.5, 0.75, 1, and 2 ng/µL. The tagged DNA fragments were then
561 amplified via PCR. The PCR samples contained 11.2 µL of KAPA polymerase mix
562 (Illumina), 4.4 µL each of the 5 µM column and row indexing primers, and 2.5 µL of
563 tagmented DNA. The thermocycler settings were as follows: preincubation (3 min, 72
564 °C), polymerase activation (5 min, 98 °C), 13 amplification cycles (denaturation at 98 °C
565 for 10 sec, annealing at 62°C for 30 sec, and extension at 72°C for 30 sec), and
566 termination (5 min, 72 °C).

567 To determine which starting DNA concentrations yielded the best fragment size,
568 3.75 µL of each amplified sample was mixed with 4 µL of 6x loading dye and run on a
569 1.5% agarose gel at 110 V. The sample with an average length closest to 500 bp was
570 chosen for each strain. Those samples were then cleaned as recommended by Illumina,
571 except that we started with 15 uL of amplified tagmented DNA and 12 µL of AMPure XP
572 beads. The concentrations of the resulting DNA samples were estimated via Qubit
573 Fluorometric Quantification (Thermo Fisher Scientific) following the manufacturer's
574 instructions. The DNA was then diluted to 1 ng/µL and pooled, and a portion of it was
575 submitted to the Harvard Biopolymer's Facility for TapeStation and qPCR quality control
576 analysis. Upon passing the quality control step, the DNA was prepared and loaded into
577 the sequencing cartridge as directed by the MiSeq Reagent Kit v3 with 150 cycles
578 (Illumina), including a 1% PhiX control spike-in prepared according to manufacturer's
579 instructions (PhiX Control v3, Illumina), and paired-end sequenced using a MiSeq
580 instrument.
581 Genomic data was then analyzed to find SNPs, insertions, and deletions.
582 MetaPhlAn2 was used to verify that the DNA was not contaminated. Sequences were
583 then aligned to the appropriate NCBI reference genome using the Burrow-Wheels
584 Aligner (BWA-MEM, v7.12) with default settings [82]. Note that NCTC 8325 was used
585 for HG003, as the HG003 genome on NCBI is not closed. Duplicate reads were marked
586 with Picard, and Pilon (v1.16) was used for variant calling, with a minimum depth of
587 1/10th of the average read depth and a minimum mapping quality of 30, unless the
588 average read depth was less than 100, in which case the minimum mapping quality was
589 set to 15 [83, 84]. The resulting VCF files were then evaluated for mutations in relevant

590 areas of the genome (*i.e.* the LTA pathway or known *ltaS* suppressors). We compared
591 the predicted protein sequences for LTA pathway members and *ltaS* suppressors in the
592 five strains using the online Clustal Omega tool from EMBL-EBI [85].
593
594 **Gene deletion and complementation**
595
596 To make an anhydrotetracycline (Atet) inducible construct of *ugtP*, a fragment
597 containing the *ugtP* and *ltaA* operon (SAOUHSC_00953-00952) and its ribosomal
598 binding site was amplified from HG003 genomic DNA using *ugtP*-F and *ugtP*-R. The
599 fragment was then cloned into pTP63 using KpnI and EcoRI to generate pTP63-*ugtP*,
600 and the pTP63-*ugtP* was transformed into a wildtype RN4220 strain containing pTP44
601 [86]. Next, the integrated inducible *ugtP* operon was transduced into wildtype HG003,
602 MW2, and USA300-TCH1516 using ϕ11 phage to generate HG003-P_{Atet}-*ugtP*, MW2-
603 P_{Atet}-*ugtP*, and USA300-TCH1516-P_{Atet}-*ugtP*. To generate inducible *ugtP* strains, the
604 wildtype P_{Atet}-*ugtP* strains were grown in TSB containing 0.3 μM Atet for 6 hours at
605 30°C, after which they were transduced with a transposon-inactivated *ugtP* marked with
606 an erythromycin resistance gene [13]. The desired mutants were selected on TSB agar
607 containing 5 μg/mL erythromycin and 0.3 μM Atet and confirmed by PCR using the
608 primers *ugtP*-CA and *ugtP*-CB.
609 To construct an Atet-inducible *ltaA* construct, the *ltaA* gene (*SAOUHSC_00952*)
610 and the *ugtP*-*ltaA* operon ribosome binding site were amplified from HG003 genomic
611 DNA using primers *ltaA*-F and *ugtP*-R and cloned into pTP63 to generate pTP63-*ltaA*.
612 As described above, the pTP63-*ltaA* was transduced into wildtype HG003 and MW2,

