




In Vitro Selection Identifies *Staphylococcus aureus* Genes Influencing Biofilm Formation

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ABSTRACT *Staphylococcus aureus* generates biofilms during many chronic human infections, which contributes to its growth and persistence in the host. Multiple genes and pathways necessary for *S. aureus* biofilm production have been identified, but knowledge is incomplete, and little is known about spontaneous mutations that increase biofilm formation as infection progresses. Here, we performed *in vitro* selection of four *S. aureus* laboratory strains (ATCC 29213, JE2, N315, and Newman) to identify mutations associated with enhanced biofilm production. Biofilm formation increased in passaged isolates from all strains, exhibiting from 1.2- to 5-fold the capacity of parental lines. Whole-genome sequencing identified nonsynonymous mutations affecting 23 candidate genes and a genomic duplication encompassing *sigB*. Six candidate genes significantly impacted biofilm formation as isogenic transposon knockouts: three were previously reported to impact *S. aureus* biofilm formation (*icaR*, *spdC*, and *codY*), while the remaining three (*manA*, *narH*, and *fruB*) were newly implicated by this study. Plasmid-mediated genetic complementation of *manA*, *narH*, and *fruB* transposon mutants corrected biofilm deficiencies, with high-level expression of *manA* and *fruB* further enhancing biofilm formation over basal levels. This work recognizes genes not previously identified as contributing to biofilm formation in *S. aureus* and reveals genetic changes able to augment biofilm production by that organism.

KEYWORDS *Staphylococcus aureus*, biofilm, evolution, genomics, infection, adaptation, cystic fibrosis

Staphylococcus aureus is a prevalent clinical pathogen worldwide that is capable of causing acute and chronic disease in a variety of organ systems (1–5). Morbidity and mortality attributable to *S. aureus* infection have increased substantially over the past decades, due in part to the rise and dissemination of community-acquired methicillin-resistant *S. aureus* (MRSA) clones and attendant challenges in treatment (6).

Among the many virulence factors *S. aureus* deploys during chronic infection is the production of biofilms or biofilm-like aggregates (7, 8). Biofilm formation has been implicated in the pathogenesis of *S. aureus* osteomyelitis, indwelling device infections, wound infections, endocarditis, and other medical conditions (7) and is believed to promote persistence through several complementary mechanisms: staphylococcal biofilms impair action of the innate immune system by expressing factors that disable immune cells and circumvent inflammatory responses (9, 10), reducing host-mediated clearance. They similarly provide resistance to antibiotic-mediated killing (11), blunting the impact of a broad range of therapeutic agents. Dispersion of cells from mature biofilms facilitates the continuance and progression of chronic infection, including the colonization of new body sites (8).

Substantial work has been conducted to identify regulatory and effector genes essential to *S. aureus* biofilm formation using various methods including examination of orthologs

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with relevant function in other species (12), saturation transposon mutagenesis screens (13), and transcriptomic (14, 15) or proteomic (16, 17) analyses. These efforts have provided an increasingly detailed picture of the pathways and genes relevant to biofilm formation in this organism, but even so, the factors involved remain incompletely enumerated (7, 8, 10, 18). Moreover, chronically infecting *S. aureus* populations are known to undergo mutational adaptation during the course of disease that selects for increased fitness in the host, including changes that promote biofilm formation phenotypes (19–24). Mutations enhancing *S. aureus* biofilm production *in vivo* are therefore likely of relevance to chronic pathogenesis, but comparatively little is known about what genomic changes are able to elevate biofilm formation beyond basal levels (25), and systematic studies to investigate that phenomenon have not yet been performed.

Here, we utilized *in vitro* selection and whole-genome sequencing to identify spontaneous chromosomal mutations associated with enhanced biofilm formation in four laboratory *S. aureus* strains (ATCC 29213, JE2, N315, and Newman). Using a combination of isogenic transposon mutant knockouts (26) and genetic complementation studies, we provide functional validation of genes not previously described as contributing to *S. aureus* biofilm formation.

