Supplementary Materials

Bacterial strains. *Staphylococcus aureus* strain HG003 was chosen as the background strain for this study because: a) it was originally isolated as an infection-derived ST8 strain, the most common sequence type associated with methicillin resistant infection in the community (1), but is not itself methicillin-resistant (2), adding an additional level of safety to animal experimentation; b) related strains have served as the basis for many studies, including a complete genome sequence determination (GenBank accession no. CP000253) (Gillaspy A.F., *et al*; "The Staphylococcus aureus NCTC8325 genome"; Direct Submission (JAN-2006) to the EMBL/GenBank/DDBJ databases.); and c) it has been verified as a fully virulent and representative of ST8 (2). Inducible antisense constructs were initially generated in *E. coli* DH5a, and subsequently tested in *S. aureus* strain RN4220.

Construction and validation of the transposon library. The mariner-based *bursa aurealis* transposon insertion system (60) was used to construct a mutagenized library in HG003 essentially as described by Bae *et al.* (57). Confirmation of independent, unique transposon insertion was achieved by screening 20 randomly selected colonies by inverse PCR, as previously described (57). Approximately 5,000 colonies from 20 plates were harvested directly using 5 mL BHI with erythromycin to wash each plate, which was mixed with 5 mL 50% glycerol to generate 20 separate 10 mL aliquots each representing a subset of the library. Before freezing at -80^{o}C, each tube was

thoroughly homogenized by vortexing and 1 mL was pooled into a single aliquot representing the entire library encompassing approximately 100,000 individual transposon insertions. Total DNA was extracted from 1 mL of the library freezer stock using the DNeasy blood and tissue kit (Qiagen, Valencia, CA), following manufacturer's specifications, and subjected to Tn-Seq analysis.

Competitive fitness in laboratory medium versus physiological fluids. To generate a common inoculum for determining which genes encode products that are critical for competitive growth of *S. aureus* in different environments, 100 µL representing 10^8 colony forming units (CFU) of the *S. aureus* transposon library stock was inoculated into 100 mL BHI without antibiotic, and incubated overnight at 37^{o}C. An overview of the experimental strategy to determine genes essential for fitness in competitive, infection-relevant contexts is illustrated in Figure S1. A 10 µL aliquot of this culture, containing 10^7 CFU was then used to inoculate 10 mL of fresh BHI (control), 10 mL of bovine aqueous or vitreous fluids buffered with 25 mM MOPS. Aqueous and vitreous fluid were aspirated from freshly enucleated bovine eyes (Sierra Medical, Whittier, CA), and filtered through a 0.45 µm HT Tuffryn membrane sterile acrodisc syringe filter (Whatman, Clifton, NJ) before storage at -80°C. Peripheral blood was freshly isolated from healthy volunteers into heparinized Vacutainer collection tubes (BD, Franklin Lakes, NJ) following Institutional Review Board approved protocol #11-093H. Preliminary studies examining culture of these mutants in blood found a decrease in viable cells following inoculation, therefore a 100 µl inoculum representing 10^8 CFU,

into 10 mL, was adopted to overcome potential population bottlenecks in the library pool. No such drop was observed in vitreous or aqueous cultures. Bacterial growth in physiological fluids occurred at 37^{o}C with shaking at 225 RPM. Biological replicates were conducted for each growth condition. Bacteria were grown for 24 hours, and 9-10 generations of outgrowth occurred in each growth condition. Cultures were harvested by centrifugation at 13,000 x g for 10 min. To facilitate the lysis and removal of eukaryotic cells from the blood, cultures were washed three times by resuspending in an equal volume of deionized water and centrifugation. DNA was then isolated from the bacteria harvested from each growth condition, using the DNeasy blood and tissue kit (Qiagen, Valencia, CA) following manufacturer's specifications.

