

# Potential Influence of *Staphylococcus aureus* Clonal Complex 30 Genotype and Transcriptome on Hematogenous Infections

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**Background.** The contemporary *Staphylococcus aureus* clonal complex (CC) 30 lineage is associated with complicated infections, including endocarditis and osteomyelitis. This lineage diverged from the phage-type 80/81 *S aureus* clone responsible for a major bacterial epidemic of the 20th century. The genome and transcriptome features that contribute to complicated infections of the CC30 lineage are unknown.

**Methods.** Twenty-nine clinical methicillin-resistant *S aureus* (MRSA) strains (8 from CC30 and 21 from other major CCs) were evaluated for virulence using murine and *Galleria mellonella* sepsis models. Genomic features of CC30 were identified by comparative genome sequencing and RNA-Seq transcriptome analysis of the 29 strains and 31 previously sequenced *S aureus* genomes.

**Results.** The CC30 isolates displayed lower virulence in the sepsis models compared with other CCs [ $P < .0001$ ]. Comparisons of orthologous proteins and transcriptome analysis identified genes (eg, nitric oxide reductase) and changes in metabolic pathways (eg, pyrimidine metabolism) that contribute to the distinct CC30 phenotype. Previously reported nonsynonymous single-nucleotide polymorphisms (SNPs) were found in accessory gene regulator C (*agrC*) and  $\alpha$ -hemolysin (*hla*), molecules important for virulence. Additional nonsynonymous SNPs conserved across clinical CC30 isolates when compared with the first sequenced contemporary CC30 clone, MRSA-16, were identified in multiple genes, suggesting continuing evolutionary divergence in this lineage.

**Conclusions.** Genomic and transcriptional analyses suggest that the CC30 lineage has acquired metabolic features that contribute to persistent and complicated infections. Absence of sepsis-induced mortality in animal models may be due in part to its unique genomic profile and suggests that specific genotypes of *S aureus* elicit distinct types of infection types.

**Keywords.** genome; genotype; hematogenous infections; *Staphylococcus aureus*; transcriptome.

*Staphylococcus aureus*, a commensal of the skin and nares, is the cause of infections ranging from

uncomplicated skin infections to serious complicated infections such as pneumonia, osteomyelitis, endocarditis, and sepsis [1–3]. The ability of *S aureus* to cause severe infections is attributed to its repertoire of virulence factors, many of which are transferred horizontally through the *S aureus* community [4, 5]. *Staphylococcus aureus* isolates are classified into different genetic lineages or clonal complexes (CCs) based on genome content, with a close association between bacterial genetic characteristics and the distinct clinical manifestations [6–12]. There are over 100 CCs of *S aureus*, and CC5, CC8, CC22, CC30, and CC45 are more frequently associated with hospital-acquired infections. *Staphylococcus aureus* CC30 is the major nasal carriage lineage, and it is associated with the majority of autologous infections

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[13]. Previous work from our group [8, 14, 15] demonstrated that the CC30 lineage is associated with hematogenous complications, including endocarditis, septic arthritis, and vertebral osteomyelitis. In addition, CC30 is more frequently associated with persistent versus resolving bacteremia, and it shows increased adhesion to endothelial cells, elevated resistance to human neutrophil peptide hNP-1, and increased membrane fluidity compared with resolving strains [16]. These attributes of CC30 isolates may potentially be responsible for invasion of endocardial surfaces and contribute to persistent infection. The genomic basis for these CC30 characteristics is unknown.

In the current investigation, we used *in vivo* sepsis models, comparative genomics, and RNA-Seq transcriptome analysis of CC30 and other CCs to (1) evaluate virulence and severity of infections and (2) identify genomic features that contribute to CC30 persistence and complicated infections. *Staphylococcus aureus* CC30 isolates were significantly less virulent compared with other CCs. The attenuated virulence of CC30 isolates in sepsis models is attributed to its distinct genomic architecture, single-nucleotide polymorphisms (SNPs) that inactivate key virulence genes, and differential regulation of metabolic and adhesin genes.

## MATERIALS AND METHODS

All animal research was approved by Duke University Institutional Review Board and Institutional Animal Care & Use Committee, as appropriate. Genome sequence and assembly, assembly validation using Opgen optical maps, genome annotation, whole-chromosome phylogenetic analysis, Jaccard Orthologous Clustering (JOC) analysis, and SNP discovery and analysis in CC30 strains are described in [Supplementary Materials and Methods](#).

### *Staphylococcus aureus* Clinical Isolates

The 379 *S aureus* isolates (125 methicillin-resistant *S aureus* [MRSA] and 254 methicillin-sensitive *S aureus* [MSSA] isolates from CC1, CC5, CC8, CC5, CC30, and CC45) used for our initial genotypic multilocus sequence typing [MLST]/Spa typing were selected using strict definitions to identify 3 clinical groups that represent a progression from healthy individuals to those who are severely infected: (1) nasal carriage only (healthy controls), (2) uncomplicated infection, and (3) bacteremia with hematogenous complications [8]. Multiple levels of array-comparative genomic hybridization analyses identified differences in gene content relative to CC, MRSA, and MSSA status as well as clinical outcome. Two separate analyses, one dependent on CC status (found in CC5 and CC30) and a second independent of CC status, identified an identical set of 14 genes associated with complicated infections [8, 14]. The 29 MRSA isolates in the Complicated Infection Group (CIG) (Table 1) used in the current study were selected from the initial 379 *S aureus* isolates based on (1) carriage and clinical severity,

(2) MRSA status, (3) presence and proportion of the 14 candidate virulence genes, and (4) CC. The CC30 CIG isolates belong to the previously described contemporary CC30 clone lineage that diverged from the phage-type 80/81 *S aureus* clone [17–19].

### *Staphylococcus aureus* Reference Strains for Comparative Genome and Transcriptome Analysis

*Staphylococcus aureus* MRSA252 (a reference strain for the contemporary CC30 MRSA-16 lineage [20]), N315 (a CC5 reference strain [21]), THC60 (an *S aureus* CC30 isolate sequenced in the Human Microbiome Project [PRJNA159859]), and UAMS-1 (a commonly used clinical CC30 strain from an osteomyelitis patient [22]) were obtained from Network on Antimicrobial Resistance in *Staphylococcus aureus* or Mark Smeltzer (University of Arkansas).