613 and the resulting strains were then transduced with a Δ*ltaA* construct marked with a
614 kanamycin resistance gene to generate the inducible *ltaA* strains [87].
615 To construct an Atet-inducible *ltaS* construct, the *ltaS* gene and its ribosome
616 binding site were PCR amplified using the primers *ltaS*-F and *ltaS*-R and cloned into
617 pTP63 to make pTP63-*ltaS*. To make an inducible *ltaS* strain, pTP63-*ltaS* and then a
618 Δ*ltaS* construct marked with an erythromycin resistance gene were transduced into
619 wildtype HG003, MW2, and USA300-TCH1516 as described above [37]. The *ltaS*
620 mutants were confirmed by PCR using the primers *ltaS*-CA and *ltaS*-CB.
621
622 **Spot dilution assays**
623
624 Overnight cultures of the relevant strains were grown in TSB at 30°C until mid-log
625 phase and diluted to an OD_{600} of 0.1. Five 10-fold dilutions of the resulting cultures were
626 prepared, and 5 μL of each dilution was spotted on TSB plates with or without 0.3 μM
627 Atet and, where indicated, 2.5 μg/mL daptomycin. Plates were imaged after
628 approximately 16 hours of incubation. For the *ltaS* experiments, plates were incubated
629 at 30°C, 37°C, and 42°C. For the *ltaA* experiments, plates were incubated at 37°C.
630
631 **LTA western blot**
632
633 LTAs from MW2, MW2-P_{Atet}-*ltaS,* and 4S5, a strain derived from RN4220 known
634 to not produce LTAs [37], were isolated and detected via western blot using a procedure
635 similar to that previously described [88]. Overnight cultures of each strain were grown

636 shaking at 30°C in TSB supplemented with 7.5% salt, with the MW2-P_{Atet}-*ltaS* grown
637 both in the presence and absence of 0.4 μM Atet. Cultures were then diluted 1:50 in the
638 same medium and incubated shaking at 30°C until the OD_{600} was between 0.6 and 0.75.
639 The equivalent of 1 mL of OD_{600} 0.8 was then harvested from each sample by
640 centrifuging at 8000 x *g* for three minutes. The cell pellets were resuspended in 50 μL of
641 a buffer composed of 50 mM Tris pH 7.5, 150 mM NaCl, and 200 μg/mL lysostaphin.
642 Samples were incubated at 37°C for ten minutes. Then, 50 μL of 4x SDS-PAGE loading
643 buffer was added and the samples were boiled for thirty minutes. After returning the
644 samples to room temperature, 100 μL water and 0.5 μL of proteinase K (New England
645 Biolabs) was added to each sample, and samples were then incubated at 50°C for two
646 hours. After cooling samples to room temperature, 2 μL of AEBSF was added to each
647 sample to quench the proteinase K. Ten microliters of each sample and 5 μL of
648 Precision Plus Protein Dual Xtra standard (BioRad) were loaded onto a 4-20% TGX
649 precast gel (BioRad) and the gel was run for 30 minutes at 200 V using a running buffer
650 that was 0.5% Tris, 1.5% glycine, and 0.1% SDS. Proteins were transferred to a
651 methanol-activated PVDF membrane using a TransBlot Turbo (BioRad) via the pre-
652 installed mixed molecular weight setting. The membrane was rinsed with TBST (0.05%
653 Tween-20) and incubated rocking overnight at 4°C in 5% milk in TBST to block. After
654 washing the membrane with TBST three times for five minutes each, the LTAs were
655 bound with a 1:750 solution of a mouse anti-LTA antibody (Hycult Biotech) in TBST for
656 45 min. The membrane was then washed an additional three times for five minutes
657 each before incubating in a 1:2000 dilution of anti-mouse horseradish peroxidase
658 conjugated antibody (Cell Signaling Technologies) in TBST. The membrane was

659 washed a final five times for five minutes each and then exposed to ECL western
660 blotting substrate (Pierce). The membrane was imaged on a FluoroChem R gel doc
661 (ProteinSimple) with a 2 min 40 second exposure for luminescence to detect the LTAs
662 and automatic exposure for red and green fluorescence channels to detect the protein
663 standard.
664

