Highly Saturated Transposon Sequencing identifies genes impacting Staphylococcus aureus pathogenesis in macrophages

HsinYu Lo

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Committee:

Stephen J. Salipante

Brad T. Cookson

Noah G. Hoffman

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Abstract

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HsinYu Lo

Chair of the Supervisory Committee:

Stephen J. Salipante

Department of Laboratory Medicine and Pathology

Staphylococcus aureus is a facultative intracellular pathogen in many host cell types, facilitating its persistence in chronic infections. The genes contributing to intracellular pathogenesis have not yet been fully enumerated. Here, we cataloged genes influencing *S. aureus* invasion and survival within human macrophages using two laboratory strains (ATCC2913 and JE2). We developed an *in vitro* transposition method to produce saturated transposon mutant libraries in *S. aureus*, and performed Tn-Seq to identify candidate genes with significantly altered abundance following macrophage invasion. While some significant genes were strain-specific, 107 were identified in common across both *S. aureus* strains, with most (n=105) being required for optimal macrophage infection. We used CRISPR interference (CRISPRi) to functionally validate phenotypic contributions for a select subset of genes. Of the 20 genes passing validation, 7 had a previously identified role in *S. aureus* virulence, and 13 were newly implicated. Validated genes frequently evidenced strain-specific effects, yielding opposing phenotypes when knocked

down in the alternative strain. Genomic analysis of *de novo* mutations occurring in groups (n=237) of clonally-related *S. aureus* isolates from the airways of chronically infected individuals with cystic fibrosis (CF) revealed significantly greater rates of *in vivo* selection in candidate genes than factors not associated with macrophage invasion. This study implicates a core set of genes necessary to support macrophage invasion by *S. aureus,* highlights strain-specific differences in phenotypic effects of effector genes, and provides evidence for selection of candidate genes identified by Tn-Seq analyses during chronic airway infection in CF patients *in vivo*

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Chapter 1. Abstract Importance

Staphylococcus aureus is an important cause of chronic infections. Its persistence in the body reflects, in part, the ability to invade and live inside human cells, where it is protected from the immune system and from the effects of antibiotics. However, the genes that *S. aureus* uses to infect human cells are not fully understood. We conducted studies to comprehensively catalog the genes allowing two *S. aureus* strains to reside within human macrophages. We identified a common set of 105 genes necessary to support full virulence, and found that many exerted strain-specific effects. We also found that genes implicated by our study are under evolutionary selection during chronic infection of human airways in individuals with cystic fibrosis. This work newly identifies genes and pathways important to *S. aureus* intracellular pathogenesis, which may enable novel therapeutic strategies able to block chronic *S. aureus* infections in humans.

Chapter 2. Introduction

Staphylococcus aureus is a prevalent and well-studied bacterium capable of causing a range of diseases in humans and animals alike, but has only recently been recognized as a facultative intracellular pathogen[1,2]. Multiple studies conducted both *in vitro* and *in vivo* have demonstrated that *S. aureus* is able to enter, replicate within, and persist inside various host cell types, including professional and non-professional phagocytes[1–4]. Intracellular pathogenesis is believed to perpetuate chronic infections by allowing *S. aureus* to evade both the human immune system and the action of extracellular antibiotics[5]. Infected host cells can consequently serve as reservoirs for quiescent *S. aureus* that later maintain persistent infection and facilitate dissemination of bacteria[2,6]. Indeed, small colony variants (SCVs) of *S. aureus,* slow-growing auxotrophic mutants that arise frequently in chronic infection, are notable for their elevated capacity for intracellular pathogenesis[7–11].

The fate of intracellular *S. aureus* depends on host and strain-dependent factors. Host cell type is one such determinant[4]. During invasion of non-professional phagocytes, *S. aureus* utilizes adhesins to trigger uptake by host cells. After internalization, *S. aureus* can escape the endosome into the cytosol and replicate. In contrast, professional phagocytes actively engulf bacteria into phagosomes, which ultimately fuse with lysosomes to mature as bactericidal phagolysosomes. *S. aureus* can persist and replicate in that compartment, and encodes molecular pathways providing protection against lysozyme, antimicrobial peptides, reactive oxygen species, and low pH environments[4]. Prior work has separately demonstrated that various *S. aureus* lineages differ in their inherent capacity for pathogenesis within various host cell types, likely due to differences in the complement or expression of relevant virulence factors[12].

Though substantial work has identified genes critical for *S. aureus* pathogenesis in host cells, knowledge of the pathways and factors involved remains incomplete[1,3,4]. Moreover, the extent to which strain-specific accessory genes or genetic backgrounds impact intracellular pathogenesis has not yet been extensively explored[4]. To address these questions, here we conducted studies to more comprehensively catalog factors contributing to the invasion and early survival of two phylogenomically distinct *S. aureus* laboratory strains (ATCC2913 and JE2) within human macrophages. We developed methods to generate high-saturation Tn5-based transposon mutant libraries for each strain and performed transposon insertion sequencing (Tn-Seq)[13] of the population remaining viable after entry into THP-1 cell-line derived human macrophages. The contributions of select candidate genes was subsequently verified using isogenic knockdowns generated by CRISPR interference (CRISPRi). The potential role of the complete set of implicated genes in chronic human infection was ascertained using mutational analysis of strains isolated from the airways of individuals with cystic fibrosis (CF).

Chapter 3. Results

3.1 Development of a facile Tn-Seq strategy for S. aureus.

Based on prior work[28], we developed a novel vector, pAureus-TnCAM, enabling transposome-mediated saturation mutagenesis in *S. aureus* (**Figure 1**). The transposon cassette is flanked by mosaic end sequences recognized by Tn5 transposase, and contains a *cat194* chloramphenicol resistance gene driven by the constitutive *sarA* promoter and *sodB* ribosome binding site[29,30]. A bidirectional *blaZ* transcriptional terminator[31] downstream of the resistance cassette limits polar effects from read-through transcription of adjacent genes[32] and interference of resistance gene expression from opposing transcripts in the bacterial genome. Transposome complexes derived from PCR-amplified transposon were manufactured *in vitro*[28] and subsequently electroporated into *S. aureus*[16] with purified phage *ocr* protein[33], facilitating bypass of the *S. aureus* type I restriction system. 19 transformations were combined for each *S. aureus* strain to generate transposon mutant libraries. We measured 545,582 unique insertions in ATCC29213 and 632,474 in JE2 libraries, about 27- and 31- fold greater, respectively, than considered saturated in prior work[34]. Based on genome size, this averaged an insertion every 5.1 bp and 4.5 bp, respectively. These estimates are likely conservative, as library complexity was not fully explored with the sequencing power allocated.

