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Transposon sequencing identifies genes impacting *Staphylococcus aureus* invasion in a human macrophage model

Hsin-Yu Lo,[1] Dustin R. Long,[2] Elizbeth A. Holmes,[1] Kelsi Penewit,[1] Taylor Hodgson,[1] Janessa D. Lewis,[1] Adam Waalkes,[1] Stephen J. Salipante[1]

AUTHOR AFFILIATIONS See affiliation list on p. 16.

ABSTRACT *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 THP-1 derived macrophages using two laboratory strains (ATCC2913 and JE2). We developed an *in vitro* transposition method to produce highly saturated transposon mutant libraries in *S. aureus* and performed transposon insertion sequencing (Tn-Seq) to identify candidate genes with significantly altered abundance following macrophage invasion. While some significant genes were strain-specific, 108 were identified as common across both *S. aureus* strains, with most (n = 106) being required for optimal macrophage infection. We used CRISPR interference (CRISPRi) to functionally validate phenotypic contributions for a subset of genes. Of the 20 genes passing validation, seven had previously identified roles 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 *in vivo* purifying selection in conditionally essential candidate genes than those 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*.

KEYWORDS *Staphylococcus aureus*, facultatively intracellular pathogens, transposons, Tn-Seq, genomics, cystic fibrosis, chronic infection, macrophages, persistence, cell invasion

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).

Editor Kimberly A. Kline, Universite de Geneve, Geneva, Switzerland

Address correspondence to Stephen J. Salipante, stevesal@uw.edu.

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The fate of intracellular *S. aureus* depends on both host and strain-encoded 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 encode 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 (14, 15). The contributions of select candidate genes were 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).

MATERIALS AND METHODS

Strains and growth conditions

S. aureus ATCC29213 was obtained from the American Type Culture Collection (ATCC, Manassas, Virginia), and JE2 was obtained from the Biodefense and Emerging Infections Research Resources Repository. *S. aureus* transposon mutants were grown using LB (ThermoFisher, Waltham, Massachusetts) supplemented with 5 µg/mL thymidine, 1 µg/mL hemin, and 1 µg/mL menadione to support auxotrophic mutants (16) 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% (vol/vol) 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% (vol/vol) 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) (Table S1).

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 (17), with some modifications. Briefly, phosphorylated primers (transposon_mosaic_F and transposon_mosaic_R) were used to amplify the transposon cassette by PCR. The PCR product was purified using Monarch PCR & DNA clean up kit (NEB), eluting in TE buffer. 1 µL transposon DNA at 400 ng/µL, 2 uL EZ-TN transposase (1 U/µL, Lucigen, Middleton, WI), and 1 µL 100% glycerol were combined, incubated at room temperature for 45 min and then at 4°C overnight, prior to long-term storage at −20°C.

Transposon mutant libraries were generated as elsewhere (17), with some modifications. Electrocompetent *S. aureus* were prepared as elsewhere (18). Per *S. aureus* transformation (~10^8 cells), 0.5 µl transposome complex was electroporated with 1 µl TypeOne restriction inhibitor (Lucigen) as elsewhere (18), except excluding the use of pellet paint and using a Bio-Rad MicroPulser set to 2.3 kV and 2.5 ms time constant. Transformants were incubated in 950 µL recovery medium (18) for 2 h. Nineteen transformations were performed per strain, and transformants were pooled after recovery. The transformant pool was cryopreserved using 75 µL DMSO per milliliter culture, and aliquots were stored at −80°C.

Transformants were then expanded by culture on solid media to generate the initial transposon mutant pools. Frozen transformants 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 on a series of 74 150 mm SupLB-CAM agar plates and incubated overnight. Colonies were harvested from each dish by applying 3 mL SupLB-CAM and resuspending colonies using a sterile cell spreader. Harvested colonies were pooled on ice and cryopreserved as above, yielding the initial transposon mutant library pools.

Macrophage invasion

All studies were conducted in quadruplicate. Forty-eight hours prior to bacterial infection, ~1 million THP-1 cells per infection were differentiated into macrophages using PMA (Sigma-Aldrich, St. Louis, MO) solubilized in DMSO at a concentration of 10 µg/mL (19–21). The PMA-containing medium was removed 24 h after treatment, and cells were washed with RPMI 1640 and incubated for 24 h. To maximize the number of transposon mutants that could be analyzed, the initial transposon mutant library pools were applied at a multiplicity of infection (MOI) of 100 in serum-free minimal essential medium (MEM) for 1 h. This condition resulted in successful internalization of 0.6% of the JE2 and 0.04% ATCC29213 bacterial inoculum while retaining 72% (JE2) and 50% (ATCC29213) THP-1 macrophage viability at the conclusion of the experiment. Following incubation, the medium was replaced with MEM containing 50 µg/mL lysostaphin (Sigma-Aldrich) for 3 h to kill extracellular bacteria (22). Host cells were washed with DPBS and lysed with 0.025% Triton X-100 in water (Sigma-Aldrich). Lysate was 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 lysostaphin 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 (17, 23). Bacterial DNA was extracted using Qiagen DNeasy UltraClean Microbial Kit. Using Covaris E220 (peak power 140 w, duty factor 10%, 200 cycles/burst, time 80 s), 1.5 µg DNA was sheared to ~300 bp. End repair was performed in a 40 µL reaction containing 5 × 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 min and 72°C for 20 min. 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 (17) except that DNA was eluted in 7.5 µL EB buffer, followed by rounds 1 and 2 of PCR (17). Multiplexing of specimens was accomplished by incorporating sample-specific indexed primers (TnSeq barcode primers 1–12) during round 2 of PCR (17). Size selected 100–500 bp fragments were purified using Monarch DNA Gel Extraction Kit (NEB). Sequencing utilized a Nextseq500 (Illumina, San Diego, CA) with "Mid-Output" flow cells and a 75 bp single-end read using a custom

read 1 sequencing primer (T26_SEQ-6). Demultiplexing was performed using bcl2fastq v2.20.0.422 (Illumina).