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666

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673

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970

971 ## Figure captions

972

973 **Fig 1. We created complex transposon libraries of similar coverage in five diverse**
974 ***S. aureus* strains.**
975 (A) Six barcoded transposon constructs were used to make sub-libraries that were
976 combined into a single transposon library. Construct 1 can drive expression only of
977 *erm*^{R} (gene within the transposon constructs). Constructs 2-6 can drive expression of
978 other genes, either by readthrough of *erm*^{R} in constructs lacking terminators or because
979 they contain an additional outward-facing promoter (P_{cap}, P_{pen}, or P_{tuf}). The libraries can
980 be sequenced to find essential genes that lack transposon insertions (red gene) or
981 treated with compound and then sequenced to find conditionally essential genes (yellow
982 gene), upregulation signatures (upstream of yellow gene), and genes with conditional
983 fitness costs (green gene). (B) High-density transposon libraries were made in five *S.*
984 *aureus* strains. MSSA = methicillin-sensitive *S. aureus;* MRSA = methicillin-resistant *S.*
985 *aureus*; ST = sequence type. Sites with insertions refers to the number of TA
986 dinucleotide sites having insertions of at least one transposon construct. The "unique
987 insertions" column sums the TA insertion counts from each transposon construct.
988 Coverage per gene is the average percent of TA sites within a gene with insertions, ±
989 standard deviation. Low-coverage genes were defined as those that had less than half
990 of the average gene coverage.
991
992 **Fig 2. The fitness of lipoteichoic acid pathway mutants varies by strain.**
993 (A) The number of essential genes per strain (horizontal bars) and the number of genes
994 found to be essential for all three, two, or one of the MRSA strains (vertical bars, with
995 black dots denoting strains for which the genes are essential). Only MRSA strains were

996 included for simplicity, but a similar plot with the MSSA strains is included in the
997 supporting information. Note that gene essentiality is functionally defined as a lack of
998 transposon insertions in a gene, but it may be possible to make knockouts of some of
999 these genes under favorable conditions. (B) A schematic of the lipoteichoic acid
1000 pathway in *S. aureus*. (C) The number of reads in each LTA pathway gene for each
1001 transposon library, expressed as a percentage of the average number of reads per TA
1002 site in the coding regions of the libraries. There are strain-dependent differences in
1003 transposon insertions in LTA pathway genes, with USA300-TCH1516 appearing reliant
1004 on most genes in the pathway while MW2 is insensitive even to inactivation of *ltaS*.
1005

1006 **Fig 3. The lipoteichoic acid pathway is crucial for fitness of USA300-TCH1516 but**
1007 **is dispensable in MW2.**
1008 (A) Tn-Seq data for the LTA pathway genes, with sequencing reads from insertions in
1009 the plus strand in purple and minus strand in teal. The reads were calculated by
1010 summing two replicate Tn-Seq experiments, only including data from transposon
1011 constructs that contained a transcriptional terminator. The data were normalized to each
1012 other by non-zero means normalization prior to plotting. The x-axis is expressed in
1013 kilobases. The y-axis is expressed on a $\log_{10}$ scale and is truncated to 500 reads. (B)
1014 Growth on agar plates for wildtype (WT) and Δ*ugtP* strains confirms that USA300-
1015 TCH1516 is more sensitive to *ugtP* deletion than MW2 is. (C) Growth on agar plates of
1016 wildtype and Δ*ltaS* strains confirms that *ltaS* is dispensable in MW2. (D) Western blot of
1017 LTAs produced by 4S5 (known to not produce LTAs), wildtype MW2, and MW2-P_{Atet}-

1018 *ltaS* confirms that MW2-P_{Atet}-*ltaS* does not produce LTAs unless expression of the
1019 exogenous copy of *ltaS* is induced.
1020
1021 **Fig 4. Transposon sequencing supports previously reported vulnerabilities in *S.***
1022 ***aureus* and reveals new ones.**
1023 (A) A comparison of the Tn-Seq hits under daptomycin exposure in five strains of *S.*
1024 *aureus*. All hits needed to have a q-value less than 0.05. Depleted genes had 10-fold
1025 fewer reads in the daptomycin-exposed sample compared to the control sample after
1026 normalization. Upregulated genes had a strand-biased enrichment of reads in the region
1027 upstream of the gene. The upper graph shows how many strains each hit was found in
1028 while the lower graph shows how many hits found in each strain were shared with at
1029 least one other strain and how many were unique to that strain. (B) Genes that were
1030 depleted of reads under daptomycin exposure in at least three of the transposon
1031 libraries, using the cutoffs described above. Hypothetical genes are listed according to
1032 their NCTC 8325 locus tag numbers. Genes meeting all cutoffs in a strain are indicated
1033 by dark pink. Those that have a significant q-value but are only depleted 5- to 10-fold
1034 are indicated in light pink. (C) Normalized Tn-Seq reads from daptomycin-exposed
1035 MSSA476 in RPMI+LB plotted against the reads from the control condition. Genes from
1036 above that were shared by at least 4 strains are highlighted.
1037
1038 **Fig 5. LTA loss sensitizes *S. aureus* to daptomycin.**
1039 (A) Normalized Tn-Seq reads from daptomycin-exposed HG003 in RPMI+LB plotted
1040 against the reads from untreated HG003 in the same growth medium. Genes in the LTA