Competitive fitness in a murine abscess. The competitive ability of various *mariner* insertion mutants of *S. aureus* to proliferate at the site of infection was tested using a murine abscess model, essentially as described (38) (Figure S3A). In order to prepare the mutant library for injection, a 100 µL aliquot of the input library freezer stock, representing 10^8 CFU, was inoculated into 100 mL of BHI without antibiotic selection and was cultured overnight at 37°C. To isolate cells in exponential growth phase, a 1:1000 dilution from the overnight was made into fresh BHI without antibiotic and bacterial growth was monitored until an $O.D._{600}$ of 0.8 was reached. A 1 mL sample was harvested, centrifuged and resuspended in 2 mL sterile phosphate-buffered saline (PBS), diluted 1:10 to achieve 10^7 CFU/mL in 1 mL sterile PBS and mixed with an equal volume of autoclaved

Cytodex-1 beads (60 – 87 μm; Sigma, St, Louis, MO). A 200 μL aliquot containing $5x10^6$ CFU was injected subcutaneously into each shaved hind flank of a 4-5 week old Swiss Webster male mouse, anesthetized with ketamine and xylazine, and allowed to form abscesses for 24 or 48 h before harvest. Groups of 5 mice were used for either 24 h or 48 h abscess growth studies (Figure S3B). For each time point, four animals were used for Tn-Seq analysis and output CFU enumeration and one for histology (Figure S3C). Abscesses were excised from each animal with flank skin attached and immediately placed on ice. Extraneous skin and tissue were carefully removed before each abscess was pulverized in 1 mL BHI in a 1.5 mL tube using a mortar, followed by a 1-3 second vortex. Bacterial load was quantified for each abscess by track dilution (3). The average bacterial load per abscess was found to be $7.75x10^6$ CFU. To enrich for bacterial cells available for Tn-seq analysis, a 0.5 mL aliquot from two suspended abscesses were pooled together, and used to inoculate 4 mL of BHI supplemented with 10 ug/ml erythromycin ($1.55x10^6$ CFU/mL). Abscesses harvested from the contralateral flanks from two animals were pooled in efforts to equalize animal-to-animal variability that might have arisen due to technical reasons (*e.g.,* variability in injection size, depth, inoculum). Pooled samples were cultured for 5 h at 37°C, allowing between 5 – 6 generations in order to enrich the bacterial profiles while minimizing potential biasing during this outgrowth phase, as well as aiding in the lysis and removal of any residual eukaryotic cells. Average bacterial outgrowth during the 5 h was found to be $6.7x10^7$ CFU/mL by track dilution plating. The top 4.5 mL from each tube enriched in bacterial cells

93 (remaining eukaryotic cells will sink to the bottom), representing $3x10^8$ CFU, was
94 harvested for DNA isolation using the DNeasy blood and tissue kit following
95 manufacturer's specifications (Qiagen, Valencia, CA), which was subsequently
96 subjected to preparation for Tn-Seq analysis as described below.
97
98 **Sequencing of libraries.** Molecular methods used to construct the Illumina
99 sequencing libraries was recently described (61). Briefly, genomic DNA (50-100
100 L at 70 ng/L) was further purified using an Edge Biosystems spin column
101 (Gaithersburg, MD), and 130 µL was sheared in a Covaris sonicator (Woburn,
102 MA) to generate products of average size of approximately 200-600 bp. PolyC
103 tails were added to 1 µg of the sheared DNA in a 20 µL reaction containing 0.5
104 µL terminal deoxynucleotidyl transferase (TdT, Promega, Madison, WI), 4 L 5x
105 TdT reaction buffer (Promega, Madison, WI), 9.5 mM dCTP / 0.5 mm ddCTP.
106 Following a 1 h at 37°C, enzyme was inactivated by incubation at 75 °C for 20
107 minutes and sheared products repurified using an Edge Biosystems spin column
108 (Gaithersburg, MD). Transposon-genome fusion fragments were specifically
109 amplified in an initial 50 µL PCR reaction that contained 5 µL C-tailed template,
110 600 nM oligo dG primer (Table S10), 600 nM transposon-specific primer (Table
111 S10), 400 M dNTPs, 5 µL 10x Easy-A buffer (Stratagene-Agilent Technologies,
112 Santa Clara, CA), and 1 µL Easy-A high fidelity PCR cloning enzyme using the
113 following thermocycler program: Initial denaturation 95 °C, 2 minutes; followed by
114 25 cycles of 95 °C denaturation for 30 seconds; 60 °C annealing for 30 seconds;
115 72 °C extension for 120 seconds; followed by a 4 °C hold. A second PCR