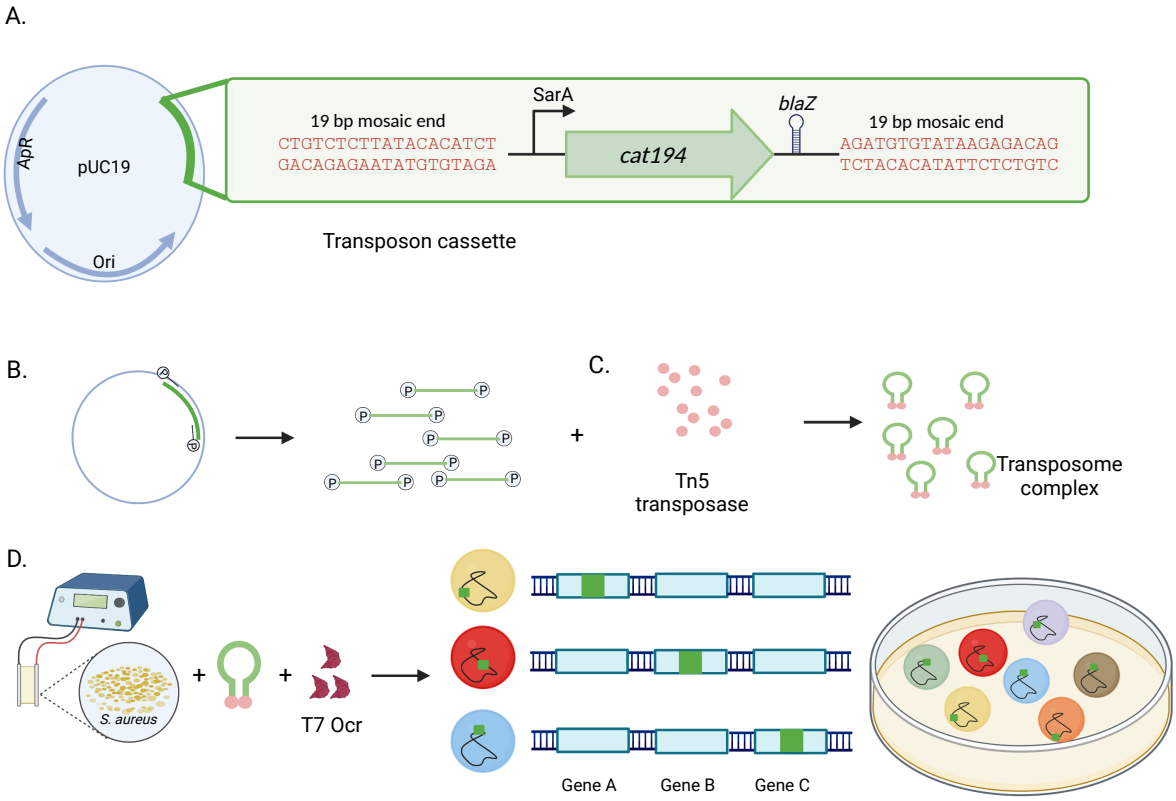


Figure 1. Transposon mutant generation in *S. aureus*. **A.** The transposon vector is built into a pUC19 backbone, and integrates a constitutively expressed chloramphenicol antibiotic resistance marker (*cat194*) with a bidirectionally-active transcriptional terminator. **B.** Transposon is amplified using phosphorylated primers and **C.** combined with Tn5 transposase *in vitro* to generate stable transposome complexes. **D.** Transposomes are electroporated into *S. aureus* with inhibitor of the type one restriction system (Ocr), and transformants selected for antibiotic resistance on solid media (**E**) to yield transposon mutant libraries.

3.2 TnSeq identifies S. aureus genes relevant to macrophage pathogenesis.

We used Tn-Seq to identify genes influencing invasion and early survival of *S. aureus* in macrophages (**Figure 2)**. We infected human macrophages derived from cultured THP-1 cells, which have previously been established as a model for *S. aureus* macrophage invasion *in vivo*[35], with sufficient quantities of transposon mutant libraries to ensure ~100-fold redundancy of each individual mutant. Following invasion, extracellular bacteria were killed[20], and intracellular organisms harvested and expanded by overnight growth on solid media. Primary analyses compared the initial library composition to that following invasion, but as additional controls for outgrowth, library aliquots were also inoculated into cell culture media without macrophages and plated after the incubation period. All experiments were performed in quadruplicate, followed by Tn-Seq analysis.

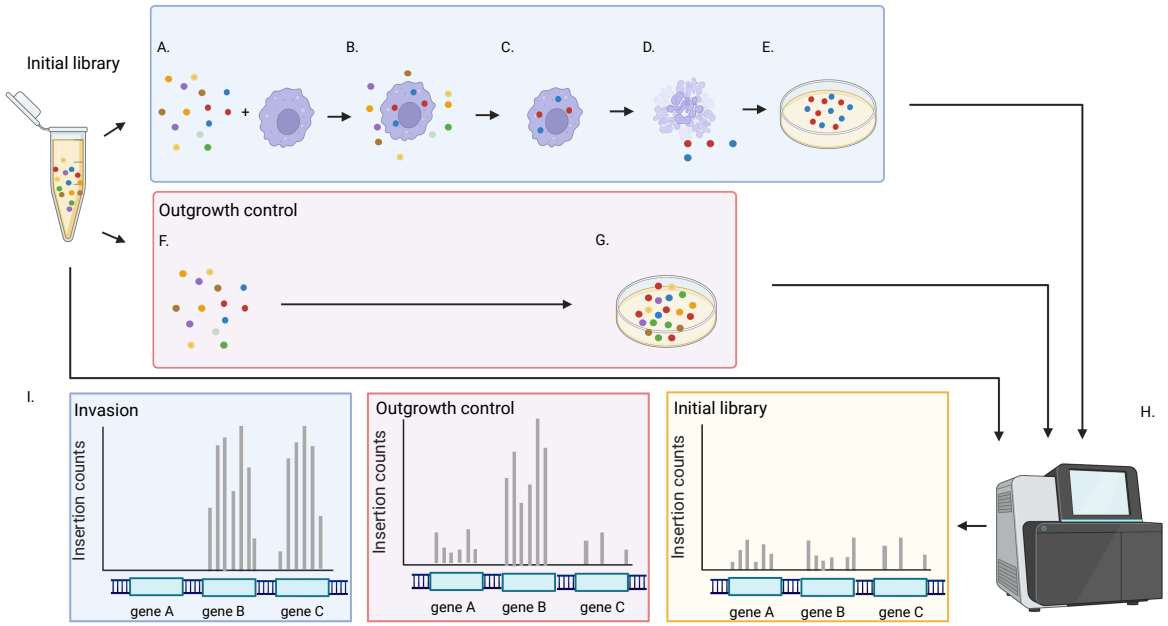


Figure 2. Experimental design. A. initial transposon mutant libraries are incubated with THP-1 derived macrophages to allow invasion (**B.**) to occur. **C.** Extracellular bacteria are killed by the application of lysostaphin. **D.** Macrophages are lysed, and surviving bacteria are expanded by

growth on solid media (**E**). **F.** To provide a control for outgrowth, initial transposon mutant libraries are incubated in cell culture media and then plated directly on solid media (**G**). **H.** Tn-Seq is performed on the initial library population, the library after macrophage invasion, and the outgrowth control. **I.** Tn-Seq output is compared across conditions to identify genes for which insertional inactivation is significantly overrepresented (gene C), underrepresented (gene A), or unchanged (gene B) in mutants successfully invading macrophages relative to counts in initial and/or outgrowth control libraries.

ANOVA identified genes (**Table 1)** exhibiting statistically significant variability (Benjamini-Hochberg adjusted $p < 0.05$) in transposon insertion counts when comparing the initial library to the mutant pool having successfully invaded host cells (**Supplementary Tables 2 and 3**). This identifies genes whose attendant transposon mutants are significantly overrepresented or underrepresented relative to the initial library state, encompassing genes whose disruption enhances or diminishes macrophage invasion, respectively. The number and identity of genes significantly affecting macrophage invasion varied substantially between strains. Comparing the initial library to the mutant pool recovered after invasion identified 321 significant genes for ATCC29213 and 215 for JE2. For both strains, the majority of genes were underrepresented (83% for ATCC29213 and 92% for JE2), suggesting they were "conditionally essential" for the phenotype. The substantially smaller proportion of overrepresented genes likely reflects the minority of factors promoting increased fitness of corresponding mutants when disrupted.

Table 1. Genes significant to macrophage pathogenesis identified by Tn-Seq.