RESULTS

***In vitro* selection of *S. aureus* for enhanced biofilm formation.** We performed selection of *S. aureus* for spontaneous mutations conferring increased capacity for biofilm production. We serially passaged replicate liquid cultures of four *S. aureus* laboratory strains (ATCC 29213, JE2, N315, and Newman) and selectively harvested the population adhering to an abiotic surface after each growth period. Thirty-seven serial passages were conducted using five separate replicates of each strain, after which single colonies were isolated from evolved populations for subsequent analysis.

Baseline phenotypes of the parental *S. aureus* strains differed significantly by crystal violet biofilm absorbance assays, with N315 having the greatest prepassaging biofilm formation capacity and Newman the least (Fig. 1A). Phenotyping of isolates from evolved populations (Fig. 1B) revealed statistically significant increases in biofilm-forming capacity relative to their matched parental strains for 18 of 20 replicates tested. The exceptions were N315 replicate 3, which showed slightly reduced biofilm formation, and N315 replicate 5, which did not change significantly. N315 exhibited the smallest relative gains in biofilm formation during passaging, with isolates having a maximum of 1.2 times the biofilm formation of their progenitor, while derivatives of other strains achieved maxima of 4- to 5-fold increases relative to their respective parents.

Mutations associated with biofilm formation *in vitro*. Whole-genome sequencing of passaged isolates having enhanced biofilm formation identified nonsynonymous coding sequence mutations in 23 different genes (Table 1 and see Table S2 in the supplemental material). Most genes ($n = 19$) were altered in a single isolate each, while three genes (*spdC*, *icaR*, and *codY*) were recurrently mutated in multiple isolates from different strains and one (*eap/map*) was mutated in two independently evolved replicates of the Newman strain. At least one gene mutation with unequivocally inactivating consequences (stop-gain, frameshift, and/or start codon loss) consistent with loss-of-function effects was noted in *spdC*, a hypothetical 5'-nucleotidase of the lipoprotein e(P4) family (pangenome gene identifier [27] SAUPAN001228000), *icaR*, and a HAD-family hydrolase (pangenome gene identifier SAUPAN002478000). Mutations in the remaining genes exclusively comprised missense variants or in-frame indels.

Structural analysis of whole-genome sequencing reads additionally revealed an ~110-kb region of copy number gain (approximate positions 2,183,746 to 2,293,258 bp of the JE2 reference genome) in a single passaged isolate, JE2 replicate 3 (Fig. 2A). The affected region encompassed 110 genes, notably including the *sigB* locus (Fig. 2B and Table S3). Verification of the copy number alteration was achieved using real-time PCR to measure DNA content within the presumptive event (*sigB* at the 5' end and a second locus at the 3' terminus) and genomic regions flanking the event and was consistent with a duplication event occurring in this region (Fig. 2C).