reaction using a nested pBursa primer was then used to provide added specificity and append the primer sequences needed for Illumina sequencing and indexing. This 50 µL reaction contained 1 µL of template from the previous PCR reaction, 600 nM transposon end-specific primer (Table S4), 600 nM indexing primer (1 of 15 available indices), 400 M dNTPs, 5 µL 10x Easy-A buffer (Stratagene-Agilent Technologies, Santa Clara, CA), and 1 µL Easy-A high fidelity PCR cloning enzyme using the same thermocycler program described above, but only for 10 – 15 cycles. Up to 15 different resulting libraries were multiplexed and sequenced in parallel for 51 cycles in a single end sequencing reaction on a single lane of an Illumina HiSeq 2000 (Tufts University Genomic Core Facility) using a custom sequencing primer and a standard Illumina index sequencing primer (Table S4).

Data analysis. Mapping of transposon-genome junction sequence reads to the HG003 genome was carried out utilizing custom scripts and programs on the Tufts University Galaxy server. The computational methods used for analysis was recently published (61). Briefly, following removal of adapter and transposon sequence, genomic sequences with a minimum read length of 25 nucleotides were aligned to the *S. aureus* reference genome of the HG003 parent strain NCTC8325 (Genebank: CP000253.1), using bowtie with its default settings. The resulting bowtie output file was then used as input for a custom script, "hopcount". Hopcount tabulates the number of times individual insertion amplicons were re-sequenced. An excel spreadsheet is generated that for each insertion site, indicates its position within the genome, gene locus to which that

position maps, the strand (positive vs. negative) associated with the site, as well as the number of each amplicon species read. Hopcount output was used to determine the complexity of transposon libraries, and to identify shifts in the representation of specific mariner insertion in input and output samples. It was also used as input for a second custom script, “Aggregate Hop Table”. The output of this script is an excel file in which all individual transposon insertion amplicon reads are tabulated by their collective frequency in each annotated gene of the genome. For each gene, the number of unique insertions sites observed, absolute count of sites in the positive strand, in negative strand, and in both strands is tabulated. Also recorded is a value, dval, which normalizes the total number of insertion amplicon reads occurring in a specific gene to the number expected for that gene bases solely on its size and assuming complete randomness of insertion. That is, dval represents the observed number of mappable reads of insertions in a gene, divided by the number of mappable reads of insertions predicted for that gene based on its size relative to the genome and the total number of mappable reads obtained for that experiment. Typically, approximately 500 amplicons from each transposon insertion site are read in a sequencing reaction, resulting in about 10,000 total reads for all transposon insertions in a 1000 bp gene. To illustrate dval, in a typical experiment, a 1 kb gene for which 10,000 reads were obtained would have a dval ratio of 1.0 (10,000 reads obtained divided by 10,000 reads expected). Obtaining only 1,000 reads for this gene would result in a dval ratio of 0.1, indicating that fewer amplicons of *mariner* fusion insertions in that gene were read than would

be predicted; and 100 reads a dval of 0.01, indicating a high level of underrepresentation of insertions in that gene.

Parameters for preliminary identification of genes encoding functions likely to be essential or making an important contribution to fitness in a particular environment. Following transposon fusion fragment sequencing and bioinformatic analysis, all HG003 genes encoding open reading frames, or known structural RNAs, were ranked by dval, and binned into three categories: 1) Candidate essential genes, dval 0≤0.01, indicating very low representation in the sequencing reaction; 2) fitness compromised, dval 0.01≤0.1; and 3) unaffected, dval >0.1. That is, candidate essential genes were identified as genes into which transposon insertion was not tolerated, and as a result no reads were mapped (dval = 0), or where sequencing reads were obtained at less than 1% of the frequency predicted based on gene size (allowing for example for a rare insertion at the 3' end of a gene). Genes encoding products that were not defined as essential, but were clearly selected against in the population (Fitness compromised), possessed reads of transposon insertions at a rate ranging from 1% of expected to 10% of expected. This group, as well as genes into which insertions occurred without large fitness cost, were re-examined to identify additional genes that, irrespective of the number of sequencing reads obtained, possessed improbably low numbers of transposons insertions. The goal was to cull out genes that may encode essential products, but into which insertions near the sequence encoding the amino terminus or C-terminus of a protein were