	S. aureus ATCC29213	*S. aureus* JE2
Overrepresented	54	17
Underrepresented	267	198
Total	321	215

3.3 Conserved and strain-specific pathways are relevant to intracellular pathogenesis in S. aureus.

Pathway analysis ascertained enrichment of significant genes sharing functionally related roles (**Figure 3**). Transposon mutagenesis may produce opposite phenotypic effects within the same pathway, for example, knocking out an effector gene compared to disrupting its repressor. Analysis was therefore performed per strain on all implicated genes, regardless of their relative over- or underrepresentation after invasion. This identified several consistently enriched pathways across strains: DNA replication, lysine biosynthetic process via diaminopimelate, tricarboxylic acid cycle, tetrahydrofolate biosynthetic process, tRNA pseudouridine synthesis, and enterobacterial common antigen biosynthetic process. Genes contributing to several other functional pathways were similarly enriched, but were highly strain-specific.

To more directly compare specific implicated genes from the two strains, we identified gene homologs on the basis of sequence identity. 299 of the genes identified from ATCC29213 (93%) and 203 genes from JE2 (94%) had homologs present in the opposing strain. 107 homologs were concordantly identified as significant in both strains (**Supplemental Table 4**). Moreover, 105 of those 107 genes were underrepresented after invasion in both strains, consistent with the majority of shared gene content being conditionally essential for macrophage invasion.

These results indicate that genes affecting macrophage pathogenesis comprise several core functions that are relevant to both strains and necessary to support the virulence phenotype, but that a significant number of genes are strain-specific.

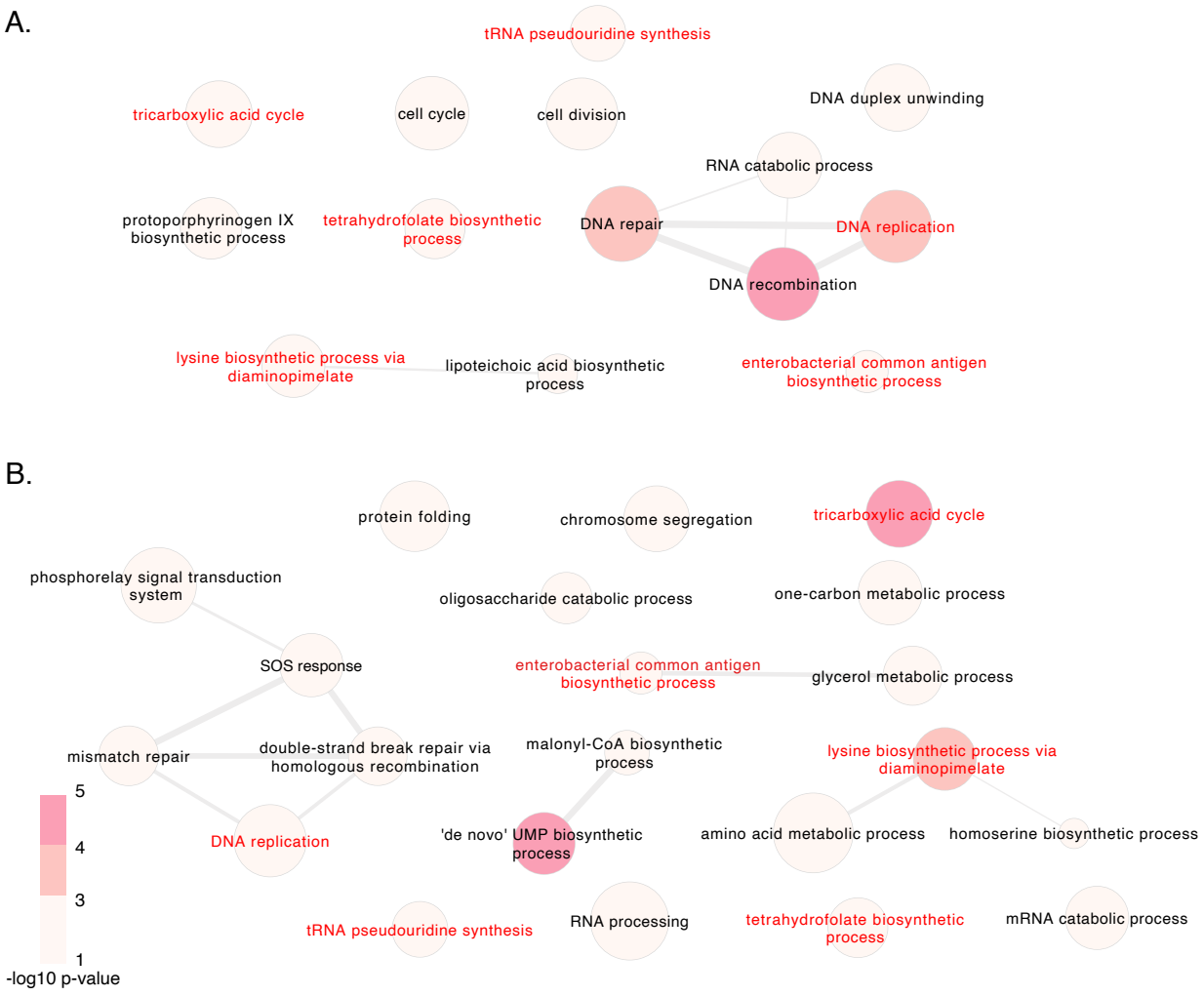


Figure 3. Pathway enrichment of genes contributing to macrophage invasion in *S. aureus*. Genes significant by Tn-Seq after macrophage invasion at adjusted p-value< 0.05 are represented. The color of circles corresponds to the p-value for each Gene Ontology (GO) category, and the size is proportional to Log_{10} size of each GO term. The thickness of connecting lines represent semantic similarity between categories, and spatial arrangement of discs approximately reflects the grouping of categories by semantic similarity. Results are separately displayed for strains ATCC29213 (**A**) and JE2 (**B**), with pathways common to both strains labeled in red.

3.4 Assessment of candidate invasion genes by isogenic CRISPRi knockdown.

We next tested the contributions of candidate macrophage invasion genes by generating gene-specific, isogenic CRISPRi knockdowns. Given the number of genes implicated, we prioritized a limited set for functional validation in each strain. We required that candidate genes achieved statistical significance by ANOVA when compared against both the initial library (**Supplemental Table 2 and 3**) and the outgrowth control (**Supplemental Table 5 and 6**), and that their relative over- or underrepresentation was consistent across both comparisons. These criteria identified 31 unique genes for functional testing (**Table 2**): 13 overrepresented mutants and 11 underrepresented mutants from ATCC29213, and 1 overrepresented mutant and 7 underrepresented mutants from JE2, with a single candidate gene, *liaR*, shared between strains.