Tn-Seq data analysis

Analysis was performed with TRANSIT (version 3.2.7) (24) using the appropriate reference genome (GenBank accession CP000255.1 for JE2 or the ATCC29213 reference genome supplied by ATCC), for all protein coding features. Preprocessing used the TPP tool. Transposon mutant library sizes were estimated for each strain by combining sequencing data from all four replicates of the initial transposon mutant pool and tallying the number of unique coding insertions using the Tn5Gaps method. ANOVA with default parameters was used 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 (25) was used to summarize and visualize pathway enrichment analyses. Gene clustering to identify homologous genes between the *S. aureus* lineages was performed as previously (26).

CRISPRi knockdown

CRISPRi knockdown of candidate genes was performed using vector pCRISRPi, as described elsewhere (D. R. Long, E. A. Holmes, H.-Y. Lo, K. Penewit, J. Almazan, T. Hodgson, N. F. Berger, Z. H. Bishop, D. J. Wolter, J. D. Lewis, A. Waalkes, and S. J. Salipante, submitted for publication). sgRNA were designed using CRISPOR (27) (Table S1). 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 (26) were utilized for analysis of *in vivo* strain adaptation. The isolates comprise 237 clonal groups related by descent (26). Gene clustering was repeated as above for reference genomes in this study against pre-existing clustering models from clonal groups (26) to identify homologous genes. The presence of disruptive (stop-gain and frameshift) and synonymous (silent) *de novo* mutations within each clonal group was tallied for candidate genes from Tn-Seq. As a control, for each strain we identified genes within the upper quartile of *P*-value (i.e., least significant) by Tn-Seq ANOVA and having average insertion counts among replicates of the initial transposon mutant pool ≥1, identified homologs shared between the two strains (n = 183), and similarly analyzed those genes. For each gene, Fisher's exact test was used to assess the proportion of clonal groups with one or more disruptive or synonymous *de novo* mutations occurring across clonal groups. The distribution of -$\log_{10}$-transformed *P*-values, corresponding to evidence of selection, was compared between candidate genes and control genes using the Wilcoxon rank-sum test.

RESULTS

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* (Fig. 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 the 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 generated *in vitro* (28) and subsequently electroporated

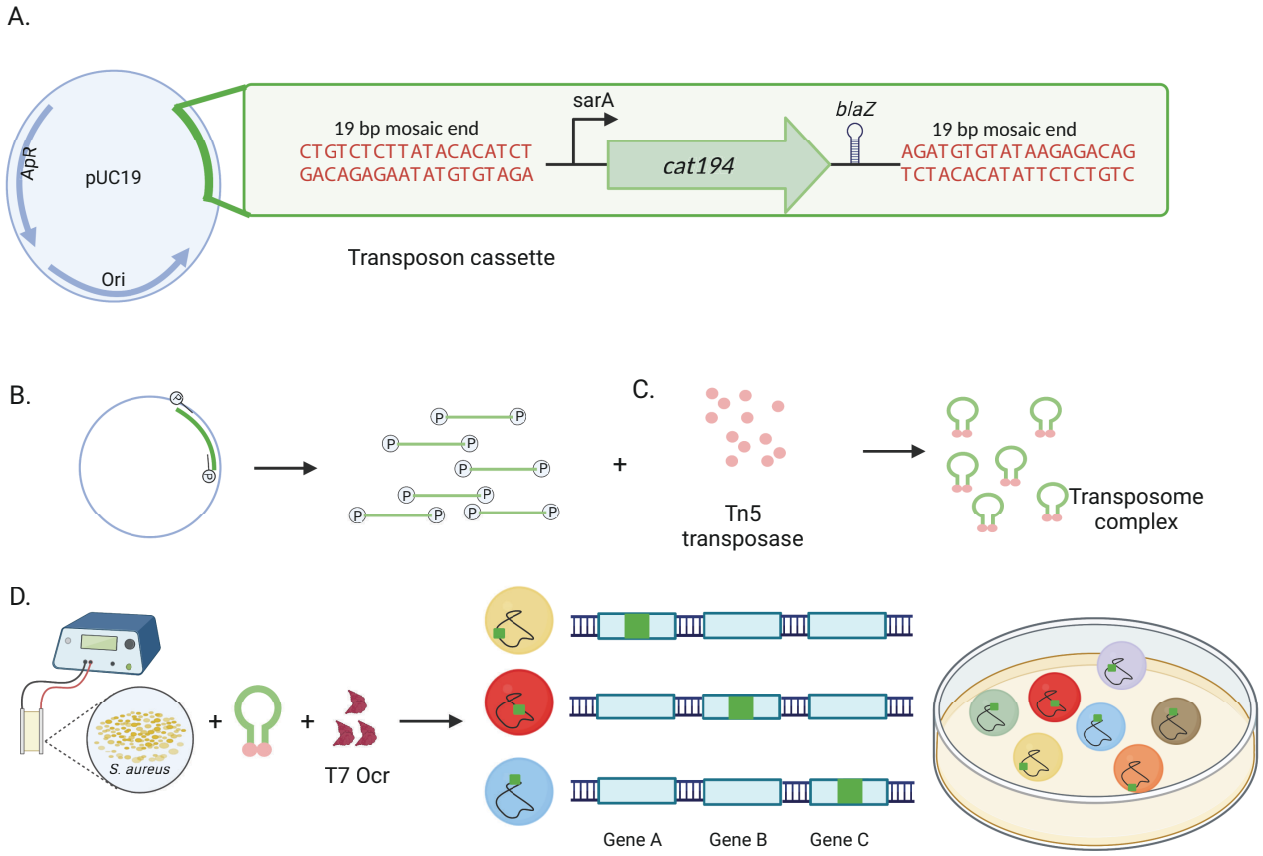

FIG 1 Transposon mutant generation in *S. aureus*. (A) The transposon vector is built into a pUC19 backbone and integrates a constitutively expressed chloramphenicol antibiotic resistance marker (*cat194*) with a bidirectionally active transcriptional terminator. (B) Transposon is amplified using phosphorylated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

into *S. aureus* (18) with purified phage *ocr* protein (33), facilitating bypass of the *S. aureus* type I restriction system. Nineteen transformations were combined for each *S. aureus* strain to generate transposon mutant libraries. Sequence analysis identified 391,070 unique insertions in ATCC29213 and 316,248 in JE2 for the initial transposon mutant library pools, about 20- and 16-fold greater, respectively, than those considered saturated in prior work (34). Based on genome size, this averaged an insertion every 7 bp and 9 bp, respectively. Library pools encompassed an average of 149 (ATCC21913) and 124 (JE2) insertions per genome feature (Tables S2 and S3). These estimated library pool sizes are expected to be conservative as they do not include insertions occurring in intergenic regions. However, the bacterial transformation rate (1 in 4,859 for ATCC29213 and 1 in 6,007 for JE2) remains low enough that the risk of more than one transposon inserted into the genome of any given bacterium is exceedingly improbable.