### Preparation of *Staphylococcus aureus* Cells

To prepare *S aureus* cells for injection, an isolated colony from a fresh tryptic soy agar (TSA) plate was inoculated to 10 mL fresh tryptic soy broth and incubated at 37°C/220 revolutions per minute (rpm) for overnight culture. An appropriate amount of overnight bacterial culture was added to 100 mL fresh tryptic soy broth (TSB) in a 500 mL Erlenmeyer flask to normalize the initial optical density ([OD] 600) to approximately 0.1. The flasks were incubated at 37°C/220 rpm to log-phase growth (OD 600 ~ 1.0). *Staphylococcus aureus* cells were harvested by centrifugation, washed twice with phosphate-buffered saline (PBS), resuspended in PBS containing 20% glycerol to a concentration of approximately 10<sup>6</sup> colony-forming units (CFUs)/μL, aliquoted into individual cryovials, and immediately stored at –80°C until further use. An aliquot of PBS-washed culture was serially diluted and plated on TSA plates to enumerate CFUs. Frozen stocks were titered for CFUs in triplicate on separate occasions before use in any experiment.

### Mouse Model of Intra-peritoneal Sepsis

Six- to 8-week-old C57BL/6J mice used in this study were purchased from The Jackson Laboratory (Bar Harbor, ME). Colony-forming units (10<sup>8</sup>) of each *S aureus* strain in 200 μL suspension in PBS were injected intraperitoneally into 5 mice in each experimental group. Mice were given supplemental care and observed continuously for survival for 8 hours postinfection and then every day for 5 days.

### Wax Moth Model of Sepsis

The wax moth model of sepsis (described previously [23, 24]) was used in this study with slight modifications. In brief, the larval form of greater wax moth (*Galleria mellonella*) was purchased from Best Bet Inc. (Blackduck, MN) and stored at 4°C in dark until used for the sepsis experiments. The larvae were incubated at room temperature overnight prior to injection with *S aureus*. Twenty healthy larvae of similar size (approximately 250–300 mg) were injected in each experimental group, using 50 μL Hamilton syringes fitted with 26-gauge needles (Hamilton Company,

**Table 1. Methicillin-Resistant *Staphylococcus aureus* Strains in the CIG**

ID <sup>a</sup>	Clonal Complex	Group	B&J	Endo	Both	Outcome <sup>b</sup>	Proportion <sup>c</sup>
1057	5	Complicated		X		1	1
1096	5	Complicated	X			2	0.9
1150	5	Complicated	X			1	0.364
1165	5	Complicated		X		3	0.92
1213	5	Complicated	X			3	0.92
1750	5	Complicated		X		3	1
1769	5	Complicated		X		3	0.7
1612	8	Complicated	X			1	0.8
1114	8	Complicated		X		1	0.55
547	8	Complicated		X		1	0.86
2018	8	Complicated	X			1	0.75
1524	45	Complicated			X	3	0.3
290	45	Complicated	X			2	0.56
1835	1	Complicated		X		3	0.88
1176	30	Complicated	X			1	0.93
1242	30	Complicated		X		1	0.93
1500	30	Complicated		X		3	1
1605	30	Complicated	X			2	0.92
1267	30	Complicated			X	1	1
1214	30	Complicated		X		3	0.9
149	30	Complicated			X	3	0.9
1233	30	Complicated	X			3	1
1770	8	Complicated	X			3	0.58
C34D	8	Nasal carriage					0.2
C34D	5	Nasal carriage					0.2
C348	5	Nasal carriage					1
C34D	30	Nasal carriage					1
C93	15	Nasal carriage					0.1
C128	1	Nasal carriage					0.6

Abbreviations: B&J, bone and joint infection; Both, both bone and joint and endocarditis; CIG, Complicated Infection Group; Endo, endocarditis; ID, identification.

<sup>a</sup> Sequence data for the CIG genomes has been deposited in GenBank under PID (Pathway Interaction Database):

<sup>b</sup> 1, cured; 2, recurrent *S aureus* infection; 3, death due to *S aureus* infection.

<sup>c</sup> Proportion of 14 potential virulence genes more frequently associated with strains causing complicated infections [14]. This value is a fraction determined by X/14, where X = the number of virulence genes in the isolate.

PRJNA60651, PRJNA60653, PRJNA60655, PRJNA60657, PRJNA60659, PRJNA60661, PRJNA60663, PRJNA60665, PRJNA60667, PRJNA60669, PRJNA60671, PRJNA60673, PRJNA60675, PRJNA60677, PRJNA60679, PRJNA60681, PRJNA60683, PRJNA60685, PRJNA60687, PRJNA60689, PRJNA60691, PRJNA60693, PRJNA60695, PRJNA60697, PRJNA60699, PRJNA60701, PRJNA60703, PRJNA60705, PRJNA60707.

Reno, NV), along with 20 uninjected and PBS-injected controls. The frozen stock of each culture (prepared as described earlier) was thawed on ice and diluted in PBS to the desired number of *S aureus* cells in the inoculum (10 µL of inoculum containing either 10<sup>5</sup> or 10<sup>6</sup> CFUs of *S aureus*). The larvae were incubated at 37°C, observed 4 hours postinfection, and then every day for 5 days to monitor survival. Dead larvae were identified as those melanized and unresponsive to stimuli.

### RNA-Seq Transcriptome Analysis of Complicated Infection Group Isolates

Whole transcriptome analysis of the 29 MRSA CIG isolates, MRSA252, N315, and UAMS-1 was determined by RNA-Seq.

To prepare isolates for RNA extraction, an overnight culture of *S aureus* was inoculated into 100 mL fresh TSB in a 500 mL Erlenmeyer flask to an OD (600) to approximately 0.1. The flasks were incubated at 37°C/220 rpm to log-phase growth (OD 600 ~ 0.6). Total RNA was extracted by mechanical lysis in acid phenol [25], and rRNA was depleted by Ribo-Zero (Illumina, San Diego, CA) and sequenced with Illumina Tru-Seq. Low complexity reads, adapter, and vector contamination were removed using Seqclean (<http://sourceforge.net/projects/seqclean/>) and the National Center for Biotechnology Information (NCBI) UniVec database. Processed reads were then mapped to the MRSA252 genome with SHRiMP version 2.2.3 [26]. Differential expression analysis was performed using

Cufflinks (cuffDiff2) version 2.0.2 [27] of the general transfer format annotation file for the reference genome and a false discovery rate of 0.05. The RNA expression levels for each gene were expressed as fragments per kilobase per million reads mapped (FPKM). Genes in which all samples did not have at least an FPKM of 1 were removed from consideration before downstream analysis. A greater than log<sub>2</sub>-fold increase or decrease in expression level was used to identify genes that were significantly different between the CCs. A custom PERL program was used to determine overlap of significant differentially expressed genes between samples within and between the CCs at a variety of levels of agreement (50%, 80%). Heat maps and dendrograms were generated by per gene z-score that was computed from log<sub>10</sub> FPKM values or hierarchical clustering of log<sub>10</sub> FPKM values. Comparison of whole transcriptome expression between CCs was determined by multidimensional scaling (MDS) of the normalized compression distance. All RNA-Seq data (raw sequence reads and primary analysis) are deposited at NCBI and Sequence Read Archive under BioProject PRJNA255909.