Table 2. Prioritized S. aureus candidate genes involved in macrophage pathogenesis

Strain of gene origin	Representation in Tn-Seq libraries	Pan-genome gene identifier	ATCC locus tag	JE2 locus tag	Common gene name	Gene function
ATCC21913	Overrepresented	SAUPAN004469000	LNEJMEBC_00229	SAUSA300_1721		putative staphylococcal protein
ATCC21913	Overrepresented	SAUPAN004146000	LNEJMEBC_00424	SAUSA300_1525	*glyQS*	glycyl-tRNA synthetase
ATCC21913	Overrepresented	SAUPAN003559000	LNEJMEBC_00728	SAUSA300_1152	*frr*	ribosome recycling factor
ATCC21913	Overrepresented	SAUPAN002319000	LNEJMEBC_01408	SAUSA300_0532	*fusA*	Translation elongation factor G
ATCC21913	Overrepresented	SAUPAN002146000	LNEJMEBC_01525	SAUSA300_0420		membrane protein
ATCC21913	Overrepresented	SAUPAN001028000	LNEJMEBC_01759	SAUSA300_0188	*brnQ_3*	Branched-chain amino acid transport system2
ATCC21913	Overrepresented	SAUPAN000035000	LNEJMEBC_01860	SAUSA300_0023	*yycI*	Two-component system WalR/WalK regulatory protein
ATCC21913	Overrepresented	SAUPAN000017000	LNEJMEBC_01874	SAUSA300_0009	*serS*	Serine--tRNAligase
ATCC21913	Overrepresented	SAUPAN006410000	LNEJMEBC_01929	SAUSA300_2599	*icaR*	Biofilm operonicaADBCHTH-typenegative
ATCC21913	Overrepresented	SAUPAN006269000	LNEJMEBC_02007	SAUSA300_2526	*pyrD*	Dihydroorotate dehydrogenase(quinone)
ATCC21913	Overrepresented	SAUPAN006235000	LNEJMEBC_02026	SAUSA300_2506	*isaA*	putative trans glycosylase
ATCC21913	Overrepresented	SAUPAN005904000	LNEJMEBC_02216	SAUSA300_2326	*NA*	hypothetical protein
ATCC21913	Overrepresented	SAUPAN005440000	LNEJMEBC_02446	SAUSA300_2096	*gmuF*	putative mannose-6-phosphate isomerase
ATCC21913	Underrepresented	SAUPAN003893000	LNEJMEBC_00550	SAUSA300_1331	*ald1*	alanine dehydrogenase
ATCC21913	Underrepresented	SAUPAN003264000	LNEJMEBC_00932	SAUSA300_0958	*lytR_1*	cell envelope-related transcriptional attenuator
ATCC21913	Underrepresented		LNEJMEBC_00949			Hypothetical protein
ATCC21913	Underrepresented	SAUPAN003193000	LNEJMEBC_00971	SAUSA300_0917	*ltaA*	major facilitator superfamily transporter
ATCC21913	Underrepresented	SAUPAN005415000	LNEJMEBC_02466	SAUSA300_2077	*qsrR*	transcriptional regulator
ATCC21913	Underrepresented	SAUPAN004592000	LNEJMEBC_00188	SAUSA300_1769	*lukEv*	Leucotoxin
ATCC21913	Underrepresented		LNEJMEBC_00223			hypothetical protein
ATCC21913	Underrepresented	SAUPAN003833000	LNEJMEBC_00574	SAUSA300_1307	*arlS*	Signal transduction histidine-protein kinase
ATCC21913	Underrepresented	SAUPAN003749000	LNEJMEBC_00627	SAUSA300_1255	*mprF*	Phosphatidyl glycerol lysyl transferase
ATCC21913	Underrepresented	SAUPAN003673000	LNEJMEBC_00670	SAUSA300_1213		hypothetical protein
JE2	Overrepresented	SAUPAN002234000	LNEJMEBC_01475	SAUSA300_0473	*purR*	puroperonrepressor
JE2	Underrepresented	SAUPAN002709000	LNEJMEBC_01182	SAUSA300_0759	*gpm1*	2,3-bisphosphoglycerate-independent phosphoglycerate mutase
JE2	Underrepresented	SAUPAN003031000	LNEJMEBC_01054	SAUSA300_0835	*dltA*	D-alanine--poly(phosphoribitol) ligase subunit 1
JE2	Underrepresented	SAUPAN003810000	LNEJMEBC_00591	SAUSA300_1290	*dapD*	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N acetyltransferase
JE2	Underrepresented	SAUPAN004782000	LNEJMEBC_00149	SAUSA300_1799	*airS*	sensor histidine kinase
JE2	Underrepresented	SAUPAN005279000		SAUSA300_1991	*agrC*	accessorygeneregulatorproteinC
JE2	Underrepresented	SAUPAN006159000	LNEJMEBC_02081	SAUSA300_2455		putativefructose-1,6-bisphosphatase
E2 and ATCC2921	Underrepresented	SAUPAN004781000	LNEJMEBC_00150	SAUSA300_1798	*liaR*	DNA-binding response regulator

Despite multiple attempts, we were unable to achieve significant knockdown for 8 of the candidate genes (LNEJMEBC_00229, LNEJMEBC_00424, LNEJMEBC_01874, LNEJMEBC_02026, LNEJMEBC_00949, LNEJMEBC_02466, LNEJMEBC_00627, and LNEJMEBC_00670). Successful isogenic knockdowns were generated for *liaR*, as well as 15 additional genes relevant for *S. aureus* ATCC29213 and 7 for JE2 (**Supplemental Table 7**). CRISPRi knockdown mutants were assessed for their capability to invade THP-1 derived macrophages relative to a strain-matched control bearing a CRISPRi vector targeting an irrelevant biological target (**Figure 4**).

Four of the nine knockdowns of ATCC29213-relevant genes that were overrepresented after selection (**Figure 4a**) recapitulated anticipated gains in invasive capacity (*frr*, *fusA*, *yycI*, and LNEJMEBC_02216), while two had no significant impact (LNEJMEBC_01525, and *brnQ3*), and three paradoxically decreased invasive capacity (*icaR*, *pyrD*, and *gmuF*). Unexpectedly, all seven underrepresented candidate genes from the ATCC29213 library produced statistically significant increases in invasiveness following their knockdown in ATCC29213 (**Figure 4b**).

Only one JE2 gene in the validation set was overrepresented after invasion (*purR*), and its knockdown accordingly resulted in a significant increase in invasiveness (**Figure 4c**). Knockdown of the 7 candidate genes underrepresented in the transposon mutant pool (**Figure 4d**) resulted in significantly decreased invasion for 3 of the genes (*gpmI*, *dltA*, *dapD*) as anticipated, did not significantly impact invasion for two genes (*liaR*, *agrC*), and produced significantly increased invasion capacity for two genes (*airS* and SAUSA300_2455).