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 (Fig. 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* (14), with sufficient quantities of transposon mutant libraries to ensure ~100-fold redundancy of each individual mutant. Following invasion, extracellular

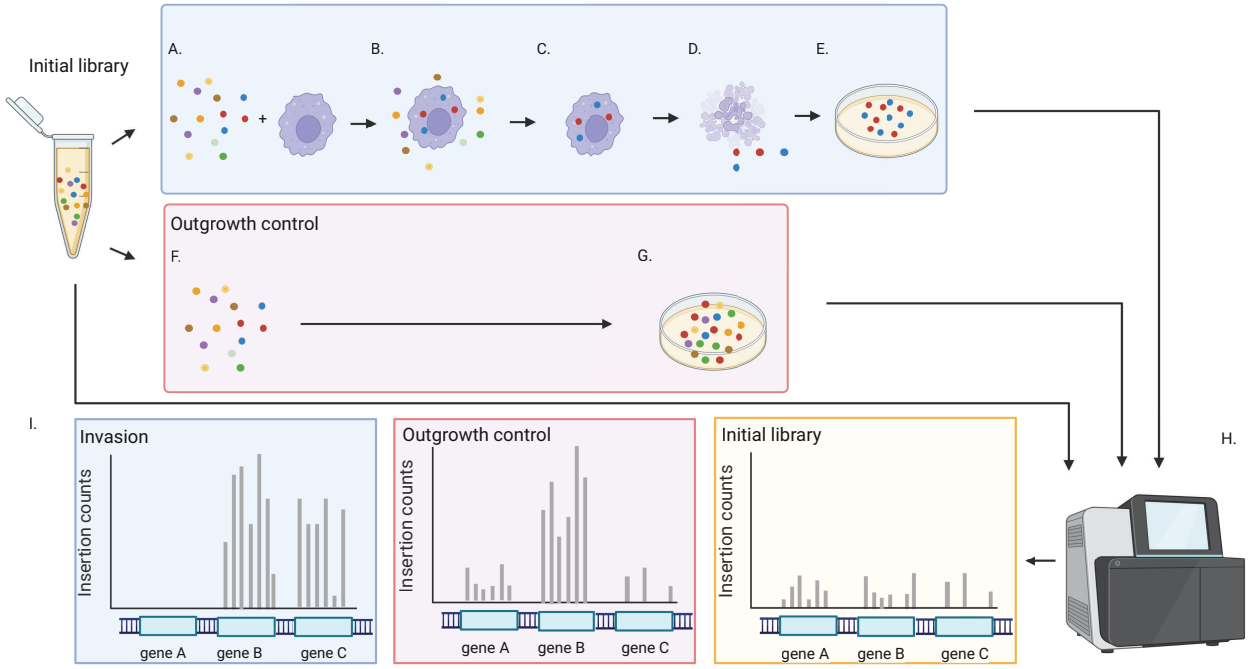

FIG 2 Experimental design. (A) Initial transposon mutant libraries are incubated with THP-1 derived macrophages to allow invasion (B) to occur. (C) Extracellular bacteria are killed by the application of lysostaphin. (D) Macrophages are lysed, and surviving bacteria are expanded by growth on solid media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

bacteria were killed (22), and intracellular organisms were harvested and expanded by overnight growth on solid media. Primary analyses compared the composition of the initial transposon mutant library pools to those following invasion, but as additional controls for outgrowth, library aliquots were also inoculated into cell culture media without macrophages and lysostaphin and plated after the incubation period. All experiments were performed in quadruplicate, followed by Tn-Seq analysis. Sequencing was performed to an average depth of 9.3 million reads per specimen and ultimately yielded an average of 4.6 million uniquely mapped reads per specimen.

ANOVA identified genes (Table 1) exhibiting statistically significant variability (Benjamini-Hochberg adjusted $P < 0.05$) in transposon insertion counts when comparing the initial transposon mutant library pools to the mutant pools having successfully invaded host cells (Tables S4 and S5). This analysis identifies populations of genes whose corresponding transposon mutants exhibit significant increases or decreases in fold change relative to the initial library state, corresponding to 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 significant genes showed decreases in fold change abundance (83% for ATCC29213 and 92% for JE2), suggesting they were "conditionally essential" for the phenotype. The substantially smaller proportion of genes having increases in fold change 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
Positive fold change abundance	54	17
Conditionally essential	267	198
Total	321	215

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

Pathway analysis ascertained enrichment of significant genes sharing functionally related roles, determined by comparing initial transposon mutant library pools to mutants surviving after invasion (Fig. 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 the direction of fold change 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. Three hundred of the genes identified from ATCC29213 (93%) and 204 genes from JE2 (94%) had homologs present in the opposing strain. One hundred eight homologs were concordantly identified as significant in both strains (Table S6). Moreover, 106 of those 108 genes had negative fold changes 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.

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 (Tables S4 and S5) and the outgrowth control (Tables S7 and S8) and that the direction of their fold change was consistent across both comparisons. These criteria identified 31 unique genes for functional testing (Table 2): 13 mutants with positive fold change in abundance and 11 conditionally essential mutants from ATCC29213 and one mutant with positive fold change in abundance and seven conditionally essential mutants from JE2, with a single candidate gene, *liaR*, shared between strains.

Despite multiple attempts, we were unable to achieve significant knockdown for eight 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 seven for JE2 (Table S9). 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 (Fig. 4).