### Statistical Analyses

For survival analysis in both animal models, the survival data were pooled together into 2 groups (CC30 vs others), and the Kaplan-Meier survival curve was generated using GraphPad Prism, version 5.0 (La Jolla, CA). The survival difference between strains was assessed using the log-rank: Mantel-Cox test. To identify genes uniquely associated with CC30 (Table 1), protein clusters of related function identified using JOC (Supplementary Table 1) were first sorted by a *t* test comparison of clusters present in CC30 vs all other CCs. All clusters with *P* < .05 were then evaluated across all 60 genomes to calculate proportion of genomes containing each cluster (expressed as percentage).

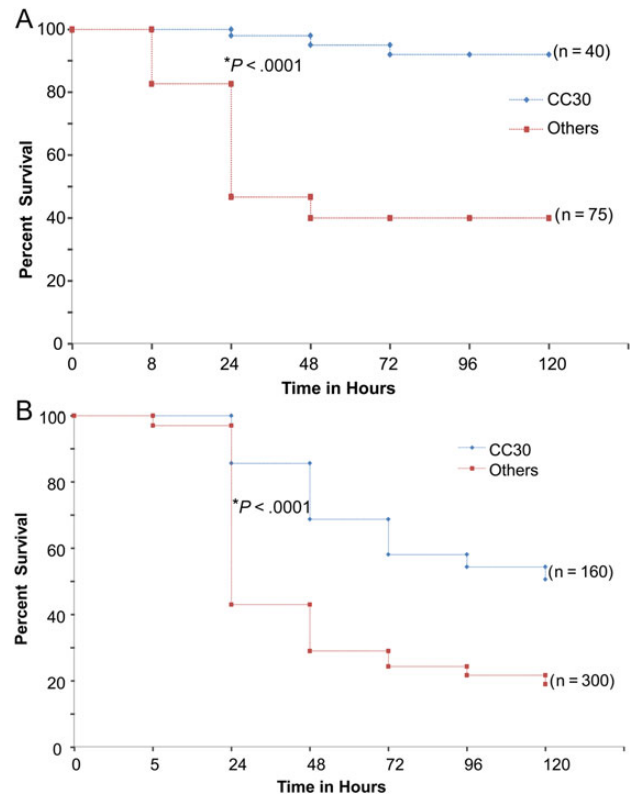
## RESULTS

### Attenuated Virulence of Clonal Complex 30 in Murine and *Galleria mellonella* Sepsis Models

When compared with all other CCs studied, CC30 isolates demonstrated significantly attenuated virulence in both the murine intraperitoneal sepsis (*P* < .0001; Figure 1A) and *G mellonella* sepsis models (*P* < .0001; Figure 1B). This attenuated virulence of CC30 persisted when alternate infective doses of *S aureus* (10<sup>6</sup> CFU instead of 10<sup>5</sup> CFU, data not shown) were used in *G mellonella* sepsis model.

### Phylogenetic Relationships Between the Clonal Complexes

Phylogenetic information included in the core genome alignments of the 29 CIG isolates and 31 closed *S aureus* genomes available in GenBank were used to determine the genomic relatedness of the CIG isolates. There is significant congruence