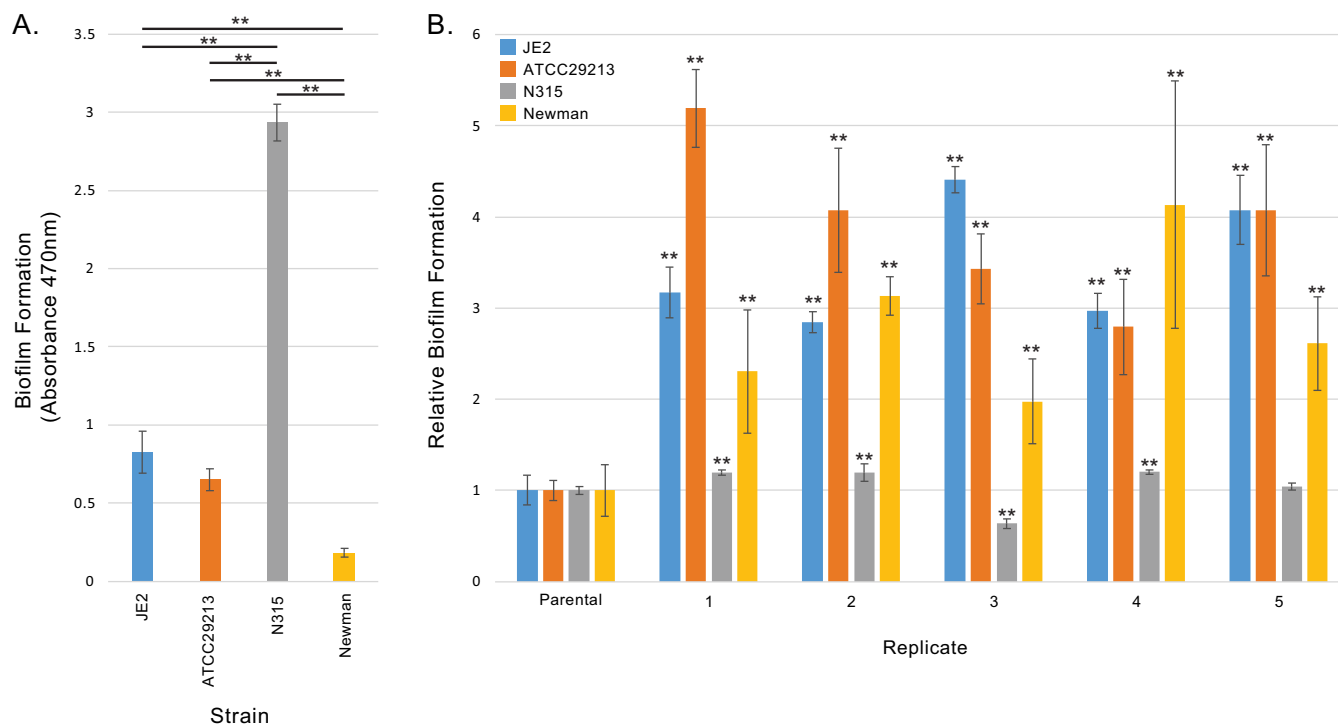


FIG 1 Biofilm formation of *S. aureus* laboratory strains and passaged derivatives. (A) Biofilm-forming capacity of *S. aureus* laboratory strains. Error bars indicate standard deviation for at least four separate measurements. ** represents a P value of <0.01 by two-tailed t test for the indicated comparison between strains (black lines). (B) Biofilm-forming capacity of laboratory strain derivatives obtained from serial passaging for increased adherence. Results are normalized to biofilm formation rates of the matched parental strain. Error bars show standard deviation from at least four different measurements. ** indicates a P value of <0.01 by two-tailed t test against the parental strain.

Validation of biofilm-associated mutations by transposon mutagenesis. We next tested the contributions of candidate gene mutations to biofilm formation using a defined, isogenic transposon knockout mutant library of strain JE2 (26). Eighteen of the 23 candidate genes had corresponding transposon mutants available for testing (Fig. 3). Of the mutants examined, six gene disruptions significantly ($P < 0.05$ by two-tailed t test) altered biofilm formation relative to the parental strain. These comprised knockouts of *spdC* (15), *icaR* (28), and *codY* (13), each previously reported to influence biofilm formation in *S. aureus*, and three newly implicated genes, *narH*, *manA*, and *fruB*. Transposon knockouts of *spdC*, *narH*, *codY*, *manA*, and *fruB* showed reduced biofilm capacity. The transposon mutant of *icaR* uniquely enhanced biofilm formation, consistent with its loss-of-function promoting that virulence phenotype.

Complementation of newly identified biofilm-associated genes. The demonstration that specific, isogenic transposon mutants measurably impacted biofilm formation is consistent with the impacted genes being involved in that phenotype. However, it is possible that additional, unlinked chromosomal mutations or polar effects resulting from transposition could have affected biofilm production (13).

To address this uncertainty, we performed genetic complementation of the three biofilm-associated genes that were newly implicated by our study (*narH*, *manA*, and *fruB*). Each gene was cloned into an expression plasmid under the control of a strong, constitutive promoter and introduced into the corresponding transposon knockout mutant. To serve as a comparator for the complemented mutants, we generated an analogous vector expressing green fluorescent protein (GFP) and transformed that plasmid into the same genetic backgrounds.