Four of the nine knockdowns of ATCC29213-relevant genes that showed positive fold change in abundance after selection (Fig. 4A) recapitulated anticipated gains in invasive capacity (*frr*, *fusA*, *yycI*, and LNEJMEBC_02216), two had no significant impact

TABLE 2 Prioritized *S. aureus* candidate genes involved in macrophage pathogenesis

Strain of gene orign	Tn-Seq result	Pan-genome gene identifier	ATCC locus tag	JE2 locus tag	Common gene name	Gene function
ATCC21913	Positive fold change abundance	SAUPAN004469000	LNEJMEBC_00229	SAUSA300_1721		putative staphylococcal protein
ATCC21913	Positive fold change abundance	SAUPAN004146000	LNEJMEBC_00424	SAUSA300_1525	*glyQS*	glycyl-tRNA synthetase
ATCC21913	Positive fold change abundance	SAUPAN003559000	LNEJMEBC_00728	SAUSA300_1152	*frr*	ribosome recycling factor
ATCC21913	Positive fold change abundance	SAUPAN002319000	LNEJMEBC_01408	SAUSA300_0532	*fusA*	translation elongation factor G
ATCC21913	Positive fold change abundance	SAUPAN002146000	LNEJMEBC_01525	SAUSA300_0420		membrane protein
ATCC21913	Positive fold change abundance	SAUPAN001028000	LNEJMEBC_01759	SAUSA300_0188	*brnQ_3*	branched-chain amino acid transport system
ATCC21913	Positive fold change abundance	SAUPAN000035000	LNEJMEBC_01860	SAUSA300_0023	*yycI*	two-component system WalR/WalK regulatory protein
ATCC21913	Positive fold change abundance	SAUPAN000017000	LNEJMEBC_01874	SAUSA300_0009	*serS*	serine—tRNAligase
ATCC21913	Positive fold change abundance	SAUPAN006410000	LNEJMEBC_01929	SAUSA300_2599	*icaR*	*ica* operon repressor
ATCC21913	Positive fold change abundance	SAUPAN006269000	LNEJMEBC_02007	SAUSA300_2526	*pyrD*	Dihydroorotate dehydrogenase(quinone)
ATCC21913	Positive fold change abundance	SAUPAN006235000	LNEJMEBC_02026	SAUSA300_2506	*isaA*	putative *trans* glycosylase
ATCC21913	Positive fold change abundance	SAUPAN005904000	LNEJMEBC_02216	SAUSA300_2326	*NA*	hypothetical protein
ATCC21913	Positive fold change abundance	SAUPAN005440000	LNEJMEBC_02446	SAUSA300_2096	*gmuF*	putative mannose-6-phosphate isomerase
ATCC21913	Conditionally essential	SAUPAN003893000	LNEJMEBC_00550	SAUSA300_1331	*ald1*	alanine dehydrogenase
ATCC21913	Conditionally essential	SAUPAN003264000	LNEJMEBC_00932	SAUSA300_0958	*lytR_1*	cell envelope-related transcriptional attenuator
ATCC21913	Conditionally essential		LNEJMEBC_00949			hypothetical protein
ATCC21913	Conditionally essential	SAUPAN003193000	LNEJMEBC_00971	SAUSA300_0917	*ltaA*	major facilitator superfamily transporter
ATCC21913	Conditionally essential	SAUPAN005415000	LNEJMEBC_02466	SAUSA300_2077	*qsrR*	transcriptional regulator
ATCC21913	Conditionally essential	SAUPAN004592000	LNEJMEBC_00188	SAUSA300_1769	*lukEv*	leucotoxin
ATCC21913	Conditionally essential		LNEJMEBC_00223			hypothetical protein
ATCC21913	Conditionally essential	SAUPAN003833000	LNEJMEBC_00574	SAUSA300_1307	*arlS*	Signal transduction histidine-protein kinase
ATCC21913	Conditionally essential	SAUPAN003749000	LNEJMEBC_00627	SAUSA300_1255	*mprF*	Phosphatidyl glycerol lysyl transferase
ATCC21913	Conditionally essential	SAUPAN003673000	LNEJMEBC_00670	SAUSA300_1213		hypothetical protein
JE2	Positive fold change abundance	SAUPAN002234000	LNEJMEBC_01475	SAUSA300_0473	*purR*	*pur* operon repressor
JE2	Conditionally essential	SAUPAN002709000	LNEJMEBC_01182	SAUSA300_0759	*gpmI*	2,3-bisphosphoglycerate-independent phosphoglycerate mutase
JE2	Conditionally essential	SAUPAN003031000	LNEJMEBC_01054	SAUSA300_0835	*dltA*	D-alanine--poly(phosphoribitol) ligase subunit 1
JE2	Conditionally essential	SAUPAN003810000	LNEJMEBC_00591	SAUSA300_1290	*dapD*	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-acetyltransferase
JE2	Conditionally essential	SAUPAN004782000	LNEJMEBC_00149	SAUSA300_1799	*airS*	sensor histidine kinase
JE2	Conditionally essential	SAUPAN005279000		SAUSA300_1991	*agrC*	accessory gene regulator protein C
JE2	Conditionally essential	SAUPAN006159000	LNEJMEBC_02081	SAUSA300_2455		putative fructose-1,6-bisphosphatase
JE2 and ATCC29213	Conditionally essential	SAUPAN004781000	LNEJMEBC_00150	SAUSA300_1798	*liaR*	DNA-binding response regulator