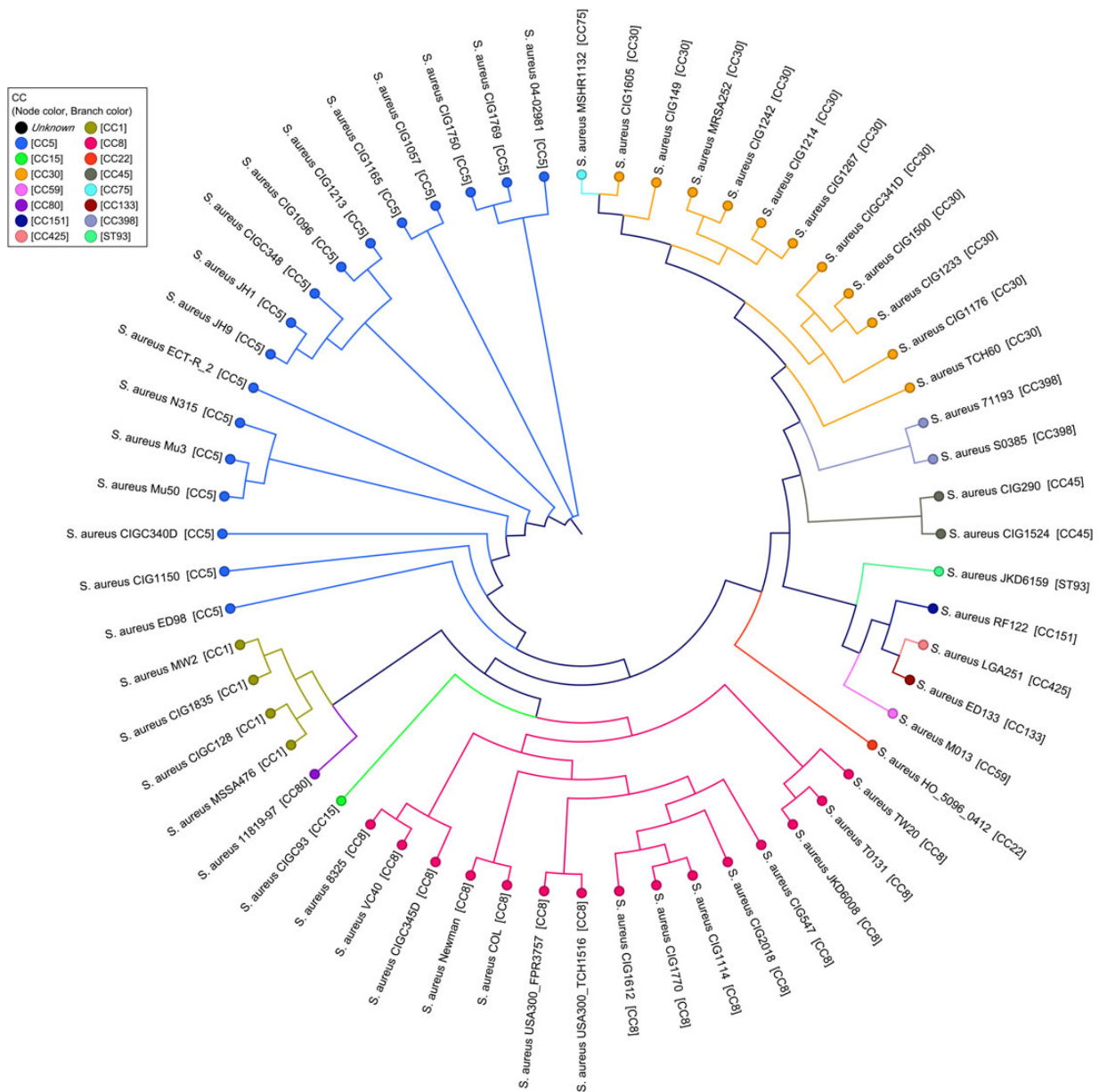


**Figure 1.** (A) Kaplan–Meier survival curve of C57BL/6J mice after infection with *Staphylococcus aureus* in the Complicated Infection Group (CIG). C57BL/6J mice were given intraperitoneal injections with 10<sup>8</sup> colony-forming units (CFUs) of each CIG *S aureus* isolates (n = 5 mice for each strain). Survival analysis was done using log-rank Mantel-Cox test in GraphPad prism. \*, *P* value (log-rank: Mantel-Cox test); clonal complex (CC) 30 strains include 1176, 1242, 1500, 1605, 1267, 1233, and 1214. Others include CC1 (1835), CC5 (1057, 1096, 1150, 1165, 1213, 1750, 1769), CC8 (1612, 1114, 547, 2018, 1770), and CC45 (1524, 290). (B) Kaplan-Meier survival curve of greater wax moth (*Galleria mellonella*) after infection with *S aureus* in the CIG. The greater wax moth (*G mellonella*) were given injections with 10<sup>5</sup> CFU of each CIG *S aureus* isolates (n = 20 worms for each strain). Survival analysis was done using log-rank Mantel-Cox test in GraphPad prism. \**P* value (log-rank: Mantel-Cox test); strains used are identical to those in A.

between the previously determined grouping by MLST into CCs and the current whole-genome phylogeny. As demonstrated by SNP whole-genome alignments (Figure 2) showing phylogenetic relationship between the CCs, all CC30 CIG isolates clustered into the same phylogroup, most closely related to the CC45 and CC398 lineages and most distant from CC5.

### Jaccard Orthologous Clustering Analysis to Distinguish Genome Features Unique to Clonal Complex 30 Isolates

Jaccard Orthologous Clustering analysis is typically used to group together highly similar proteins within a single genome/organism of interest and allows for one-to-many orthology. Comparisons of protein families between the 29 CIG genomes sequenced in this study (Table 1) and 31 additional



**Figure 2.** Maximum likelihood single-nucleotide polymorphism (SNP) tree for 60 *Staphylococcus aureus* isolates, representing relationships between clonal complexes (CCs) in the Complicated Infection Group (CIG) and previously sequenced *S aureus* genomes. Single nucleotide polymorphisms were identified in pairwise genome comparisons between the predicted genes in all genomes. Single nucleotide polymorphisms for each genome were concatenated to form SNP pseudosequences and used to generate a phylogenetic tree using the HKY93 algorithm [28] with 500 bootstrap replicates.

complete *S aureus* genomes available in the GenBank were used to identify genomic features that distinguished the CC30 lineage from the other predominant lineages. A total of 2300 proteins were present among all tested CCs. The CC30 isolates possessed a distinct set of proteins, including 25 proteins encoded in all CC30 strains and absent in greater than 90% of all other CCs (Table 2 and Supplementary Table 1). Twenty-six proteins were uniquely associated with CIG CC30 isolates but were absent in CC30 isolates MRSA252 and THC60 (contemporary CC30 reference clones [20]). Functions of these 26 proteins

suggested ongoing evolutionary changes among the CIG isolates. Twenty-eight proteins unique to CC30 were previously identified in islets of the MRSA252 genome [20].

### Single-Nucleotide Polymorphisms in Clonal Complex 30 Complicated Infection Group Isolates

The CIG genomes were compared with the contemporary CC30 reference strain, MRSA252, and the N315 (CC5) genomes (described in Materials and Methods) to identify specific SNPs potentially associated with unique phenotypic characteristics of

**Table 2. Proteins Identified by Jaccard Orthologous Clustering as Associated with *Staphylococcus aureus* CC30 Isolates**

Representative Protein ID in Cluster (LOCUS Name)	Representative MRSA252 Protein in Cluster (LOCUS Name)	Common Name	CC30 Average Number of Proteins in Cluster <sup>a</sup>	Present in All CC30 Strains and Absent in >90% of Other CC	Present in >50% of CC30 and Absent in >90% of Other CC	Present in CC30 CIG and Absent in MRSA252 or THC60	Absent in >50% of CC30 and Present in >90% of Other CC	Absent in >90% of CC30 and Present in >90% of Other CC	t Test CC30 vs Others
CIG1176_2319	SAR1587 <sup>+b</sup>	PfkB Carbohydrate Kinase Family Protein	1.0	X					1.97E-30
SAR0638	SAR0638 <sup>+</sup>	Hypothetical (Putative Membrane Protein)	1.0	X					1.97E-30
CIGC341D_1278	SAR0717	Bacterial Regulatory Helix-Turn-Helix, LysR Family Protein	0.9			X			1.38E-29
CIG1500_1282	SAR0721	Multicopper Oxidase <i>mco</i>	0.9			X			1.38E-29
CIG1267_1237	SAR0715	Glyoxalase	0.9			X			1.38E-29
CIG1267_1239	SAR0718	Conserved Hypothetical 698 Family Protein	0.9			X			1.38E-29
CIG1214_1043	A <sup>c</sup>	Pemk-Like Family Protein	0.9			X			1.38E-29
CIG1214_2795	A	Phosphoribosylformylglycinamide Synthase Subunit PurS	0.9			X			1.38E-29
CIG1233_343	SAR2591 <sup>+</sup>	LysE Type Translocator Family Protein	1.0	X					1.09E-22
SAR2592	SAR2592 <sup>+</sup>	Fatty Acid Desaturase	1.0	X					1.09E-22
HMPREF0772_10409	SAR0091 <sup>+</sup>	Insertion Sequence Protein	1.0	X					1.09E-22
CIG1500_1207	A	Putative Inorganic Diphosphatase	0.8			X			4.42E-21
CIGC341D_1257	A	Putative Membrane Protein	0.8			X			4.42E-21
CIG1267_2996	A	Putative Membrane Protein	0.8			X			4.42E-21
CIG1605_2630	A	FRG Domain Protein	0.8			X			4.42E-21
CIG149_1264	A	HAD Hydrolase, IA, Variant 1 Family Protein	0.8			X			4.42E-21
CIG1500_1272	A	HTH Domain Protein	0.8			X			4.42E-21
CIG1267_646	SAR0087 <sup>+</sup>	5-Methylcytosine-Specific Restriction Enzyme B Domain Protein	1.0	X					1.70E-18
CIG1500_647	SAR0088	McrBC 5-Methylcytosine Restriction System Component Family Protein	1.0	X					1.70E-18
SATW20_26470	A	ABC Transporter ATP-Binding Protein	0.0					X	1.70E-18
SAUSA300_0078	SAR0720	ATPase Copper Transport	0.9			X			4.96E-17
CIG149_961	SAR0368	DNA Binding , Excisionase Family Domain Protein	0.9			X			4.96E-17
SAB0348	SAR0372	Pathogenicity Island Protein	1.8	X					5.58E-17
CIG1769_494	A	ABC-2 Transporter Family Protein	0.0					X	1.01E-15
CIGC348_980	A	Beta-Grasp Domain Toxin Protein	0.0					X	1.01E-15
SAA6008_02763	SAR2788 <sup>+</sup>	Hypothetical (Putative Exported Protein)	1.0	X					1.01E-15
CIG1176_934	SAR2149 <sup>+</sup>	Hypothetical (Putative Exported Protein)	1.0	X					1.01E-15