Transposon mutant strains transformed with GFP expression vectors recapitulated the expected deficiencies in biofilm mutation we observed earlier (Fig. 4). In all cases, transformation of transposon mutants with plasmids expressing the corresponding gene fully compensated for those deficiencies. Moreover, complementation with *manA*

TABLE 1 Gene mutations associated with increased biofilm formation *in vitro*

Pangenome gene identifier	Common gene name	Gene function	Mutant count per selected strain (total replicates)			
			Newman (n = 5)	JE2 (n = 5)	N315 (n = 3)	ATCC 29213 (n = 5)
SAUPAN000916000	<i>sbnC</i>	lucA/lucC family siderophore biosynthesis protein	1	0	0	0
SAUPAN005823000	<i>spdC</i>	Lysostaphin resistance protein A	0	2	3	0
SAUPAN006410000	<i>icaR</i>	Biofilm operon <i>icaADBC</i> HTH ^a -type negative transcriptional regulator	2	5	0	0
SAUPAN005927000	<i>narH</i>	Nitrate reductase subunit beta	1	0	0	0
SAUPAN003492000	<i>rpoZ</i>	DNA-directed RNA polymerase subunit omega	0	1	0	0
SAUPAN004777000		DUF445 domain-containing protein	1	0	0	0
SAUPAN003350000		Hypothetical protein	1	0	0	0
SAUPAN003016000		Nitronate monooxygenase	0	0	0	1
SAUPAN003553000	<i>codY</i>	GTP-sensing pleiotropic transcriptional regulator	3	5	3	3
SAUPAN005440000	<i>manA</i>	Mannose-6-phosphate isomerase	0	0	1	0
SAUPAN001024000	<i>argC</i>	<i>N</i> -Acetyl-gamma-glutamyl-phosphate reductase	0	0	1	0
SAUPAN006300000		Amino acid permease	0	0	0	1
SAUPAN003545000	<i>dprA</i>	DNA processing protein	1	0	0	0
SAUPAN002478000		HAD family hydrolase	0	0	0	1
SAUPAN006252000	<i>gbaA</i>	TetR family transcriptional regulator	0	0	1	0
SAUPAN001228000		5'-Nucleotidase, lipoprotein e(P4) family	0	1	0	0
SAUPAN006369000	<i>isaB</i>	Immunodominant antigen B	0	0	0	1
SAUPAN002607000		ABC transporter ATP-binding protein	1	0	0	0
SAUPAN002583000	<i>fruB</i>	1-Phosphofructokinase	1	0	0	0
SAUPAN000959000		Bifunctional metallophosphatase/5'-nucleotidase	1	0	0	0
SAUPAN000382000	<i>dusB</i>	tRNA-dihydrouridine synthase	0	0	1	0
SAUPAN005009000	<i>eap/map</i>	MAP domain-containing protein	2	0	0	0
SAUPAN000011000	<i>gyrB</i>	DNA gyrase subunit B	0	1	0	0

^aHTH, helix-turn-helix.

and *fruB* under regulation by a strong constitutive promoter resulted in significantly increased biofilm formation levels relative to those of the original parental strain.

Collectively, these results provide strong evidence that *narH*, *manA*, and *fruB* contribute to biofilm formation in *S. aureus* and that high-level constitutive expression of *manA* and *fruB* can promote enhanced biofilm formation in that organism.

DISCUSSION

S. aureus biofilms are composed of an extracellular matrix that incorporates a heterogeneous mixture of proteins, polysaccharides, and extracellular DNA and that follows a stereotyped pattern of development from initial formation to maturation and eventual dispersal (18, 29). The two major biochemical pathways responsible for aggregating *S. aureus* into biofilm matrices (7) comprise one dependent on the production of polysaccharide intercellular adhesin (PIA), which is biosynthesized by the *ica* operon (12), and a second, PIA-independent pathway mediated by adhesion of particular cell wall-associated proteins (7, 30). A number of genes directly contributing to, regulating, or providing accessory functions supporting those pathways have been catalogued, but the process of biofilm formation is complex (18), and additional effectors, including those involved in adaptive responses during chronic infection (19–24), have yet to be uncovered (7, 10, 18).