1041 pathway that were significantly depleted are highlighted. (B) Growth on agar plates of
1042 HG003 wildtype, Δ*ltaA,* and inducible *ltaA* complementation strains in the presence or
1043 absence of daptomycin and the inducer confirms that *ltaA* is required for *S. aureus*
1044 growth under daptomycin exposure.
1045

1046 ## Supporting information

1047

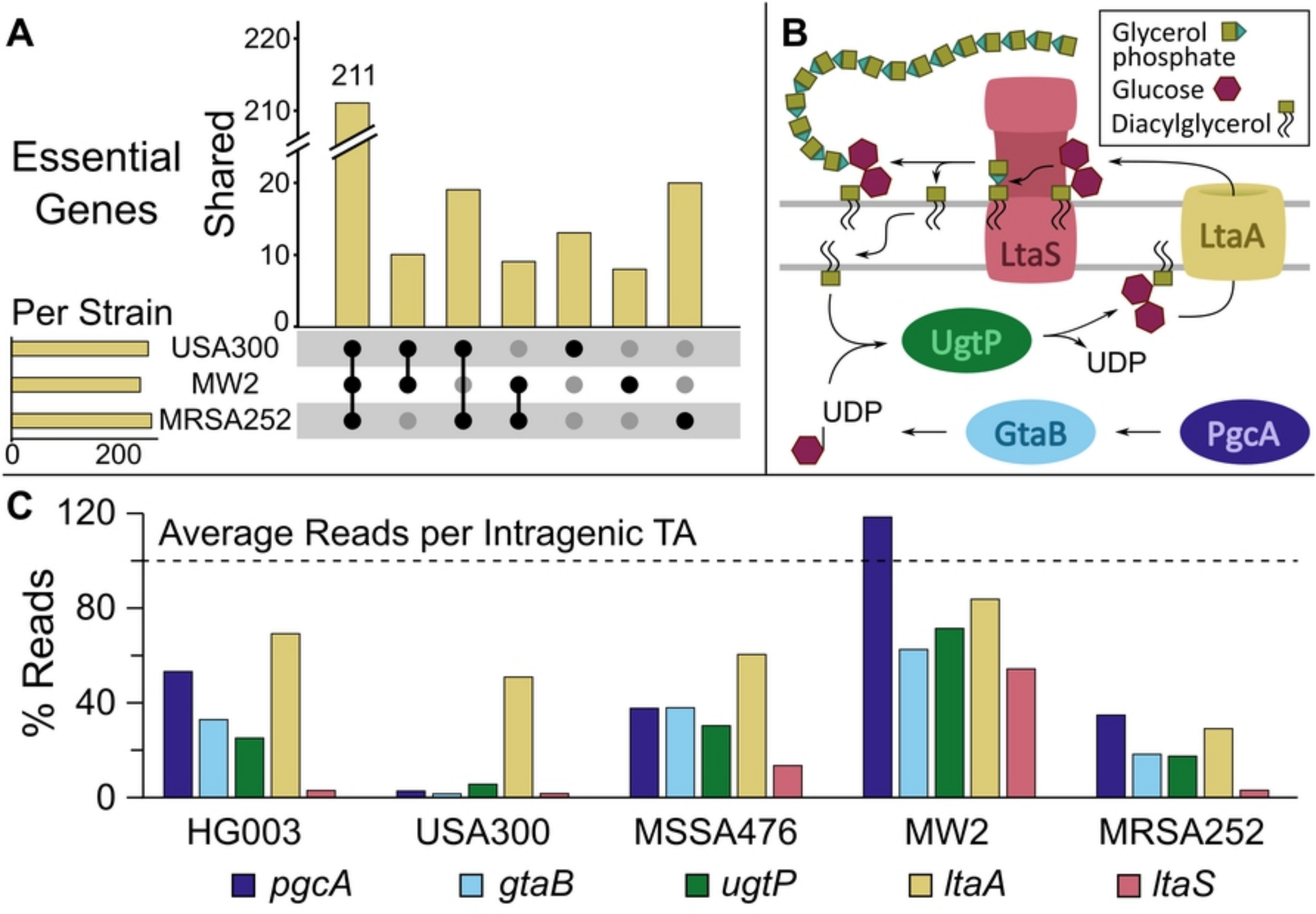
1048 **S1 Fig. Gene essentiality varies by strain.** The number of essential genes per strain
1049 (horizontal bars) and the number of genes found to be essential for all five or a subset
1050 of the *S. aureus* strains (vertical bars, with black dots denoting strains for which the
1051 gene is essential).
1052