Table 2 continued.

Representative Protein ID in Cluster (LOCUS Name)	Representative MRSA252 Protein in Cluster (LOCUS Name)	Common Name	CC30 Average Number of Proteins in Cluster <sup>a</sup>	Present in All CC30 Strains and Absent in >90% of Other CC	Present in >50% of CC30 and Absent in >90% of Other CC	Present in CC30 CIG and Absent in MRSA252 or THC60	Absent in >50% of CC30 and Present in >90% of Other CC	Absent in >90% of CC30 and Present in >90% of Other CC	t Test CC30 vs Others
CIG149_981	SAR0383	Abi-Like Family Protein	1.9	X					9.77E-15
CIG1176_1377	SAR0704	Bacteriophage CI Repressor Helix-Turn-Helix Domain Protein	0.9			X			2.81E-14
CIG1524_2315	SAR0697	Bacteriocin Export ABC Transporter	0.9			X			2.81E-14
ECTR2_1030	A	Haemolytic Family Protein	0.0					X	1.05E-13
CIG1165_915	SAR0395	Putative Membrane Protein	0.4					X	8.92E-13
CIG1605_357	A	Transposase	1.3			X			8.92E-13
CIG149_1454	SAR0838 <sup>+</sup>	Hypothetical (Putative Membrane Protein)	1.0	X					1.05E-13
SAA6008_02764	SAR2789 <sup>+</sup>	Subtilase Family Protease	0.9		X				2.60E-12
CIG1605_650	A	Acetyltransferase	0.9			X			2.60E-12
CIG1605_1279	SAR0723	Copper-Translocating P-Type ATPase	0.9			X			2.60E-12
CIG290_643	A	Arylamine N-Acetyltransferase Family Protein	0.9			X			2.60E-12
SAMSHR1132_23190	A	HTH-Type Transcriptional Regulator SarU	0.0					X	3.72E-12
CIG1605_203	SAR2451 <sup>+</sup>	Bacterial Regulatory S, TetR Family Protein	1.0	X					3.72E-12
HMPREF0772_10825	SAR2453 <sup>+</sup>	Multidrug ABC Superfamily ATP Binding Cassette Transporter Permease Protein	1.0	X					3.72E-12
CIG1214_1283	SAR0696	Conserved Hypothetical Family Protein	0.8		X				2.96E-11
CIG290_224	SAR2452 <sup>+</sup>	ABC Transporter Family Protein	1.0	X					6.43E-11
SAEMRSA15_23960	A	Putative Staphylococcal Accessory Regulator	0.0					X	6.43E-11
SAR0097	SAR0097 <sup>+</sup>	DNA-Binding Protein	1.0	X					6.43E-11
SATW20_01030	SAR0093 <sup>+</sup>	Hypothetical (Putative Membrane Protein)	1.0	X					6.43E-11
CIG1233_404	A	Putative Membrane Protein	0.7		X				1.68E-10
M013TW_0379	SAR2299	Putative Transcriptional Regulator	1.0	X					5.52E-10
CIG1176_846	SAR0287	Putative Transposase	1.0	X					6.68E-10
CIG1165_437	SAR2683	Putative Membrane Protein	0.2				X		9.32E-10
CIG1612_299	A	Prevent-Host-Death Family Protein	0.1				X		1.22E-09
SAB0782	A	Leukocidin Chain <i>lukM</i> Precursor	0.0					X	4.03E-09
CIG290_888	A	Virulence Factor EsxB Family Protein	0.0					X	4.76E-09

Table 2 continued.