The most appropriate systems for modeling *S. aureus* biofilm growth *in vivo* are unknown (31), but studies (20) have indicated that clinical isolates from chronically infected cystic fibrosis patients show enhanced biofilm formation using standard microtiter plate assays (32), suggesting that the models employed in our study reasonably emulate relevant *in vivo* evolutionary pressures. Despite their initial, basal differences in biofilm formation capacity (Fig. 1A), all strains tested were able to evolve enhanced biofilm formation capacity with serial passaging (Fig. 1B). As reflected in our data, strain Newman has limited inherent capacity for biofilm formation due to a *saeS* mutation that actively represses multiple factors relevant to that phenotype (33). Interestingly, *saeS* reversion mutations were not recovered in any Newman mutants exhibiting enhanced biofilm formation, indicating that compensatory changes in other genes are more readily selected. N315, the strain with the greatest initial

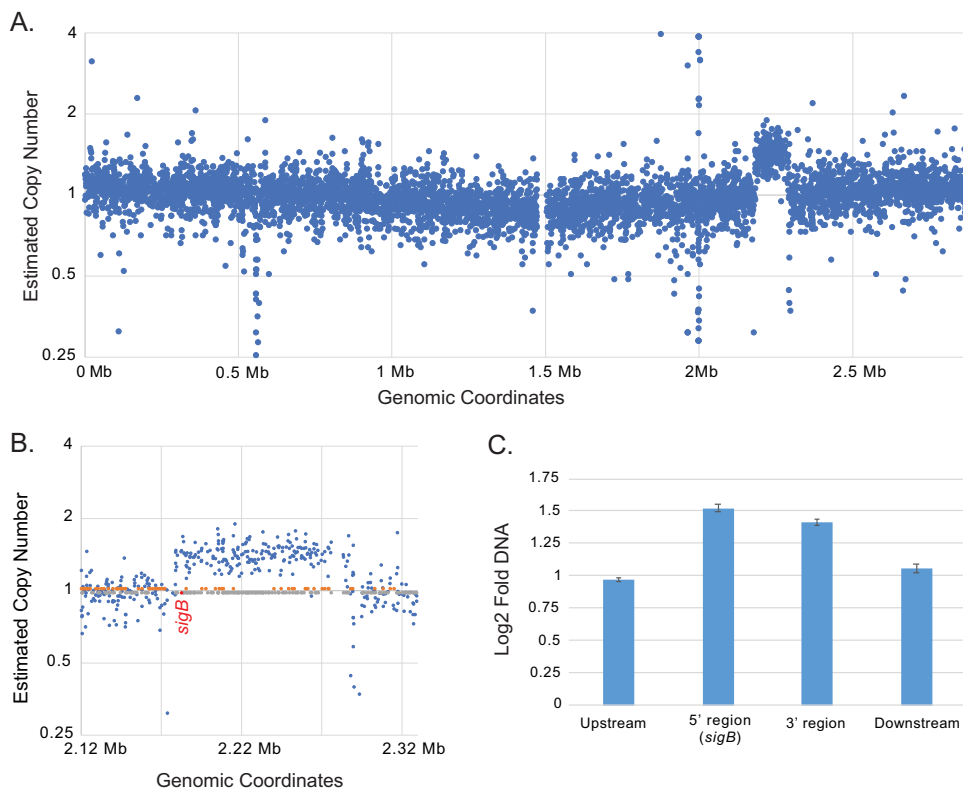


FIG 2 Copy number gain of the *sigB* locus in JE2 replicate 3. (A) Plot of estimated copy number for regions across the JE2 reference genome. (B) Enlargement of the region affected by copy number gain. Locations of genes carried in the forward orientation (orange dots) and reverse orientation (gray dots) are indicated along the central axis, with the location of *sigB* (reverse orientation) highlighted in red. (C) Confirmation of copy number gain by real-time PCR. Analysis was performed using primers designed upstream and downstream of the presumptive amplification and within both ends of the amplification. DNA copy number was estimated by the $\Delta\Delta C_T$ method, using an uninvolved region of the genome as a comparator. Error bars indicate standard deviation from three separate measurements.

biofilm capacity, achieved the smallest relative gains in biofilm formation with passaging, suggesting that phenotype may already be close to a biological maximum for N315.