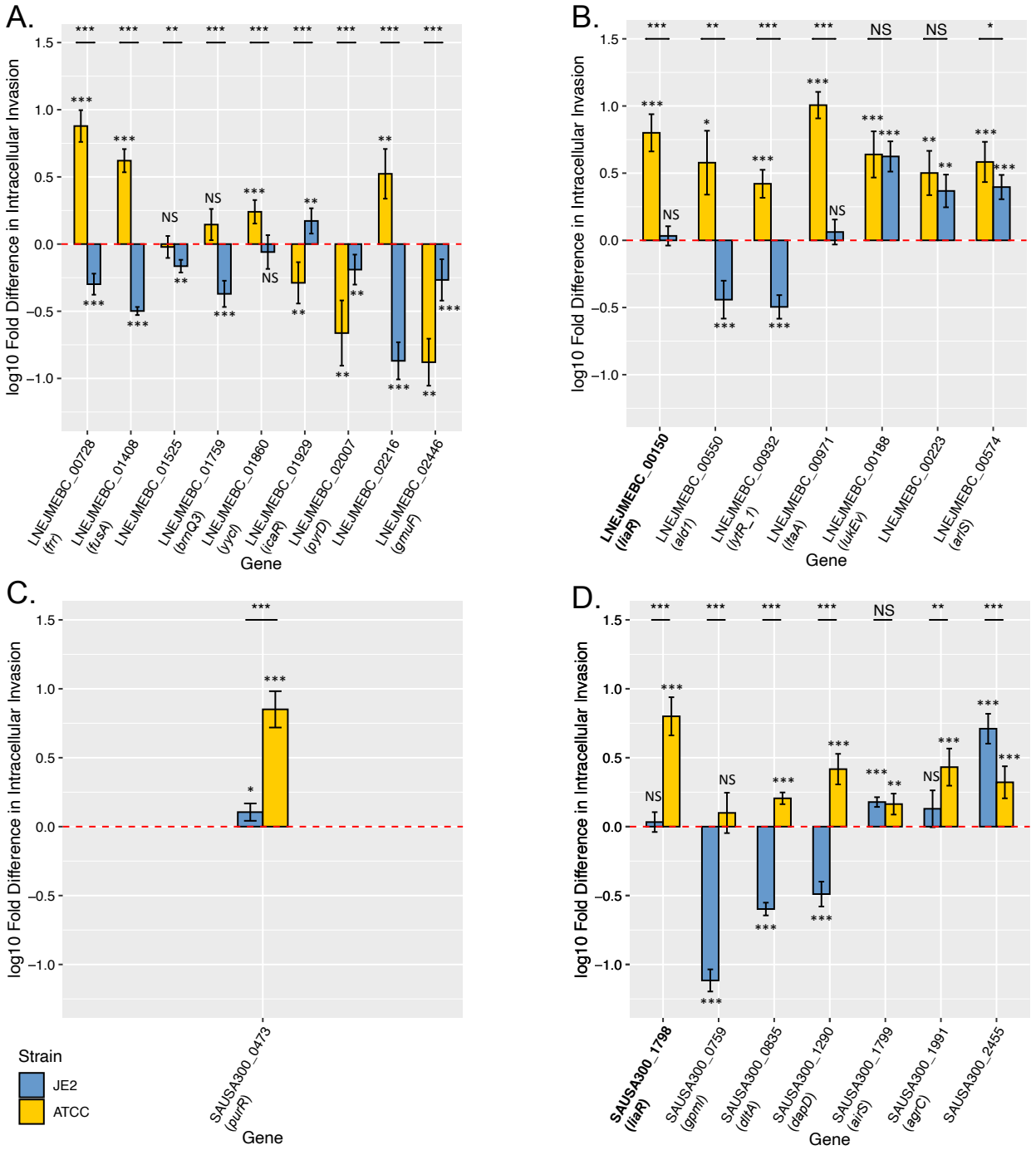


Figure 4. Intracellular invasion phenotypes of isogenic CRISPRi knockdowns. Results are shown for knockdown of candidate genes from Tn-Seq libraries that were **A.** overrepresented in ATCC29213, **B.** underrepresented in ATCC29213, **C.** overrepresented in JE2, and **D.** underrepresented in JE2. All results are normalized to invasion activity of the matched parental

strain carrying a silencing vector targeted to an irrelevant biological target (GFP), represented by a dashed line (red, at 0). Error bars indicate SEM. Measured values that are significantly different (by 2-tailed t test) are indicated by asterisks: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, either against the parental strain (displayed above each bar plot), or for the indicated comparisons between strains (black lines). NS, not significant. Common gene names, where applicable, are indicated in parentheses below each gene ID. *liaR*, relevant to both strains, is in bold.

We conclude that most candidate genes prioritized for functional validation measurably affect intracellular invasion, however, enhancement or reduction of the invasion phenotype do not always match predictions based on Tn-Seq analysis.

3.5 Strain-specific effects of effector genes contributing to S. aureus cell invasion.

Given the paucity of observed overlap in prioritized candidate genes between the two *S. aureus* strains, we next evaluated whether those genes could impact intracellular pathogenesis in a strain-dependent fashion. We therefore transformed CRISPRi vectors targeting candidate genes identified from one strain into the opposing *S. aureus* strain, and again assessed the invasion capacity of transformants relative to a strain-matched neutral CRISPRi control. The effects of individual gene knockdowns were then compared between the two strains (**Figure 4**).

Knockdown of 7 overrepresented candidate genes identified from ATCC29213 resulted in significantly decreased invasiveness for JE2, while one had no significant impact, and one significantly increased invasive capacity (**Figure 4a**): for two genes (*gmuF* and *pyrD*) were these results consistent between the two strains. Similarly, while knockdown of all seven underrepresented ATCC29213 candidate genes resulted in enhanced invasiveness in that

background, only three genes showed concordant effects in JE2, while two significantly decreased invasiveness in that strain and the remaining two showed no effect (**Figure 4b**). Inversions of phenotypic effect were also seen for JE2 candidate genes knocked down in ATCC29213. While knockdown of the single overrepresented candidate gene from JE2 (**Figure 4c**) augmented the invasion phenotypes of both strains, five of seven the underrepresented candidate genes (**Figure 4d**) showed discordant phenotypes between JE2 and ATCC29213.

These findings indicate that multiple genes contributing to macrophage invasion exert disparate functional effects, depending on the *S. aureus* strain background being evaluated.

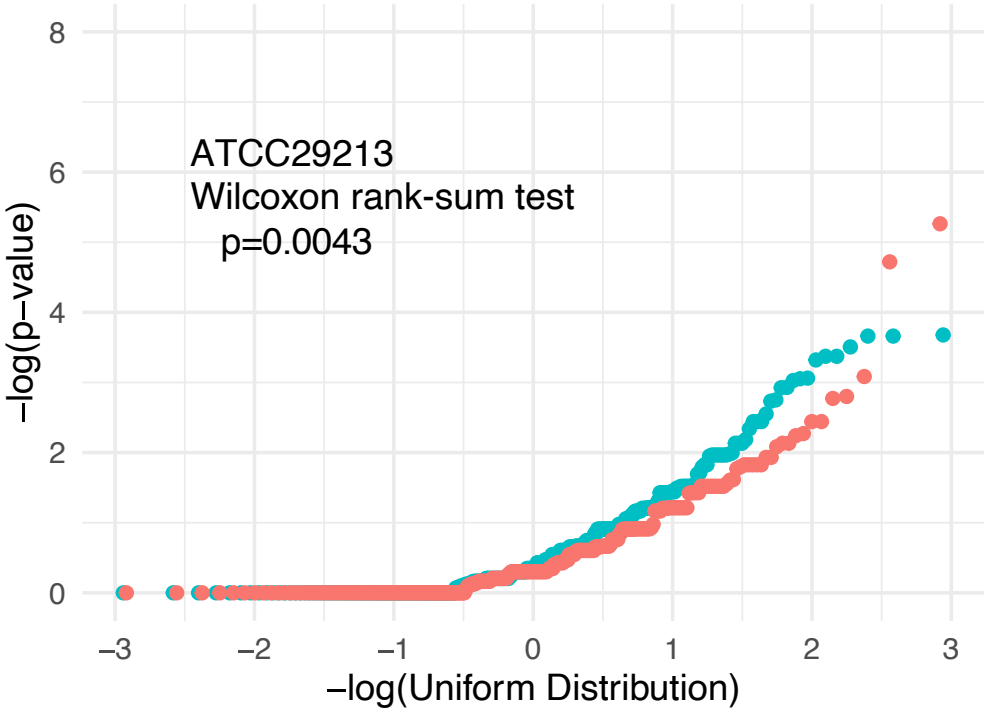
3.6 Selection of candidate intracellular invasion genes occurs in chronic cystic fibrosis airway infections in vivo.