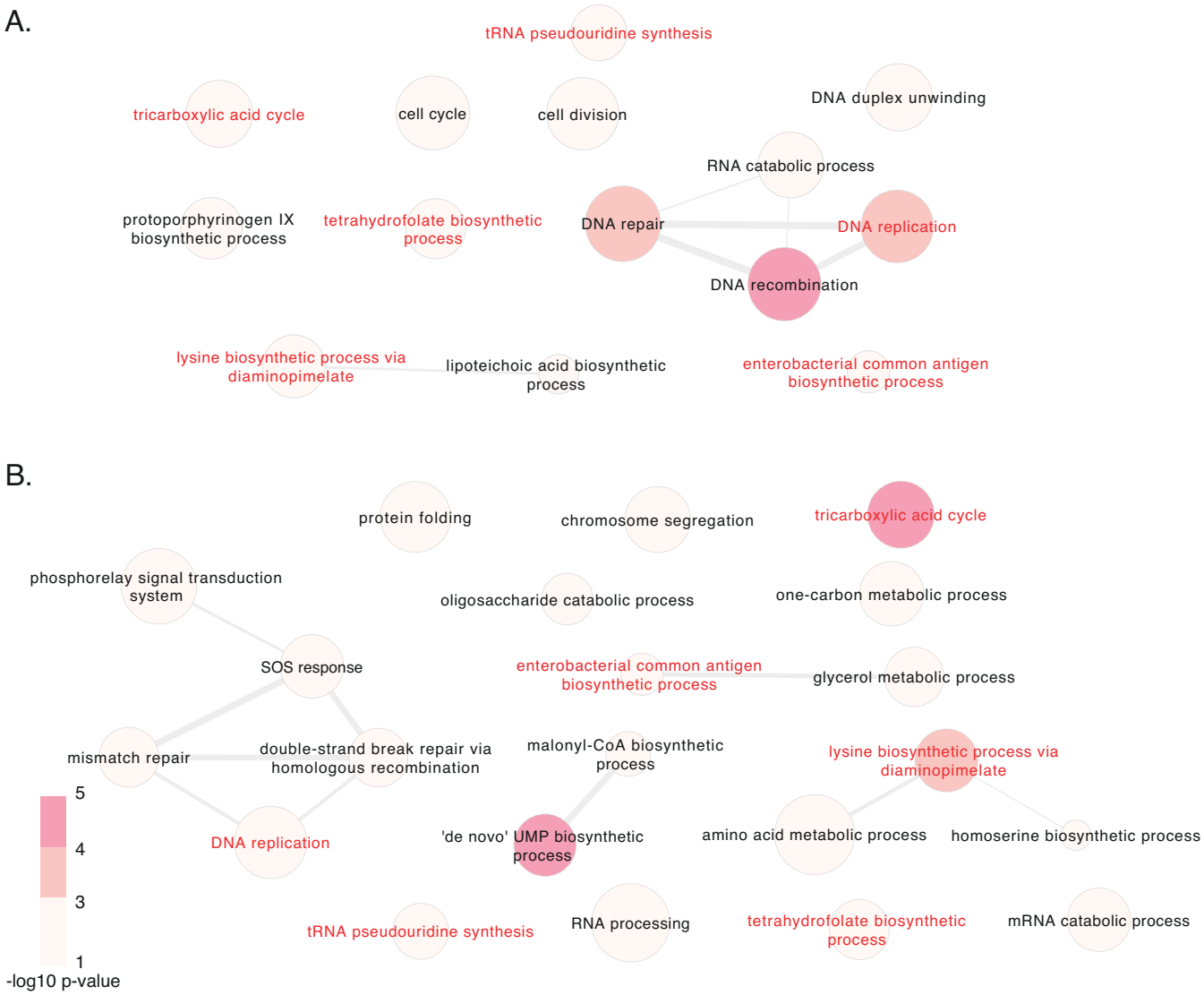

FIG 3 Pathway enrichment of genes contributing to macrophage invasion in *S. aureus*. Genes significant by Tn-Seq after macrophage invasion at adjusted *P*-value < 0.05 are represented, as determined by comparing initial transposon mutant library pools to mutants surviving following invasion. The color of circles corresponds to the *P*-value for each Gene Ontology (GO) category, and the size is proportional to the Log_{10} size of each GO term. The thickness of connecting lines represents semantic similarity between categories, and the 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.

(LNEJMEBC_01525 and *brnQ3*), and three paradoxically decreased invasive capacity (*icaR*, *pyrD*, and *gmuF*). Unexpectedly, all seven conditionally essential candidate genes from the ATCC29213 library produced statistically significant increases in invasiveness following their knockdown in ATCC29213 (Fig. 4B).

Only one JE2 gene in the validation set showed positive fold change in abundance after invasion (*purR*), and its knockdown accordingly resulted in a significant increase in invasiveness (Fig. 4C). Knockdown of the seven conditionally essential candidate genes in the transposon mutant pool (Fig. 4D) resulted in significantly decreased invasion for three of the genes (*gpmI*, *dltA*, and *dapD*) as anticipated, did not significantly impact invasion for two genes (*liaR* and *agrC*), and produced significantly increased invasion capacity for two genes (*airS* and SAUSA300_2455).