Representative Protein ID in Cluster (LOCUS Name)	Representative MRSA252 Protein in Cluster (LOCUS Name)	Common Name	CC30 Average Number of Proteins in Cluster <sup>a</sup>	Present in All CC30 Strains and Absent in >90% of Other CC	Present in >50% of CC30 and Absent in >90% of Other CC	Present in CC30 CIG and Absent in MRSA252 or THC60	Absent in >50% of CC30 and Present in >90% of Other CC	Absent in >90% of CC30 and Present in >90% of Other CC	t Test CC30 vs Others
HMPREF0772_10238	SAR0261 <sup>+</sup>	Nitric Oxide Reductase	1.0	X					4.76E-09
CIG1267_2788	A	Endodeoxyribonuclease RusA Family Protein	0.6		X				6.03E-09
SAR0724	SAR0724	Cadmium Efflux System Accessory Protein	0.9			X			1.11E-08
CIG1233_2758	A	Helix-Turn-Helix Domain Protein	0.8			X			1.35E-08
CIG149_1032	A	Toxic Shock Syndrome Toxin-1	0.8			X			1.35E-08
CIG1214_1405	SAR0824	Malic Enzyme, NAD Binding Domain Protein	1.0	X					2.54E-08
CIG1176_970	A	Helix-Turn-Helix Family Protein	0.9			X			7.02E-08
CIGC128_313	A	Surface Protein G	0.0				X	X	3.42E-07
SAT0131_00223	SAR0228 <sup>+</sup>	Glutamine Amidotransferase Class-I	1.0	X					3.42E-07
CIG1176_657	SAR0098	Acetyltransferase Family Protein	1.0	X					3.84E-07
CIG1267_946	A	Phage Integrase Family Protein	0.8		X				6.68E-07
CIG1214_965	A	DNA Binding, Excisionase Family Domain Protein	0.8			X			6.68E-07
CIG1835_198	SAR2774	Collagen Adhesin	1.0	X					1.18E-06
CIGC341D_1691	A	Seryl-tRNA Synthetase	0.5		X				1.59E-06
SAA6008_00068	SAR0092 <sup>+</sup>	Amidohydrolase	1.0	X					2.23E-06
CIG1057_526	A	Putative Membrane Protein	0.0					X	3.19E-06
CIG1233_2421	SAR1826	ATP-Binding Protein	0.9			X			4.11E-06
SAOV_2044	A	Drug/Metabolite Transporter Permease	0.0					X	7.12E-06
CIG290_550	SAR2748	Intracellular Adhesion Protein D	0.2				X		1.03E-05
CIG1057_701	A	Bacterial Transferase Hexapeptide Family Protein	0.0				X	X	3.36E-05
CIG1769_827	A	O-Antigen Ligase Family Protein	0.0				X	X	3.36E-05
ECTR2_116	A	Capsular Polysaccharide Synthesis Protein Cap5K	0.0				X	X	3.36E-05
SARLGA251_01310	A	Capsular Polysaccharide Synthesis Enzyme	0.0				X	X	3.36E-05
CIGC348_1890	A	MAP Domain Protein	0.0					X	7.11E-05

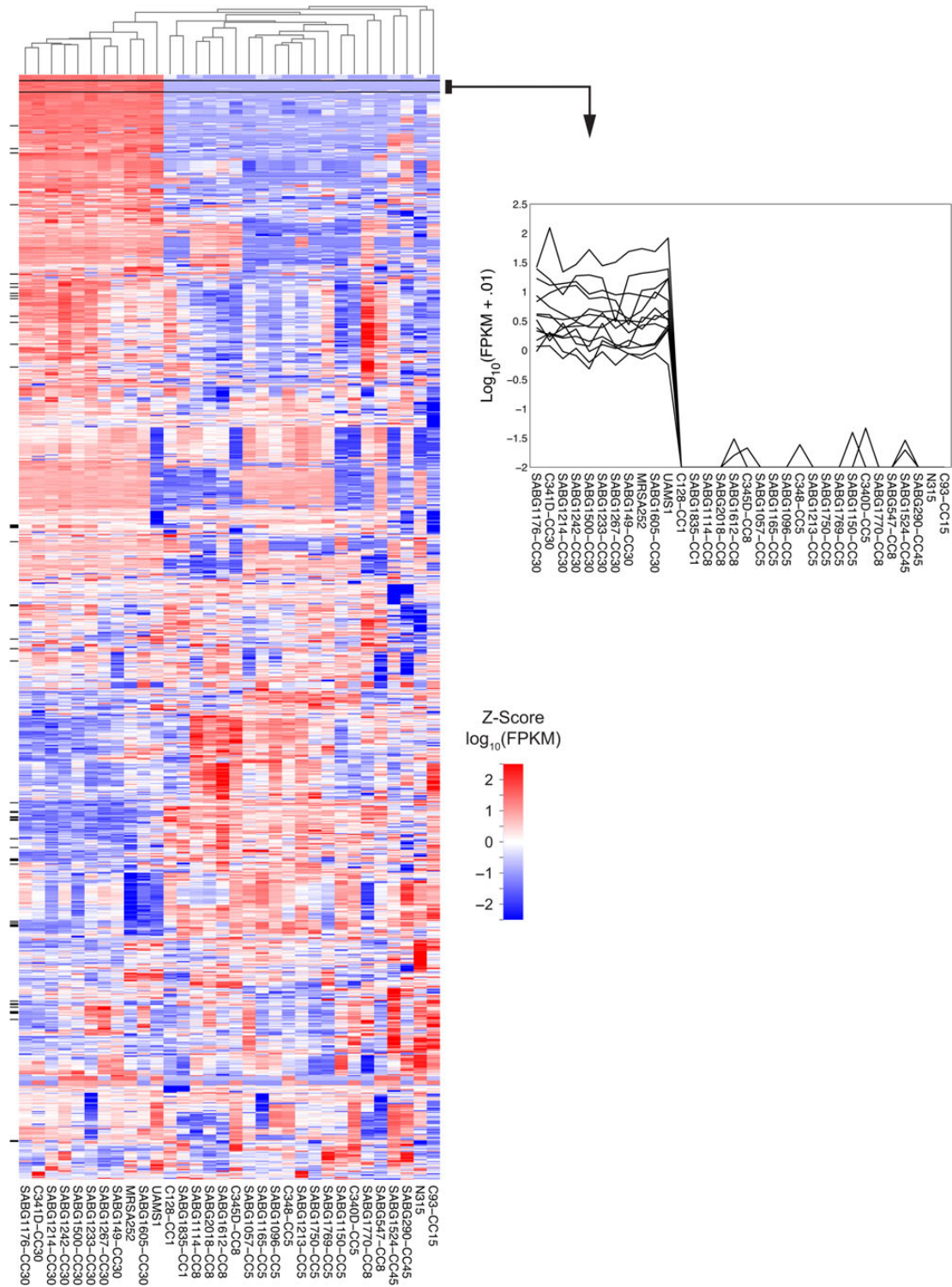
Abbreviations: CIG, Complicated Infection Group; ID, identification.

<sup>a</sup> Average number of proteins in cluster was determined by  $X/11$ , where  $X$  = total number of proteins in clusters identified in all CC30 included in the comparison and 11 = total number of CC30 isolates included in the comparison (Supplementary Table 2).