1053 **S2 Fig. Tn-Seq data reveals variable dependence on the lipoteichoic acid pathway**
1054 **among *S. aureus* strains.** Each column of graphs represents a gene in the LTA
1055 pathway, with rows representing strains. Reads in the Tn-Seq data, expressed on a
1056 $\log_{10}$ scale, are plotted against the position in the genome indicated on the x-axis with a
1057 depiction of the gene context underneath each plot. The y-axis is truncated to 500
1058 reads. Purple lines indicate plus-strand reads. Teal lines indicate minus-strand reads.
1059

1060 **S3 Fig. MW2Δ*ltaS* mutants are temperature sensitive.** Growth on agar plates of
1061 wildtype and *ltaS* complementation strains for HG003, MW2, and USA300-TCH1516
1062 with and without inducer at 30°C, 37°C, and 42°C.
1063

1064 **S4 Fig. *ltaA* is conditionally essential in USA300-TCH1516 and MW2 in the**
1065 **presence of daptomycin.** Growth on agar plates of wildtype, Δ*ltaA*, and inducible *ltaA*
1066 complementation strains for MW2 and USA300-TCH1516 in the presence or absence of
1067 daptomycin and the complementation inducer confirms that *ltaA* is required for *S.*
1068 *aureus* growth under daptomycin exposure.
1069
1070 **S1 Table. Comparison of essential genes in *S. aureus* strains.** A table of all genes
1071 that are essential for at least one strain, with essentiality designations for each gene in
1072 each strain for the TRANSIT Gumbel analysis and the subsequent permutation test
1073 which was used to determine whether differences between strains seen in the Gumbel
1074 results were significant. If differences were not significant, essential (E) and
1075 nonessential (NE) designations were converted to uncertain (U).
1076
1077 **S2 Table. Core essential genes in *S. aureus.*** A list of the genes that were determined
1078 to be essential in all five *S. aureus* strains.
1079
1080 **S3 Table. Gene ontology analysis reveals central dogma pathways to be**
1081 **universally essential in *S. aureus*.** Red text highlights those processes mentioned in
1082 the main text. DNA, RNA, and protein metabolic processes are significantly over-
1083 represented among the genes essential for all five strains of *S. aureus* studied.
1084 Phospholipid and monosaccharide synthesis were likewise over-represented, while
1085 genes of unknown function were under-represented.
1086