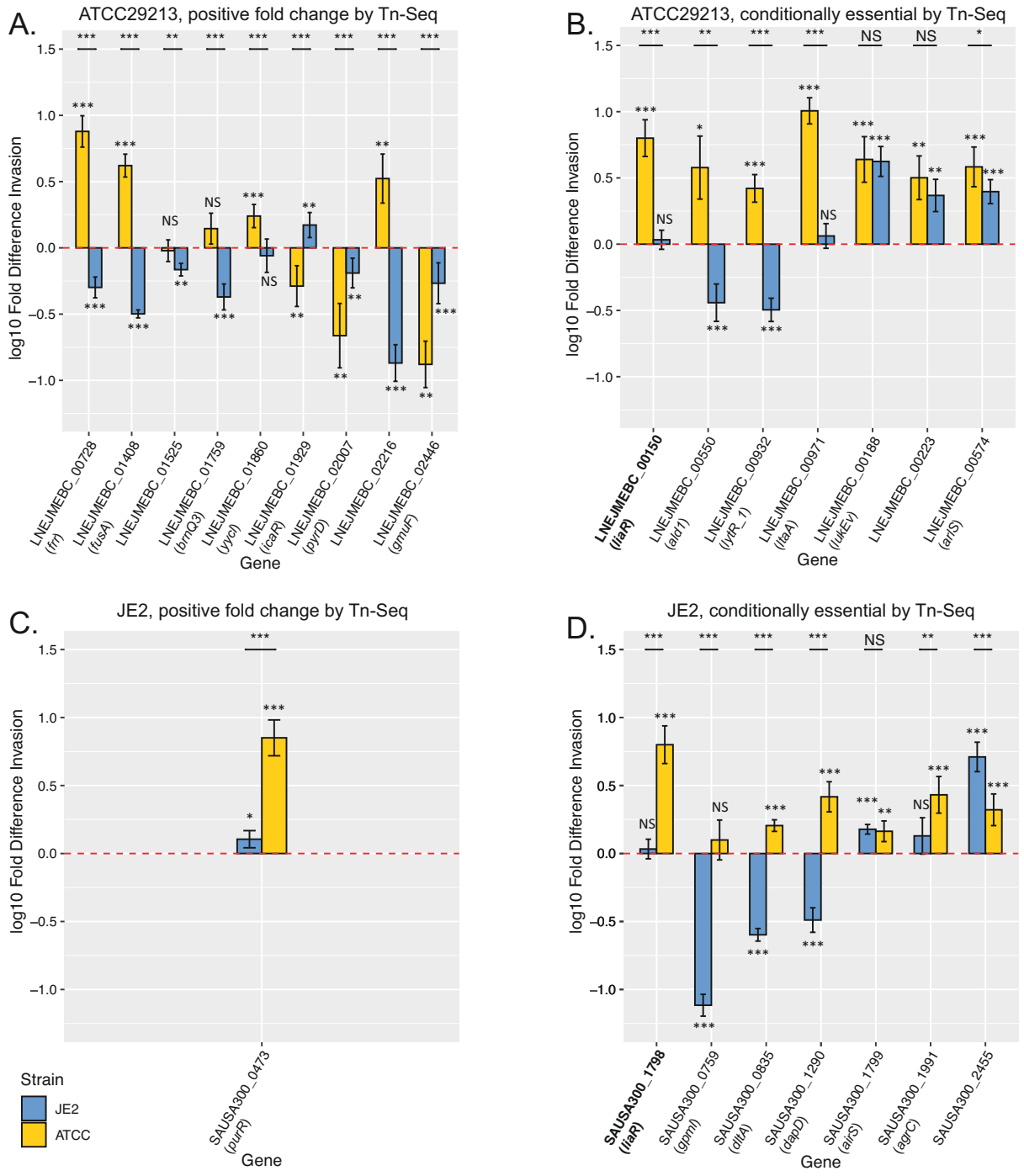

FIG 4 Intracellular invasion phenotypes of isogenic CRISPRi knockdowns. Results are shown for the knockdown of candidate genes from Tn-Seq analysis of post-macrophage invasion mutant pools that were identified as significant and showing consistent effects relative to both the initial transposon mutant library pool and the outgrowth control. Panels indicate genes identified by Tn-Seq as: (A) ATCC29213 genes with positive fold change in abundance, (B) conditionally essential ATCC29213 genes, (C) JE2 genes with positive fold change in abundance, and (D) conditionally essential JE2 genes. Y-axes indicate the fold difference in THP-1 derived macrophage invasion for isogenic CRISPRi knockdowns in both *S. aureus* strains relative 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 does not always match predictions based on Tn-Seq analysis.

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 (Fig. 4).

Knockdown of seven candidate genes with positive fold change in abundance identified from ATCC29213 resulted in significantly decreased invasiveness for JE2, while one had no significant impact and one significantly increased invasive capacity (Fig. 4A). For only two genes (*pyrD* and *gmuF*) were these results consistent between the two strains. Similarly, while knockdown of all seven conditionally essential 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 (Fig. 4B). Inversions of phenotypic effect were also seen for JE2 candidate genes knocked down in ATCC29213. While knockdown of the single candidate gene with positive fold change in abundance from JE2 (Fig. 4C) augmented the invasion phenotypes of both strains, five of the seven conditionally essential candidate genes (Fig. 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.

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 during chronic *S. aureus* infection *in vivo*. We used respiratory infection in CF as a model, as *S. aureus* is postulated to undergo selection for increased macrophage pathogenesis in that environment (10, 35). We examined 237 groups of clonally related *S. aureus* isolates longitudinally collected from children with CF over a period of several years (26). Studies from our group using the isolate collection have accordingly shown that the capacity of strains for macrophage invasion increases during the course of chronic infection *in vivo* (D. R. Long, E. A. Holmes, H.-Y. Lo, K. Penewit, J. Almazan, T. Hodgson, N. F. Berger, Z. H. Bishop, D. J. Wolter, J. D. Lewis, A. Waalkes, and S. J. Salipante, submitted for publication). We therefore hypothesized that, in chronically infecting *S. aureus* populations, natural selection would be stronger in genes involved in macrophage invasion than in genes not contributing to that phenotype.

Individuals with CF become infected with a variety of *S. aureus* lineages (26). We consequently considered genes identified by Tn-Seq as having significant effects in both ATCC29213 and JE2 (Table S6), which are most likely to affect consistent phenotypes among a diverse population of *S. aureus* strains. As comparators, we included 183 control genes least associated with the macrophage invasion phenotype. Tn-Seq identifies gene disruptions that are conditionally essential for macrophage invasion as those showing negative fold changes in abundance after invasion, which are expected to be under purifying selection *in vivo*. Conversely, genes whose inactivation enhances macrophage invasion are identified as those having positive fold changes in abundance and should be subject to positive selection. This set contained a paucity of genes expected to be under positive selection (n = 2), with nearly all expected to be subjected to purifying selection (n = 106). For this reason, and because missense mutations may produce

either gain-of-function or loss-of-function effects, which are difficult to predict, analysis was based on *de novo* mutations which unequivocally disrupted gene function (i.e., frameshift and stop-gain mutations, comparable to insertional inactivation), and the two candidate genes predicted to be under positive selection were not included.

We assessed the frequencies of *de novo* disruptive versus silent mutations in candidate and control genes occurring within clonally related *S. aureus* lineages from patient specimens (Table S10). For each gene, we ascertained whether there was a statistically significant deficit of disruptive relative to synonymous mutations observed across clonal groups, which would be indicative of purifying selection *in vivo* (36). The distribution of resultant *P*-values, showing the strength of evidence for selection in each gene, was then compared between candidate genes and control genes. This analysis showed significantly lower frequencies of disruptive mutation in candidate invasion genes relative to control genes (Fig. 5, $P = 0.002$, Wilcoxon rank-sum test, Table S10), consistent with predictions from Tn-Seq analysis.

These studies provide evidence that conditionally essential macrophage invasion genes implicated by Tn-Seq were collectively under greater purifying selection during chronic infection in CF patient airways *in vivo* than genes not associated with that phenotype, highlighting their relevance in human disease.

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, Fig. 1).