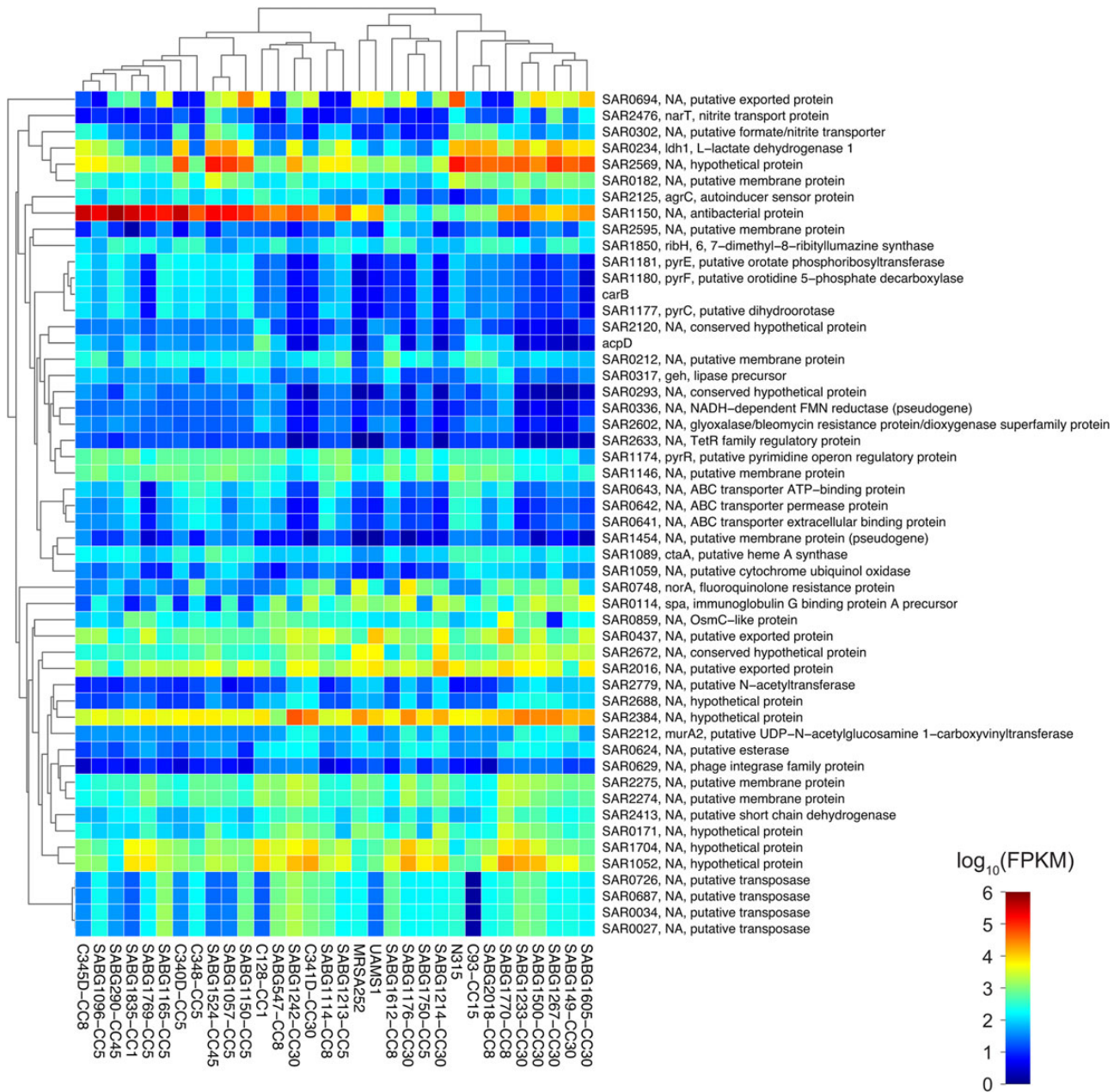
<sup>b</sup> +, within genomic islets of MRSA252.

<sup>c</sup> A, not in MRSA252.





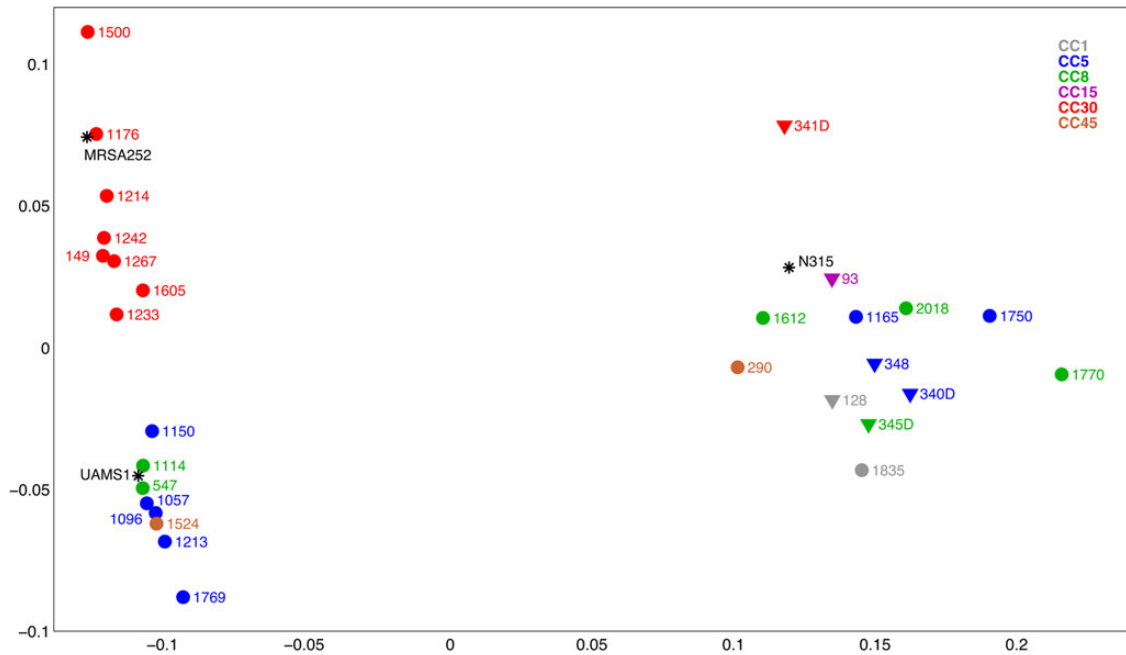
**Figure 3.** Heat map of RNA-Seq transcriptome analysis for 1259 selected genes from the *Staphylococcus aureus* Complicated Infection Group (CIG) and reference strains. Total RNA was extracted from log-phase cultures (optical density, 600 ~ 0.6), sequenced, and analyzed as described in Materials and Methods. Genes that were identified as significantly different between the clonal complexes (CCs) had fragments per kilobase per million reads mapped (FPKM) values of at least 1 and a greater than  $\log_2$ -fold increase or decrease in expression level. The heat map shows  $\log_{10}$  FPKM values for 1259 selected genes (rows) and 32 samples (columns). Color corresponds to per-gene z-score that is computed from  $\log_{10}$  FPKM (after adding 0.01). Genes were omitted from the original list of 2807 if the per-gene variance was less than 0.05 (low-variability genes) or if the FPKM expression was less than 10 for all 32 samples (uniformly, low-expressed genes). Based on these criteria, approximately 45% of the genes are shown. Genes and samples were hierarchically clustered based on Euclidean distance of z-score data and average linkage (dendrogram not shown for genes). Tick marks to the left of the heat map indicate genes in Figure 4. The figure on the right shows data expanded for 13 genes in cluster near top of heat map indicate FPKM expression of genes in CC30 relative to other CCs.