tolerated. For this manual curation, genes identified as “fitness compromised” and “unaffected” were re-sorted based upon transposon density (tn_density), which was calculated by dividing the number of unique transposon insertions by the length of the open reading frame. Genes possessing a tn_density below 0.00285 (1 insertion every 350 bp, approximately 1/10th the expected rate of insertion) were manually added to the candidate essential gene list. Therefore, the candidate essential gene list consists of genes for which transposon insertion reads occurred at ≤1% compared to the overall library pool (dval), and those with a transposon density ≤10% of the predicted frequency (tn_density). This analysis was conducted for the sequencing analysis of DNA obtained from each culture condition. To insure against anomalies deriving from irreproducible variation in a given analysis, dval and tn_density values were averaged for 2 independent biological replicates prior to analysis and curation. Since considerable variance between abscess replicates was seen, essential genes were curated for each replicate individually and only those genes which were found in both replicates have been further analyzed. Candidate essential gene sets were then compared across growth conditions. The web-based software tool Essentials, was used to determine the statistical validity of Tn-seq results (4).

Xylose inducible antisense RNA. To verify Tn-seq results, select candidate essential genes were individually examined by expressing an inducible antisense RNA, and testing for growth inhibition. Target genes of interest were specifically amplified and cloned in the antisense orientation into a xylose inducible vector.

Depending on gene length, either the entire length, if <1kb, or the first 1 – 1.4 kb of an open reading frame, and the native ribosome-binding site, were amplified by PCR, using specific primers that included either EcoRI or KpnI restriction sites for cloning (Table S4). Efforts were taken to avoid any overlap between adjacent open reading frames. Following restriction digest, amplicons were cloned into the pEPSA5 vector (13), for expression in the antisense direction under a xylose inducible promoter. Ligated constructs were initially transformed into *E. coli* DH5-alpha and verified by sequence analysis. Purified recombinant plasmid was then transformed into the transformable *S. aureus* NCTC8325-4 derivative, RN4220, and plated on LB agar supplemented with 0.2% glucose (LBG) and 15 µg/mL chloramphenicol. Growth curves were carried out utilizing RN4220 strains with gene-specific antisense fragment or empty vector as follows. Overnight cultures were diluted 1:100 in LB supplemented with 0.2% glucose and 60 µg/mL chloramphenicol. At an approximate $O.D._{600}$ of 0.2, cultures were diluted such that approximately 100 cells were inoculated in triplicate into inducing (4% xylose) or non-inducing LB supplemented with 0.2% glucose and 60 µg/mL chloramphenicol in 200 µL total volume in a microtiter plate. Growth was followed for 20 h by measuring $O.D._{600}$ on a Biochrom WPA spectrophotometer (Biochrom, Holliston, MA).

References

1. **Diep BA, Gill SR, Chang RF, Phan TH, Chen JH, Davidson MG, Lin F, Lin J, Carleton HA, Mongodin EF, Sensabaugh GF, Perdreau-**

Remington F. 2006. Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant *Staphylococcus aureus*. Lancet **367:**731-739.

2. **Herbert S, Ziebandt AK, Ohlsen K, Schafer T, Hecker M, Albrecht D, Novick R, Gotz F.** 2010. Repair of global regulators in *Staphylococcus aureus* 8325 and comparative analysis with other clinical isolates. Infect Immun **78:**2877-2889.

3. **Jett BD, Hatter KL, Huycke MM, Gilmore MS.** 1997. Simplified agar plate method for quantifying viable bacteria. Biotechniques **23:**648-650.

4. **Zomer A, Burghout P, Bootsma HJ, Hermans PW, van Hijum SA.** 2012. ESSENTIALS: software for rapid analysis of high throughput transposon insertion sequencing data. PLoS One **7:**e43012.