We performed analyses to ascertain the relevance of candidate genes identified from Tn-Seq (**Supplemental Tables 2 and 3**) during chronic *S. aureus* infection *in vivo*. We used respiratory infection in CF as a model, as *S. aureus* undergoes selection for increased macrophage pathogenesis in that environment[10,36]. We examined 237 groups of clonally related *S. aureus* isolates longitudinally collected from children with CF over a period of several years[25]. For each clonal group, we cataloged the presence or absence of one or more nonsynonymous (conferring an amino acid change) and synonymous (silent) *de novo* mutations in each significant gene identified by Tn-Seq. Most genes from ATCC29213 (n=306) and JE2 (n=213) had an identifiable homolog among the *in vivo S. aureus* population, and could be examined by this analysis. A comperable number of control genes (ATCC29213 n=285, JE2 n=209) that Tn-Seq found least significantly associated with macrophage pathogenesis (**Supplementary Tables 8 and 9**) were included as

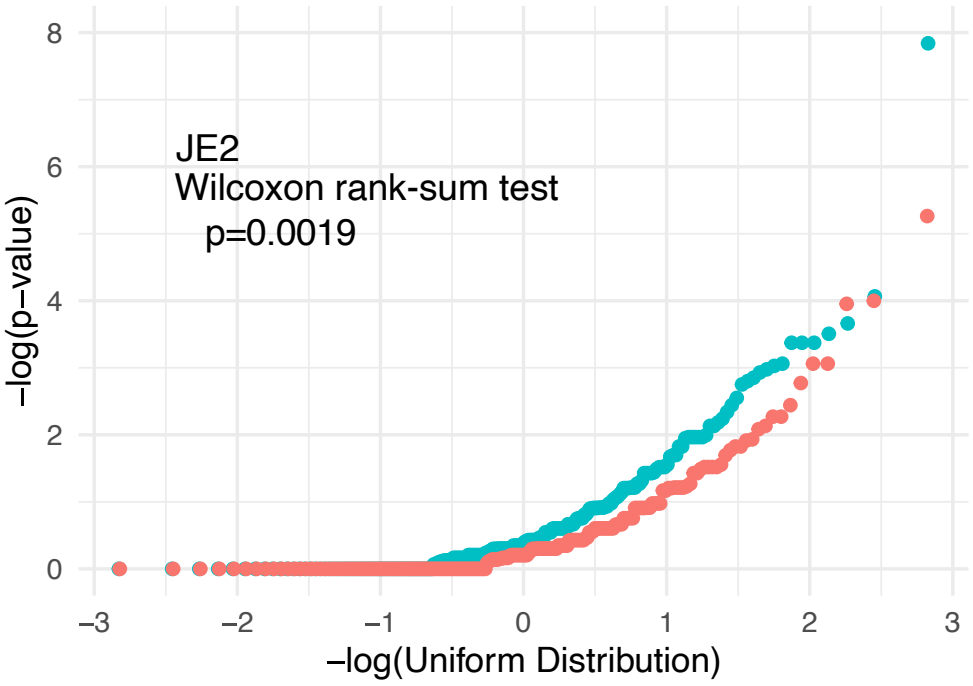
comparators. The per-base mutation rate was equivalent when comparing test and control gene groups for either strain (ATCC29213 p=0.324, JE2 p=0.119, 2-tailed T test).

Testing was performed for each gene to determine whether there was a statistically significant difference in the proportion of nonsynonymous to synonymous mutations observed across the collection of clonal groups, which would suggest evolutionary selection[37]. The distribution of resultant p values, indicating the strength of evidence selection in each gene, was then compared between candidate genes and comparator genes for each strain (**Figure 5**). Relative to strain-matched control genes, we observed a significant difference in the proportion of synonymous and non-synonymous *de novo* mutations in candidate genes identified from both ATCC29213 (**Figure 5a**, p = 0.0043, Wilcoxon rank-sum test) and JE2 (**Figure 5b**, p = 0.0019, Wilcoxon rank-sum test).

A.



B.



Significant Genes from Tn-Seq
Comparator Genes

Figure 5. Selection of candidate macrophage invasion genes in chronic CF respiratory infections *in vivo*. QQ-plots of -$\log_{10}$ transformed p-values testing the proportion of nonsynonymous versus synonymous mutations from genes identified from **A.** ATCC29213 and **B.** JE2. In each panel, results are shown for candidate macrophage invasion genes and corresponding control genes that were not implicated in the invasion phenotype. Significance of differences between the distributions of those gene categories by Wilcoxon rank-sum test are reported.

These studies provide evidence that genes significantly associated with macrophage invasion by Tn-Seq were, collectively, under greater evolutionary selection during chronic infection in CF patient airways *in vivo* than genes not associated with that phenotype.

Chapter 5. Discussion

We sought to comprehensively identify genes relevant to *S. aureus* invasion of human macrophages using Tn-Seq. To this end, we developed a novel system for generating saturation-level transposon mutant libraries in *S. aureus* through electroporation of transposon-transposase complexes (transposomes, **Figure 1**).

Our transposition system offers several advantages over prior approaches for *S. aureus*. The earliest methods involved co-transformation of two temperature sensitive plasmids that separately encode *mariner*-based *bursa aurealis* transposon and the *mariner* transposase gene, resulting in random transposition into the *S. aureus* genome when combined[34,38–43]. After transposition, high temperature plasmid-curing steps are required to remove the vectors, however, plasmid curing may be incomplete[44], resulting in ongoing or unstable transposition events, and

heat stress can inadvertently select for temperature sensitive mutants or otherwise bias the mutant pool[45]. Alternatively, Φ11 bacteriophage has been used to transduce transposon cassette-bearing plasmids with a conditional replication origin into transgenic *S. aureus* recipient strains expressing transposase[45–47]. This eliminates the need for temperature-dependent plasmid curing, and the high efficiency of phage transduction allows ultra-high density mutant libraries[45–47]. However, not all *S. aureus* isolates are susceptible to Φ11 transduction[48]. Labor intensive manipulations are also required to remove Φ11 family prophages from recipient strains to prevent non-transposase-catalyzed insertions mediated by phage-encoded integrases or homologous recombination[45]. Moreover, recipient strains in this system retain the transposase expression vector, preventing them from becoming fully isogenic.

In contrast, electroporation of transposon-transposase complexes (transposomes) is a simple and efficient way to generate transposon mutant libraries[28]. Such methods have been applied to multiple bacterial species[49–52], but until this work, have not been adapted for use in *S. aureus*. Our transposon encodes a robust selectable marker and a bidirectional transcriptional terminator intended to limit polar effects, and is electroporated into target strains in the presence of type I restriction enzyme inhibitor to bypass *S. aureus* DNA restriction systems[16]. Because transposition is mediated by complexes prepared *in vitro,* there is no dependence on host-encoded factors and no need to remove of transgenic elements, and transposition events are stable after genomic integration. The approach can be applied to any *S. aureus* strain for which electrocompetent cells can be prepared, which is readily achievable following published protocols[16]. We rapidly generated highly saturated *S. aureus* transposon mutant libraries in two strains, comprising ~500,000 to 600,000 unique insertions each.

We used Tn-Seq to compare initial transposon mutant libraries to populations having successfully invaded host macrophages, thereby identifying genes whose disruption positively or negatively impacts intracellular pathogenesis (**Table 1**). A substantial pro portion of *S. aureus*' total gene complement was implicated by Tn-Seq, corresponding to ~12% and 8% of coding sequences in ATCC29213 and JE2, respectively. This observation supports prior studies indicating that complex epistatic interactions govern many virulence traits in *S. aureus*[53]. In both strains, the majority of significant genes were underrepresented following invasion, consistent with most relevant factors being required for effective invasion and early survival in macrophages. Virtually all (98%) candidate genes which had identifiable homologs between the two strains were consistently underrepresented in Tn-Seq experiments in both strains, identifying a core set of 105 genes that are necessary to support macrophage invasion in *S. aureus* (**Supplementary Table 4**). A smaller proportion of knockouts were overrepresented in the transposon mutant pools following macrophage invasion, indicating that spontaneous chromosomal mutations in *S. aureus* can enhance this virulence phenotype[54]. Physiological and metabolic functions consistently contributing to macrophage invasion in both strains (**Figure 3**) may reflect the association between intracellular pathogenesis and the SCV phenotype[7–11], as disruption of many such pathways have been associated with SCVs [55–58].