In vitro selection for mutants with enhanced biofilm production (Table 1) identified eight affected genes previously documented as involved in *S. aureus* biofilm formation

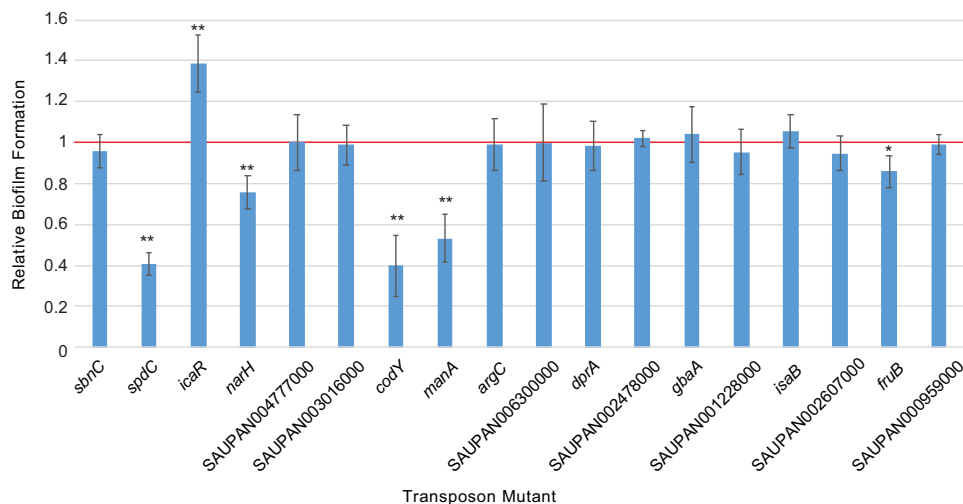


FIG 3 Biofilm formation of isogenic transposon mutant strains. Relative biofilm formation of isogenic JE2 transposon knockouts in the indicated genes, normalized to biofilm production of the parental JE2 strain (red line). Error bars indicate standard deviation from at least four separate measurements. * indicates a *P* value of <0.05 and ** indicates a *P* value of <0.01 by two-tailed *t* test against the parental strain.

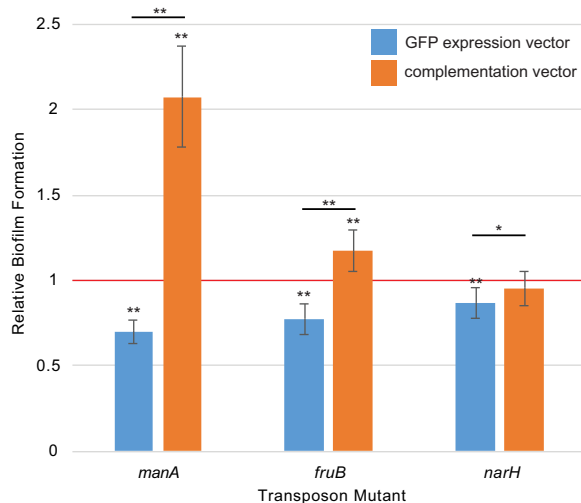


FIG 4 Biofilm formation of complemented transposon mutant strains. Biofilm formation of isogenic JE2 transposon mutants after complementation with the corresponding gene or a control vector expressing GFP. Results are normalized to biofilm production of the parental JE2 strain carrying the control vector (red line). Error bars indicate standard deviation from at least four separate measurements. * indicates a *P* value of <0.05 and ** indicates a *P* value of <0.01 by two-tailed *t* test against the parental strain (displayed above each bar plot) or for the indicated comparisons between transposon mutants carrying control or complementation vectors (black lines).

(*sigB*, *argC*, *icaR*, *gbaA*, *rpoZ*, *eap/map*, *spdC*, and *codY*), three of which (*icaR*, *spdC*, and *codY*) significantly impacted biofilm formation as isogenic transposon mutants (Fig. 3). Expression of the virulence regulator *sigB* has previously been shown to drive biofilm formation in a dose-dependent fashion (34, 35). However, to the best of our knowledge, prior to this study spontaneous chromosomal duplication of the *sigB* locus (Fig. 2) has not been associated with enhanced *S. aureus* biofilm formation. Loss of *icaR* or *gbaA*, each a regulator of the *ica* polysaccharide intracellular adhesin operon (28, 36), results in overexpression of the canonical enzymes that manufacture major constituents of PIA-based biofilms (37) and correspondingly enhance biofilm production (28, 36). Although it was not possible to functionally assess *argC*, *eap/map*, or *rpoZ* in this study, prior work has linked these genes to biofilm phenotypes (38–41).