1087 **S4 Table. Genes depleted under daptomycin exposure.** A table of the genes that
1088 were 10-fold depleted of reads in the normalized daptomycin file compared to the
1089 control file with q-values less than 0.05 and at least 100 reads in the control file.
1090
1091 **S5 Table. Genes upregulated under daptomycin exposure.** A table of the genes that
1092 had upregulation signatures under daptomycin exposure. See methods for details of
1093 analysis.
1094
1095 **S6 Table. Genes enriched under daptomycin exposure.** A table of the genes that
1096 were 10-fold enriched in reads in the normalized daptomycin file compared to the
1097 control file with q-values less than 0.05 and at least 100 normalized reads in the
1098 daptomycin file.
1099
1100 **S7 Table. Primers, plasmids, and strains used in this study.**
1101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Essential Genes
Shared
220
210
20
10
0
211
Per Strain
USA300
MW2
MRSA252
0
200
B
Glycerol phosphate
Glucose
Diacylglycerol
LtaS
LtaA
UgtP
UDP
UDP
GtaB
PgcA
C
Average Reads per Intragenic TA
% Reads
120
80
40
0
HG003
USA300
MSSA476
MW2
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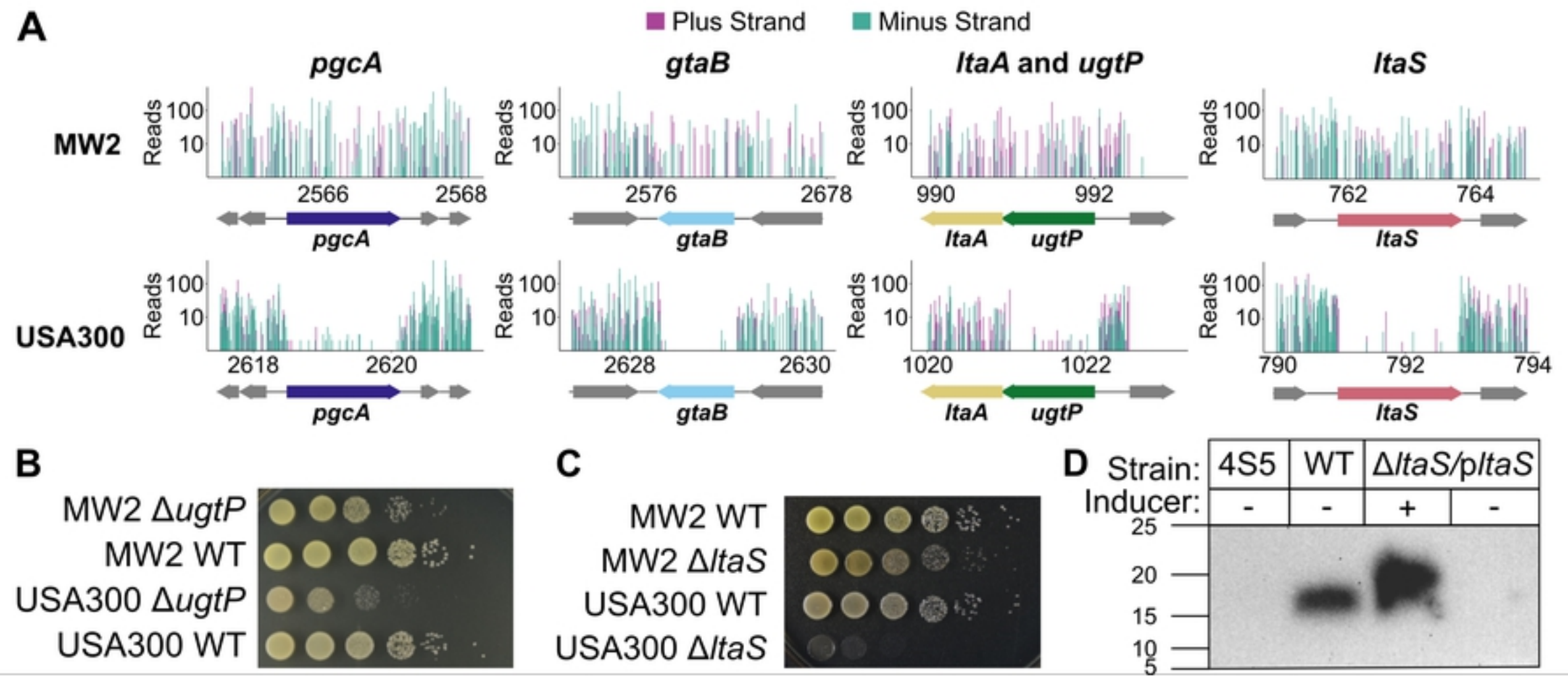
pgcA
gtaB
ugtP
ltaA
ltaS