Comparing initial and selected transposon mutant libraries is used in most Tn-Seq studies of complex bacterial phenotypes[34,42,43,59,60] and is generally considered robust. However, this approach does not account for population dynamics during the selection period, and may introduce bias from differences in the fitness or growth of mutants during that time[28]. Given these uncertainties, we prioritized genes for functional validation in each strain as those which remained significant after comparing to a secondary population controlling for outgrowth. It should be noted

that this outgrowth control is also expected to be biased, but we reasoned that genes having the strongest contributions to intracellular pathogenesis would be independently identified by comparison against both the initial and outgrowth control populations. As a case in point, disruptions of purine biosynthesis pathway genes were overrepresented in both strains after invasion relative to the outgrowth control (**Supplemental Table 5 and 6**), but not in comparison to the initial library (**Supplemental Table 2 and 3**). Previous studies have found that purine biosynthesis supports intracellular pathogenesis in *S. aureus*[54,61], making the outgrowth control result consistent with a growth-related artifact.

These conservative criteria identified 31 unique candidate genes for functional validation, 24 in ATCC29213 and 8 in JE2, with *liaR* shared in common (**Table 2**). 23 genes could be empirically tested as isogenic CRISPRi knockdown mutants. 20 significantly impacted macrophage invasion when knocked down by CRISPRi in their strain of origin and were considered validated, although we note that all 23 genes significantly affected invasion in at least one strain, after testing was conducted in both ATCC29213 and JE2 (**Figure 4**). Unexpectedly, 12 of the 20 functionally validated mutants produced phenotypic effects opposing expectations based on the relative over- or underrepresentation of genes observed by Tn-Seq in their corresponding strain of origin. Discordance between gene knockdown and knockout mutants has been well documented for eukaryotic organisms[62,63], and similar discrepancies have also been reported in studies of *Salmonella*[64], which could reflect activation of compensatory networks buffering against deleterious mutations[62], insufficient gene knockdown, or unintended off-target silencing effects, although gRNA design algorithms limit this possibility[27]. Moreover, we found that many validated genes identified from one strain (**Figure 4**) produced opposite phenotypic

effects when knocked down in the other, indicating that effects of such factors are strongly strain-specific.

Five of the validated genes (*purR*[54,61], *icaR*[65], *yycI*[66], *arlS*[67], and *ltaA*[68]) have previously described roles in *S. aureus* virulence, while two others (*lukEv*[69] and *airS*[70]) specifically impact survival against professional phagocytes, offering encouraging external validation of our findings. The remaining 13 validated genes are pathogenesis factors newly implicated by this study. Two such genes (LNEJMEBC_02216, LNEJMEBC_00223) are functionally uncharacterized, however, the described roles of other factors highlight several key pathways that mimic the higher-level functional roles enriched within the total set of genes identified by Tn-Seq (**Figure 2**), and provide greater insight into specific contributory roles.

First, the largest functional group of validated genes participate in metabolism and biosynthesis. Ribosome recycling factor *frr*[71] and elongation factor-encoding *fusA*[72] have functions in protein synthesis. Genes involved in the biosynthesis (*dapD*[73]) or catabolism of amino acids (*ald1*[74]) also impacted macrophage invasion, as did three genes involved in glycolysis (*gpmI*[75], *gmuF*[76], and putative fructose-1,6-bisphosphatase SAUSA300_2455), and one relevant to pyrimidine biosynthesis (*pyrD*[77]). Of note, several of such genes have known roles in *S. aureus* persistence (*frr*[78] and *dapD*[73]) or SCV (*fusA*[72]) phenotypes, or adaptation during respiratory infection (*ald1*[74]), phenotypes which are consistent with enhanced intracellular pathogenicity and which lend greater credence to the findings of the present study. Separately, one gene encoded functions relevant to survival within the toxic intracellular environment presented by macrophages: *gpmI,* primarily identified as a metabolic gene, above, has separately been identified as an important contributor to *S. aureus* fitness during nitric oxide exposure[79]. Third are regulatory effectors. Like members of other two-component regulatory systems, *liaR*[80] and *lytR*[81,82], serve to

translate external sensory signals to appropriate cytoplasmic responses within *S. aureus*. The responses elicited by such systems are frequently pleiotropic, and may encode multiple phenotypes relevant to invasion and survival within macrophages. The final category encompasses bacterial cell wall regulation. Flippase *ltaA* has been shown to adaptively modify *S. aureus* cell wall teichoic acids under acidic conditions[83], and which previous Tn-Seq studies have identified it as contributing to metastatic *S. aureus* bloodstream infections[68]. *dltA* has a similar role in teichoic acid alteration[84]. Implication of these genes in macrophage pathogenesis is concordant with findings from other groups identifying *S. aureus* cell wall modification as important for bacterial survival during infection *in vivo*[85,86].

Excitingly, genomic studies of isolates collected from individuals with CF provide evidence for importance of genes identified by Tn-Seq during chronic *S. aureus* infection of human hosts *in vivo*. Studies of *S. aureus* obtained from individuals with CF have indicated there is selection for mutants having enhanced capacity for intracellular pathogenesis of host cells, especially airway macrophages[10,36]. In a large, longitudinally banked collection of isolates obtained from individuals with CF[25], we found genes implicated by Tn-Seq to impact macrophage intracellular pathogenesis *in vitro* were under significantly greater selection than control genes least significantly associated with that phenotype (**Figure 5**). This is particularly striking given the diversity of adaptive phenotypes that selected in *S. aureus* during chronic respiratory infections in CF, including antibiotic resistance or tolerance, increased formation of biofilms or biofilm-like aggregates, and metabolic adaptations[10,25,87–89]. It is likely that at least some control genes used for this comparison are themselves under selection for other phenotypes relevant to chronic infection, but even so, genes associated with intracellular pathogenesis are under significantly greater selective pressures. Collectively, these findings offer evidence that genes identified by this study

are relevant to chronic human infection *in vivo*, and support the importance of macrophage pathogenesis in contributing to *S. aureus* persistence in CF[10,36].

Although illuminating, our study is subject to several limitations. We considered two distinct laboratory strains from different phylogenomic backgrounds, but it is likely that multiple strain-specific macrophage invasion genes have yet to be identified in additional lineages. Tn-Seq will only identify genes that impact the phenotype of interest through loss-of-function, and those imparting effects by overexpression or gain-of-function will be missed by our analysis. Due to the large number of genes identified by this study we were unable to subject all to empiric validation, leaving their biological contributions unverified. Lastly, use of CRISPRi as an orthologous validation method did not allow interrogation of all targets of interest, and valid effector genes which were insufficiently knocked down might not have imparted measurable phenotypic effects.

These data provide a detailed catalog of the factors influencing *S. aureus* invasion and initial survival within a host cell type important to chronic infection. Our analyses identify conserved and strain-specific genes and pathways used by *S. aureus* when invading macrophages, and reveal that individual genes may have opposing effects when disrupted in different *S. aureus* lineages. Future work will seek to better understand the mechanisms by which implicated genes influence intracellular pathogenesis, and to further explore the diversity of *S. aureus* genetic factors that facilitate human cell invasion. Genes and pathways implicated here may present novel therapeutic targets that could be leveraged to disrupt persistent *S. aureus* infection *in vivo*.