The remaining two genes, *spdC* and *codY*, are pleiotropic regulatory factors that indirectly influence biofilm formation (15, 42) and whose contributions are thereby multifactorial. Point mutations in *codY* were the most frequently selected genetic changes in passaged isolates, observed in a total of 11 replicates and in all four strains. All *codY* variants identified were single amino acid substitutions, most clustering within the helix-turn-helix motif of the DNA binding domain (residues 203 to 227 [43]) and three involving one of four essential DNA binding residues (44) (see Table S2 in the supplemental material). In contrast, only unequivocally inactivating mutations in *spdC* (two JE2 isolates with start-loss mutations and one N315 isolate with a stop-gain mutation) were recovered after *in vitro* selection for enhanced biofilm formation. Nevertheless, transposon disruption of both genes contrarily reduced biofilm formation (Fig. 2), a finding consistent with prior reports (13, 15). Together, these results suggest that mutations of *spdC* and *codY* could exert biofilm-enhancing effects when occurring concordantly with other relevant mutations or regulatory changes but could impair biofilm formation when introduced as isogenic mutants. This model is supported by our observation that all clearly inactivating mutations in *spdC* occur in concert with mutations in other candidate genes (Table S2) and would also explain inconsistencies reported in the phenotypic consequences of *codY* mutation, which in some studies have reduced biofilm formation and in others augmented it (13, 42, 45).

Excitingly, our *in vitro* evolution strategy newly identified and permitted functional validation of *manA*, *narH*, and *fruB* as genes involved in *S. aureus* biofilm formation

(Fig. 3 and 4). Mannose-6-phosphate isomerase *manA* supports biofilm generation in other species by contributing to extracellular polysaccharide production (46), and our results are consistent with the gene serving a similar role in *S. aureus*. The contributory mechanisms of either *fruB*, encoding fructose-1-phosphate kinase, or *narH*, encoding nitrate reductase subunit beta, are less clear. However, we note that expression of both *fruB* and *narH* differs significantly between *S. aureus* grown in stationary phase and in biofilms (14) and that both genes contribute to biofilm production in other species (47, 48), providing orthologous support for their involvement in that phenotype. Both genes support anaerobic respiration, and it is plausible that their action may reflect the affinity of *S. aureus* to generate biofilms during anaerobic growth (49). Inactivation of *manA*, *narH*, and *fruB* by transposon mutagenesis each resulted in biofilm deficiencies, inconsistent with a loss-of-function model promoting biofilm production. Nevertheless, genetic complementation of those mutant strains (Fig. 4) overcame those deficiencies and in the case of *manA* and *fruB* indicates that constitutive, high-level expression can elevate biofilm production beyond basal levels. It is therefore possible that mutations observed in these genes after selection result in greater protein expression or activity, by means of derepression, increased stability, or elevated kinetics.

In summary, this effort demonstrates that *S. aureus* readily accumulates spontaneous genomic mutations in multiple genes that affect capacity for biofilm formation and that high-level expression of several novel factors results in enhanced biofilm production. Although our study has identified and validated three previously undescribed factors relevant to biofilm formation in *S. aureus*, it nonetheless remains subject to several limitations. First, it is possible that some candidate genes which failed functional validation legitimately contribute to biofilm formation. Given the complex epistatic interactions that govern many virulence traits in *S. aureus* (50), some variants may exert effects only when occurring in the presence of other genetic variants or in specific strain backgrounds. Effector genes whose contributions to biofilm production are not quantitatively large may also have been overlooked by our conservative functional validation criteria. Second, although we have considered four laboratory strains from different phylogenomic backgrounds, our work does not explore the scope of causative changes that could impact biofilm formation across all *S. aureus* lineages. Similarly, biofilm mutant screening is influenced by the conditions used and the substrate tested (51, 52), and any single experiment cannot identify all factors which could potentially impact that phenotype under particular circumstances. Third, our work has limited power to identify effector mutations that occur infrequently. Future work will seek to illuminate the mechanisms by which newly implicated genes influence biofilm formation and will further probe the range of mutations and mechanisms by which *S. aureus* is able to augment its capacity for biofilm formation as an adaptive phenotype during chronic infection.