Our transposition system offers several advantages over prior approaches used in *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, 37–42). After transposition, high temperature plasmid-curing steps are required to remove the vectors, however, plasmid curing may be incomplete (43), resulting in ongoing or unstable transposition events, and heat stress can inadvertently select for temperature sensitive mutants or otherwise bias the mutant pool (44). 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 (44–46). This eliminates the need for temperature-dependent plasmid curing, and the high efficiency of phage transduction allows ultra-high density mutant libraries (44–46). However, not all *S. aureus* isolates are susceptible to Φ11 transduction (47). 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 (44). 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 (48–51), 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 (18). Because transposition is mediated by complexes prepared *in vitro,* there is no dependence on host-encoded factors and no need to remove 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 (18). We rapidly generated highly saturated *S. aureus* transposon mutant libraries in two strains, comprising ~300,000 to 400,000 unique insertions each.

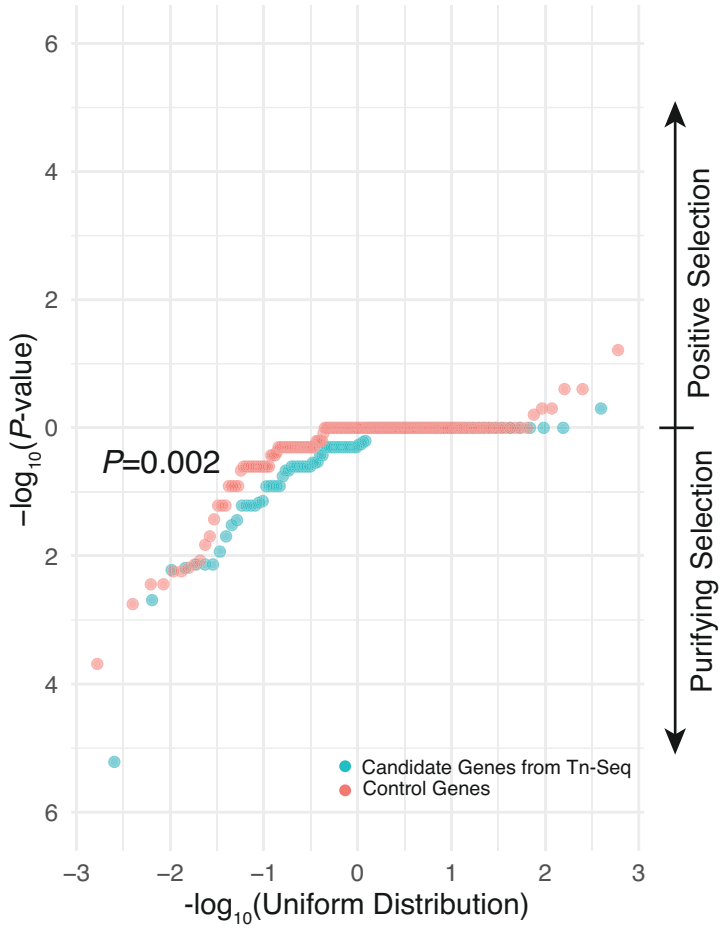

FIG 5 Selection of candidate macrophage invasion genes in chronic CF respiratory infections *in vivo*. QQ-plots of -$\log_{10}$ transformed *P*-values testing the proportion of disruptive versus synonymous mutations in candidate genes Tn-Seq identified as having significant effects on macrophage invasion for both ATCC29213 and JE2 and corresponding control genes not implicated in that phenotype. *P*-values in ranges consistent with positive (more disruptive than synonymous mutations) and purifying selection (more synonymous than disruptive mutations) are indicated by the arrows at right. Significance of difference between the distributions of candidate and control genes by Wilcoxon rank-sum test is shown.

We used Tn-Seq to compare initial transposon mutant library pools to populations having successfully invaded host macrophages, thereby identifying genes whose disruption positively or negatively impacts intracellular pathogenesis (Table 1). A substantial proportion 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* (52). In both strains, the majority of genes identified as significant by Tn-seq showed negative fold change in abundance following invasion relative to the initial transposon library pool, consistent with most relevant factors being required (or conditionally essential) for effective invasion and early survival in macrophages. Virtually all (98%) candidate genes which had identifiable homologs between the two strains consistently evidenced negative fold change in abundance from Tn-Seq experiments of both strains, identifying a core set of 106 conditionally essential genes that are necessary to support macrophage invasion in *S. aureus* (Table S6). A smaller proportion of knockouts showed positive fold change in abundance from the initial transposon mutant pools after macrophage invasion, indicating that spontaneous chromosomal mutations in *S. aureus* can also enhance this virulence phenotype (53) and

may facilitate adaptation in chronic infection. Collectively, physiological and metabolic functions consistently contributing to macrophage invasion in both strains (Fig. 3) may reflect the association between intracellular pathogenesis and the SCV phenotype (7–11), as disruption of many such pathways has been identified in SCVs (54–57).

Comparing initial and selected transposon mutant libraries is used in most Tn-Seq studies of complex bacterial phenotypes (34, 41, 42, 58, 59) 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, only differently so. As a case in point, in both strains, disruptions of purine biosynthesis pathway genes showed significant increases in fold change after invasion relative to the outgrowth control (Tables S7 and S8) but not in comparison to the initial library (Tables S4 and S5). Previous studies have found that purine biosynthesis supports intracellular pathogenesis in *S. aureus* (53, 60), making the outgrowth control result consistent with a growth-related artifact. Nevertheless, 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.

These conservative criteria identified 31 unique candidate genes for functional validation, 24 in ATCC29213, and eight in JE2, with *liaR* shared in common (Table 2). 23 genes could be empirically tested as isogenic CRISPRi knockdown mutants. Twenty of these 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 (Fig. 4). Unexpectedly, 12 of the 20 functionally validated mutants produced phenotypic effects opposing expectations based on the direction of fold change of genes observed by Tn-Seq in their corresponding strain of origin. Phenotypic discordance between knockdown and knockout mutants of specific genes has been well documented for eukaryotic organisms (61, 62), with similar discrepancies reported in studies of *Salmonella* (63) and could reflect activation of compensatory networks buffering against deleterious mutations (61), insufficient gene knockdown, or unintended off-target silencing effects, although gRNA design algorithms limit that possibility (27). Moreover, we found that many validated genes identified from one strain (Fig. 4) produced contradicting phenotypic effects when knocked down in the other. These well-controlled studies, where the same knockdown vector transferred to two different *S. aureus* strains resulted in opposing consequences to macrophage invasion, provide strong evidence that effects of such factors are strain-specific.