Chapter 6. Materials and Methods

Strains and growth conditions. S. aureus ATCC29213 was obtained from the American Type Culture Collection (ATCC, Manassas, Virginia), and JE2 from the Biodefense and Emerging Infections Research Resources Repository. *S. aureus* transposon mutants were grown using LB (ThermoFisher, Waltham, Massachusetts) supplemented with 5μg/ml thymidine, 1μg/mL hemin, and 1μg/mL menadione, to support auxotrophic mutants[14], and containing 10 μg/ml chloramphenicol (SupLB-CAM). *Escherichia coli* DH5-alpha was from NEB (Ipswich, MA), and grown in LB containing 100 μg/ml ampicillin to maintain plasmids. All strains were cultured at 37°C. THP-1 cells were obtained from ATCC and cultured at 37 °C in a humidified 5% (v/v) CO_2 air atmosphere in RPMI 1640 medium (ThermoFisher) supplemented with 0.1 mg/ml l-glutamine, 0.1 mg/ml streptomycin, 100 U/ml penicillin and 20% (v/v) Nu-Serum Serum Replacement (Corning, Corning, NY).

Transposon vector and transposon mutant libraries. Oligonucleotides and synthetic gene sequences (gBlocks) were synthesized by IDT (Coralville, IA) (**Supplementary Table 1**).

The transposon vector (pAureus-TnCAM) was generated by joining two gBlocks (Transposon_CAM_part I and Transposon_CAM_part II) into pUC19 vector. Transposomes were generated using this vector as previously[15], with some modifications. Briefly, phosphorylated primers (transposon_mosaic_F and transposon_mosaic_R) were used to PCR amplify the transposon cassette. PCR product was purified using Monarch PCR&DNA clean up kit (NEB), eluting in TE buffer. 1 μl transposon DNA at 400 ng/μl, 2ul EZ-TN transposase (1U/μl, Lucigen, Middleton, WI), and 1μl 100% glycerol were combined, incubating at room temperature for 45 minutes and then 4°C overnight, prior to long-term storage at -20°C.

Transposon mutant libraries were generated as elsewhere[15], with some modifications. Electrocompetent *S. aureus* were prepared as elsewhere[16]. 0.5 µl transposome complex was electroporated per *S. aureus* transformation (~10^8 cells) with 1 µl TypeOne restriction inhibitor (Lucigen). Electroporation was as elsewhere[16], except excluding the use of pellet paint, and using a Bio-Rad MicroPulser set to 2.3 kV and 2.5 ms time constant. Strains were incubated in 950 µl recovery medium for 2 hours. 19 transformations were performed per strain, and transformants pooled after recovery. Transformants were cryopreserved using 75 µl DMSO per mL culture and storing aliquots at -80°C.

Transformants were then expanded by culture on solid media to yield initial transposon mutant pools. Frozen transposon libraries were thawed on ice for 30 min then at room temperature for 15 min. One aliquot was initially plated onto SupLB-CAM to determine the titer of viable transposon insertion mutants. Bacteria were then plated at an approximate density of 600 colonies per plate on a series of 74 150 mm SupLB-CAM agar plates, and then incubated overnight. Colonies were harvested from each dish by applying 3 mL SupLB-CAM and resuspending colonies using a sterile cell spreader. Harvested material was pooled on ice and cryopreserved as above to generate the initial transposon mutant library pools.

Macrophage invasion. All studies were conducted in quadruplicate. 48 h prior to bacterial infection, ~1 million THP cells per infection were differentiated into macrophages using PMA (Sigma-Aldrich, St. Louis, MO) solubilized in DMSO at a concentration of 10 µg/ml[17–19]. PMA-containing medium was removed 24 h after treatment, and cells were washed with RPMI 1640 and incubated for 24 h. Bacteria were applied at a multiplicity of infection (MOI) of 100 in serum-free minimal essential medium (MEM) for 1 h. Medium was then replaced with MEM containing

50 μg/ml lysostaphin (Sigma-Aldrich) for 3h to kill extracellular bacteria[20]. Host cells were washed with DPBS and lysed with 0.025% Triton X-100 in water (Sigma-Aldrich). Lysate was then plated onto SupLB-CAM for overnight expansion of viable bacteria, then pooled and harvested as above. As an outgrowth control, smaller quantities of the initial library pools (10^5 bacteria) were inoculated into cell culture media in the absence of macrophages, and plated directly after 4 h incubation.

Quantitative measures of invasion were conducted as above, but serial dilutions of inoculum and cell lysate were plated onto LB to evaluate the count of viable bacteria, and 10 μg/ml chloramphenicol was included in all culture media.

Tn-Seq. Tn-Seq library preparation followed existing protocols[15,21]. Bacterial DNA was extracted using Qiagen DNeasy UltraClean Microbial Kit. 1.5 μg DNA was sheared to ~300 bp using Covaris E220 (Peak power 140 w, Duty factor 10%, 200 cycles/burst, Time 80 s). End repair was performed in 40 μl reaction containing 5x Quick Ligation Buffer (NEB), 1.675 mM each dNTP (NEB), 3 μl *E. coli* DNA Polymerase I (NEB), 0.5 μl T4 PNK (NEB), incubated at 37°C for 30 minutes and 72°C for 20 minutes. DNA was purified using Monarch PCR & DNA Cleanup Kit (NEB), using two sequential elutions of 10 μl EB buffer each. C-tailing was as described previously[15] except that DNA was eluted in 7.5 μl EB buffer, followed by rounds 1 and 2 of PCR[15]. Size selection of 100-500 bp fragments was performed using Monarch DNA Gel Extraction Kit (NEB). Sequencing utilized a Nextseq500 (Illumina, San Diego, CA) with a 75 bp single-end read and a custom index primer (T26_SEQ-6).

TnSeq data analysis. Transposon mutant library sizes were estimated for each strain by combining sequencing data from all four replicates of the initial transposon pool, aligning reads to the appropriate reference genome (GenBank accession CP000255.1 for JE2 or the ATCC29213 reference genome supplied by ATCC) with BWA-MEM[22], and tallying the number of unique transposon insertion sites. Analysis was performed using TRANSIT (version 3.2.7)[23]. Pre-processing used the TPP tool, followed by ANOVA with default parameters to perform pairwise comparisons across conditions, with Benjamini-Hochberg adjusted $p<0.05$ considered significant. Pathway enrichment analysis of significant genes was performed using TRANSIT. REVIGO[24] was used to summarize and visualize pathway enrichment analyses. Gene clustering to identify homologous genes between the *S. aureus* lineages was performed as previously[25].

CRISPRi knockdown. CRISPRi knockdown of candidate genes was performed using vector pCRISRPi, as described elsewhere[26]. sgRNA were designed using CRISPOR[27] (**Supplementary Table 1**). Gene knockdown was assessed using gene-targeted real-time PCR of cDNA prepared from mid-log phase growth cells, normalizing expression to that of *gyrA*. Gene expression levels in individual CRISPRi mutants were compared to relative gene expression for a pCRISRPi vector targeted to a neutral gene sequence (GFP) using the ΔΔCt method.

Analysis of gene selection in vivo. Whole-genome sequencing data from 1,382 *S. aureus* isolates longitudinally collected from 246 children with CF[25] were utilized for analysis of *in vivo* strain adaptation. The isolates comprise 237 clonal groups related by descent[25]. Gene clustering was repeated as above for reference genomes in this study against pre-existing clustering models from clonal groups[25] to identify homologous genes. The presence of nonsynonymous and synonymous

de novo mutations within each clonal group was tallied for candidate genes from Tn-Seq and, as a control, approximately the same number of non-essential genes that were least significantly associated with macrophage invasion in the same analyses. For each gene, Fisher's exact test was used to assess the proportion of clonal groups with one or more nonsynonymous or synonymous *de novo* mutations occurring across clonal groups. The distribution of -log10-transformed p-values, corresponding to evidence of selection, was compared between candidate genes and comparator genes using the Wilcoxon rank-sum test.

Data availability. Sequence data from this study are available from the NCBI Sequence Read Archive under accession PRJNA942332.

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