Fig 2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Plus Strand
Minus Strand
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gtaB
ltaA and ugtP
ltaS
MW2
USA300
Reads
100
10
2566
2568
2576
2678
990
992
762
764
2618
2620
2628
2630
1020
1022
790
792
794
pgcA
gtaB
ltaA
ugtP
ltaS
B
MW2 ΔugtP
MW2 WT
USA300 ΔugtP
USA300 WT
C
MW2 WT
MW2 ΔltaS
USA300 WT
USA300 ΔltaS
D
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WT
ΔltaS/pltaS
Inducer:
-
-
+
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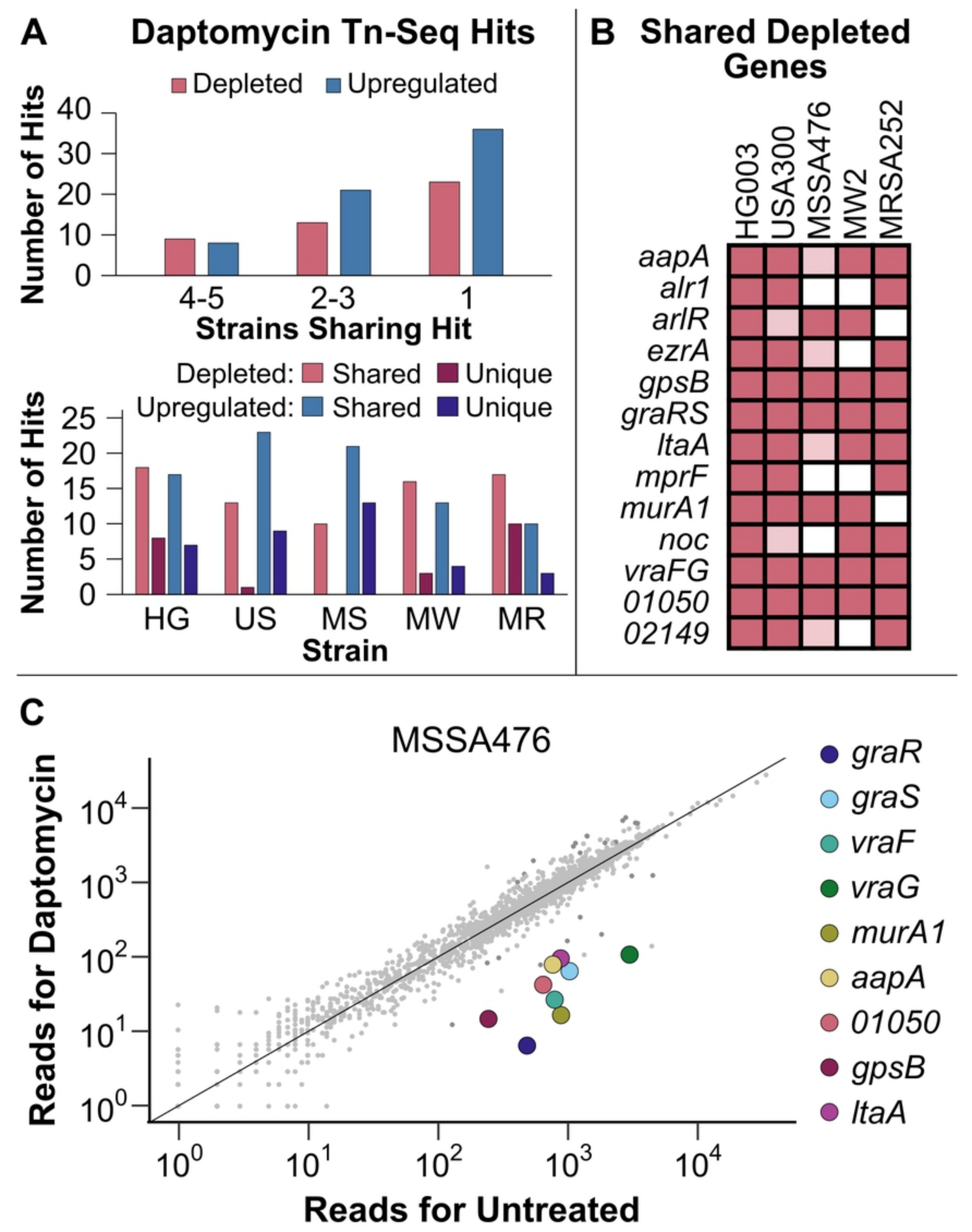
Fig 3


A
Daptomycin Tn-Seq Hits
Depleted
Upregulated
Number of Hits
40
30
20
10
0
4-5
2-3
1
Strains Sharing Hit
Depleted: Shared Unique
Upregulated: Shared Unique
Number of Hits
25
20
15
10
5
0
HG
US
MS
MW
MR
Strain
B
Shared Depleted Genes
HG003
USA300
MSSA476
MW2
MRSA252
aapA
alr1
arlR
ezrA
gpsB
graRS
ltaA
mprF
murA1
noc
vraFG
01050
02149
C
MSSA476
Reads for Daptomycin
Reads for Untreated
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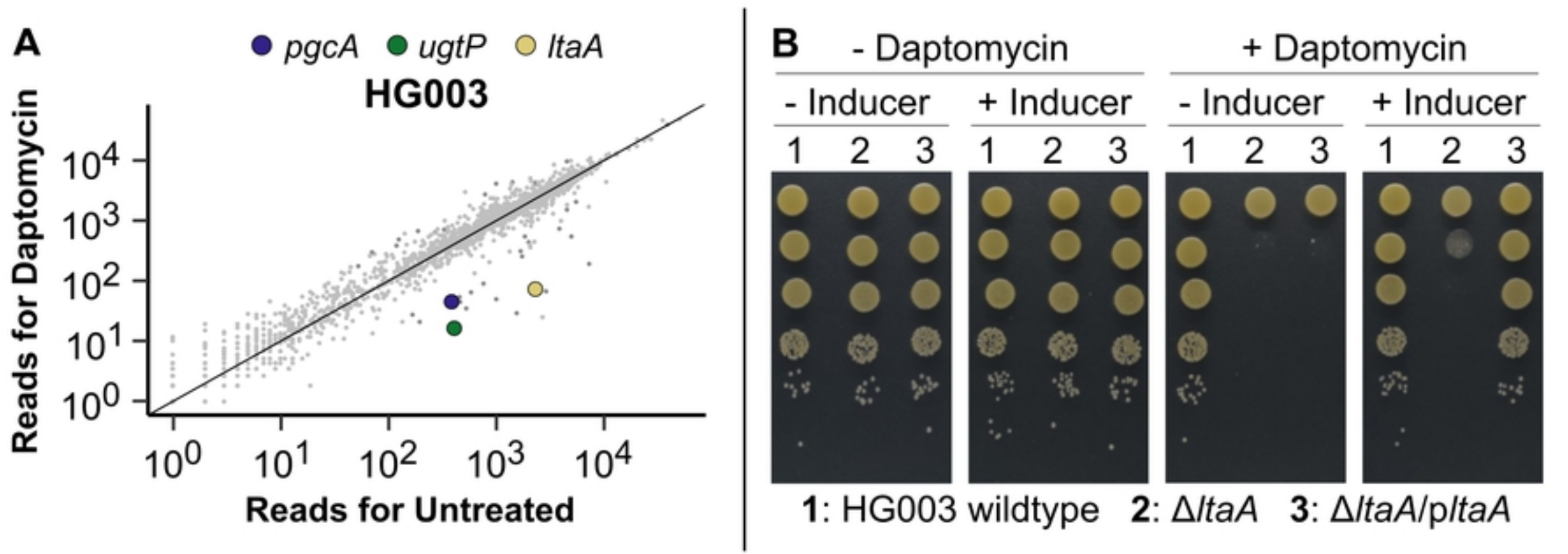
graS
vraF
vraG
murA1
aapA
01050
gpsB
ltaA