Five of the 20 validated genes [*purR* (53, 60), *icaR* (64), *yycI* (65), *arlS* (66), and *ltaA*(67)] have previously described roles in *S. aureus* virulence, while two others [*lukEv*(68) and *airS*(69)] 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 and 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 (Fig. 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* (70) and elongation factor-encoding *fusA* (71) have functions in protein synthesis. Genes involved in the biosynthesis [*dapD* (72)] or catabolism of amino acids [*ald1* (73)] also impacted macrophage invasion, as did three genes involved in glycolysis [*gpmI* (74), *gmuF* (75), and putative fructose-1,6-bisphosphatase SAUSA300_2455] and one relevant to pyrimidine biosynthesis [*pyrD* (76)]. Of note, several of such genes have known roles in *S. aureus* persistence [*frr* (77) and *dapD*

(72)] or SCV [*fusA*(71)] phenotypes or adaptation during respiratory infection [*ald1*(73)], phenotypes which are consistent with enhanced intracellular pathogenicity and which lend greater credence to the findings of the present study. Second are genes relevant to survival within the toxic intracellular environment presented by macrophages. *airS* comprises half of the two-component *airSR* regulatory system, which controls resistance to reactive oxygen species (69), while *gpmI,* primarily identified as a metabolic gene, also contributes to *S. aureus* fitness during nitric oxide exposure (78). Third are regulatory effectors. Previously described genes *purR* (53, 60), *yycI* (65), and *arlS* (66) are thought to influence virulence by this mechanism. Like members of other two-component regulatory systems, *liaR* (79) and *lytR*, (80, 81) 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 fourth category encompasses bacterial cell wall regulation. Flippase *ltaA* has been shown to adaptively modify *S. aureus* cell wall teichoic acids under acidic conditions (82), and previous Tn-Seq studies have identified its contributions to metastatic *S. aureus* bloodstream infections (67). *dltA* has a similar role in teichoic acid alteration (83). 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* (84, 85), with some studies further suggesting that these factors promote adherence of bacteria to host cells (86, 87). The two remaining genes, *icaR* and *lukEv*, provide unique functions within this set, likely impacting macrophage intracellular pathogenesis by host cell adhesion (64) and attenuation of cytotoxicity (68), respectively.

Excitingly, genomic studies of isolates collected from individuals with CF provide evidence for the 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 that there is a selection for mutants having enhanced capacity for intracellular pathogenesis of host cells, especially airway macrophages (10, 35). In a large, longitudinally banked collection of isolates obtained from individuals with CF (26), we found genes implicated by Tn-Seq as conditionally essential for macrophage intracellular pathogenesis *in vitro* were collectively under significantly greater purifying selection *in vivo* than control genes least significantly associated with that phenotype (Fig. 5). This is particularly striking given the diversity of adaptive phenotypes that selected *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, 26, 88–90). 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. For example, the candidate gene with the strongest signal of purifying selection *in vivo (adsA*) plays a critical role in *S. aureus* escape from phagocytic clearance and survival in human blood (91). 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, 35).

Although illuminating, our study is subject to several limitations. Cell lines cultured *in vitro* are not physically or biochemically identical to analogous cells or tissues found *in vivo* (12), making our Tn-Seq assay necessarily contrived. However, our finding that clinical isolates evidence selection in many candidate genes identified using the THP-1 model employed here and by others (11, 14, 15) offers encouraging support that it reasonably approximates *in vivo* conditions. 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 monitors count of insertions that disrupt gene function; therefore, it will only identify genes that impact a phenotype by loss of function. Genes able to contribute to a process of interest through overexpression or gain-of-function mutations may exist but will

not be identified by Tn-Seq 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. Finally, use of CRISPRi as an orthologous validation method did not allow interrogation of all targets of interest, and valid effector genes that 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, to further explore the diversity of *S. aureus* genetic factors that facilitate human cell invasion, to explore the basis of strain-specific gene effects, and to identify genes necessary for the maintenance of chronic infection within macrophages following initial host cell invasion. Genes and pathways implicated here may present novel therapeutic targets that could be leveraged to disrupt persistent *S. aureus* infection *in vivo*.

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AUTHOR AFFILIATIONS

[1]Department of Laboratory Medicine and Pathology, University of Washington School of Medicine, Seattle, Washington, USA
[2]Division of Critical Care Medicine, Department of Anesthesiology and Pain Medicine, University of Washington School of Medicine, Seattle, Washington, USA

AUTHOR ORCIDs

Stephen J. Salipante http://orcid.org/0000-0001-8355-6992

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Dustin R. Long, Formal analysis, Software, Visualization, Writing – review and editing | Elizbeth A. Holmes, Formal analysis, Investigation, Methodology | Kelsi Penewit, Investigation, Methodology | Taylor Hodgson, Investigation | Janessa D. Lewis, Investigation | Adam Waalkes, Formal analysis, Investigation, Software | Stephen J. Salipante, Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Writing – review and editing, Software, Visualization, Investigation.

DATA AVAILABILITY

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

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Table S1 (IAI00228-23-s0001.xlsx). Oligonucleotide sequences.
Table S2 (IAI00228-23-s0002.xlsx). Composition of initial transposon mutant pool for ATCC29213.
Table S3 (IAI00228-23-s0003.xlsx). Composition of initial transposon pool JE2.
Table S4 (IAI00228-23-s0004.xlsx). Tn-Seq results for ATCC29213, initial vs invasion library.
Table S5 (IAI00228-23-s0005.xlsx). Tn-Seq results for JE2, initial vs invasion library.
Table S6 (IAI00228-23-s0006.xlsx). Gene homologs identified as significant for invasion in both *S. aureus* strains.
Table S7 (IAI00228-23-s0007.xlsx). Tn-Seq results for ATCC29213, outgrowth control vs invasion library.
Table S8 (IAI00228-23-s0008.xlsx). Tn-Seq results for JE2, outgrowth control vs invasion library.
Table S9 (IAI00228-23-s0009.xlsx). CRISPRi gene knockdown results.
Table S10 (IAI00228-23-s0010.xlsx). Mutational characteristics of candidate macrophage invasion genes in vivo.

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