MATERIALS AND METHODS

Strains and growth conditions. *S. aureus* strain ATCC 29213 was purchased from the American Type Culture Collection. Strains JE2 and N315 were obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI Resources). JE2 transposon mutants in defined genes (26) were also obtained from BEI Resources. *S. aureus* strain Newman was a generous gift from Lucas Hoffman (University of Washington).

Strains were routinely maintained at 37°C using tryptic soy broth (TSB) medium, except for those transformed with temperature-sensitive complementation vectors, which were cultured at 32°C with the addition of 10 µg/mL chloramphenicol to select for retention of plasmids.

Selection for biofilm formation and biofilm formation assays. Passaging to select for mutants with enhanced biofilm formation was conducted using modifications of existing protocols (53, 54). Briefly, overnight cultures of *S. aureus* were grown in tryptic soy broth (TSB), diluted 1:100 in fresh medium, and deposited in 450-µL aliquots into 48-well tissue culture plates containing sterile glass coverslips (German glass round 5-mm coverslips, supplied by VWR, Radnor, PA) (54). Following overnight incubation, coverslips were removed with sterile forceps and rinsed with sterile Dulbecco's phosphate-buffered saline (DPBS), and the biofilm was dispersed into 1 mL fresh TSB by vortexing. Cells were diluted to an optical density at 600 nm (OD_{600}) of 0.07 and were applied to fresh culture plates with coverslips to initiate the next round of passaging.

To quantitatively assess biofilm formation, overnight cultures were grown in TSB, diluted to an OD_{600} of 0.063 in 450 µL TSB, and deposited into the wells of a 48-well tissue culture plate. Following 18 h of incubation, biofilm formation was assessed by crystal violet staining as described elsewhere (53). At least four replicates of each specimen were used to quantitate biofilm formation levels, with readings normalized to in-batch controls of the appropriate parental strain.

Whole-genome sequencing and genomic analysis. Whole-genome sequencing and analysis were performed as previously described (55) using a MiSeq platform with 300 cycle chemistries (Illumina, San Diego, CA). Briefly, variant calling utilized reference genomes identified as those most closely matching the *de novo* assemblies of sequenced isolates. Mutations were manually verified using the Integrative Genomics Viewer (56) prior to annotation of sequence features. Common gene names and pangenome identifiers were established using BLAST (57) searches of target gene sequences against *S. aureus* reference genomes and by cross-correlating gene identifiers against the AureoWiki database (27). Chromosomal copy number analysis was performed using CNOGpro (58).

Real-time PCR for copy number verification. Real-time PCR was performed to verify a presumptive copy number alteration in one evolved strain. Primers (see Table S1 in the supplemental material) targeted 5' and 3' regions within the alterations, regions ~1 kb upstream and downstream of the alteration, and a chromosomal locus distant to the copy number change which was used as a comparator for copy number estimation using the threshold cycle ($\Delta\Delta C_T$) method. All oligonucleotides used in this study were synthesized by IDT (Coralville, IA).

Genetic complementation. Genes of interest and an *S. aureus* codon-optimized SuperfolderGFP gene (59) (serving as a control) were synthesized (gBlocks; IDT) under the control of the strong, constitutive *S. aureus* Pcap promoter (60) (Table S1). The erythromycin resistance determinant of temperature-sensitive shuttle vector pCN50-ermc-E194ts (61) was replaced with the chloramphenicol resistance cassette of pCN50-BSA1 (61) to yield pCNpE194ts-cat, and each expression cassette was subsequently cloned upstream of that vector's transcriptional terminator to yield the final complementation vectors. Complementation vectors and the GFP-expression control were transformed into matched JE2 transposon mutant strains as described elsewhere (61). Biofilm formation of complemented mutants was measured as described above, with the addition of 10 μ g/mL chloramphenicol to all culture media, with readings normalized to in-batch controls of the parental JE2 strain transformed with the GFP-expression control vector.

Data availability. Sequence data generated from this study are available from the NCBI Sequence Read Archive under accession PRJNA860616.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.01 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.01 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.01 MB.

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