Fig 4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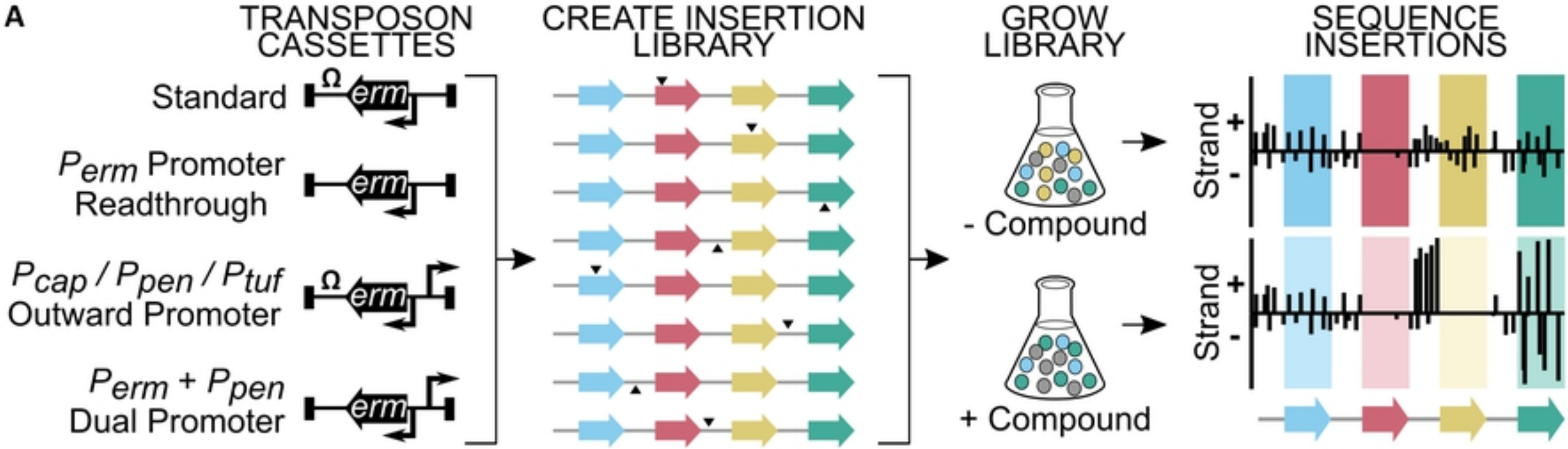
pgcA
ugtP
ltaA
HG003
Reads for Daptomycin
Reads for Untreated
10^0
10^1
10^2
10^3
10^4
B
- Daptomycin
+ Daptomycin
- Inducer
+ Inducer
- Inducer
+ Inducer
1 2 3
1 2 3
1 2 3
1 2 3
1: HG003 wildtype
2: ΔltaA
3: ΔltaA/pltaA

Fig 5



B

	MSSA/ MRSA	Associated Environment	Isolation Location	ST	Sites with Insertions		Unique Insertions	Coverage Per Gene	% Genes with Low Coverage
HG003	S	Laboratory	-	8	186,736	69%	447,835	68 ± 18%	9%
USA300	R	Community	USA	8	265,871	95%	1,193,971	88 ± 19%	8%
MSSA476	S	Community	UK	1	215,867	81%	746,933	75 ± 22%	12%
MW2	R	Community	USA	1	167,322	62%	388,101	61 ± 16%	10%
MRSA252	R	Hospital	UK	36	201,229	72%	527,404	72 ± 18%	9%

Fig 1