**Figure 4.** Heat map of RNA-Seq transcriptome analysis for 52 selected genes from the *Staphylococcus aureus* Complicated Infection Group (CIG). The heat map shows  $\log_{10}$  fragments per kilobase per million reads mapped (FPKM) values for 52 selected genes indicated by tick marks in Figure 3 (rows) and 32 samples (columns). Color corresponds to per-gene z-score that is computed from  $\log_{10}$  FPKM (after adding 0.01). Genes and samples were hierarchically clustered based on Jensen-Shannon divergence distance using non-log FPKM data (after adding 1) and average linkage (dendrogram not shown for genes). Abbreviation: CC, clonal complex.

the CC30 lineage. When compared with MRSA252, nonsynonymous SNPs were identified among 131 unique open reading frames (ORFs) in the 9 CC30 CIG isolates (Supplementary Table 2). Stop codons were identified in 5 unique ORFs, and 42 SNPs were identified in intergenic regions of the CC30 CIG genomes. Thirty-five synonymous and nonsynonymous SNPs were present in all CIG CC30 isolates when compared with MRSA252. Similar to the previous analysis of the historic

and contemporary CC30 lineage [17–20, 29], SNPs were identified in the following: (1) *agrC* (G→A) that predicts a Gly-to-Arg substitution at residue 55 (*agrC*<sub>G55R</sub>); (2) *hla*, where a premature stop codon is introduced; and (3) PSM $\alpha$ 3, which results in lower cytolytic and chemotactic activity. The ratio of nonsynonymous to synonymous SNPs (90:64 from a total of 154 SNPs) (Supplementary Table 2) across all CC30 isolates relative to MRSA252 through the entire genome suggests



**Figure 5.** Multidimensional scaling of transcriptome data for the *Staphylococcus aureus* Complicated Infection Group (CIG) isolates and reference strains. Multidimensional scaling was performed using a Jensen-Shannon divergence sample-sample distance matrix using fragments per kilobase per million reads mapped (+1) values for 2807 available genes. The first 2 coordinates are shown. Circles are CIG isolates from complicated infections and triangles are healthy controls (nasal carriage isolates). Clonal complex is indicated by color (see key). Reference *S aureus* strains (UAMS-1, MRSA252, and N315) are black asterisks.

continuing evolutionary divergence or adaptation in this lineage. Further details of SNPs identified in the CC30 CIG genomes are listed in [Supplementary Table 2](#).

### Transcriptomic Analysis of the Complicated Infection Group Isolates

To test the hypothesis that transcriptome level differences in the CIG isolates were associated with differences in virulence and fitness, we conducted RNA-Seq whole transcriptome analysis of the 29 CIG isolates, MRSA252, N315, and UAMS-1 (a CC30 osteomyelitis isolate and a commonly used laboratory strain [22]). Our analysis included the following: (1) identification of strain-specific transcriptional differences within and between CCs (Figures 3 and 4) and (2) MDS (Figure 5) of all CIG isolates to evaluate overall transcriptional differences between the CCs. The CC30 CIG clinical isolates and MRSA252 share a common transcriptome, whereas the CC1, CC5, CC8, CC15, and CC45 randomly cluster into 3 distinct transcriptome groups. The transcriptome of UAMS-1 is distinct from the CC30 CIG strains and MRSA252. Genes with higher expression levels in CC30 relative to all other CC examined in our study include protein A (*spa*), multiple putative membrane proteins (SAR2274, SAR2275), and multiple putative exported proteins (SAR2016, SAR0437, and SAR0694). Genes with lower expression levels in CC30 relative to all other CC include the following (Figure 4):

(1) several encoding metabolic functions, such as multiple members of the pyrimidine biosynthesis pathway [Carbamoyl-phosphate synthase large chain(*carB*), Dihydroorotase (*pyrC*), Bifunctional protein(*pyrR*), Orotate phosphoribosyltransferase (*pyrE*), and Orotidine 5'-phosphate decarboxylase(*pyrF*)]; (2) 3 genes [ABC transporter extracellular binding protein (SAR0641), ABC transporter permease protein(SAR0642), and ABC transporter ATP-binding protein(SAR0643)] encoding an operon similar to a *Staphylococcus epidermidis* iron repressible ABC transport system; and (3) an azoreductase (*acdD*). Comparison within the CC30 isolates identified 38 genes (excluding ribosomal proteins and transposases) that are differentially expressed within this CC (data not shown). These are primarily metabolic genes, including genes that participate in lactate metabolism (L-lactate dehydrogenase [*ldh1*] and L-lactate permease [*lldP2*]) and riboflavin biosynthesis (riboflavin biosynthesis protein [*ribA*], bifunctional riboflavin biosynthesis protein [*ribD*], riboflavin synthase alpha chain [*ribE*], and 6,7-dimethyl-8 ribityllumazine synthase [*ribH*]).

### DISCUSSION

Previous studies have shown that the *S aureus* CC30 lineage is associated with an increased risk for hematogenous complicated infections, including endocarditis, septic arthritis, vertebral

osteomyelitis [8, 14, 15], and persistent bacteremia [30]. The current study sought to evaluate this association by genotype and severity of infections using in vivo infection models, comparative genome sequencing, and transcriptome analysis.

One intriguing finding from this study is the diminished virulence phenotype of the CC30 isolates in the animal sepsis models. This outcome is in variance with our previous results showing an association of CC30 with severe human infections [8], but it is in agreement with a recent report from DeLeo et al [17] where contemporary CC30 isolates displayed attenuated virulence in mouse sepsis model. The underlying basis for these differences can be attributed to either the host background or virulence potential of the CC30 isolates. Infections caused by CC30 occurred in a hospital setting, with individuals having existing risk factors for infection. The association of CC30 with bloodstream infections and persistent bacteremia suggests that they are adapted to long-term colonization and persistence, resulting in a high colonization burden in the human host [8, 31]. This is supported by recent data from Cheung et al [29], who demonstrates that the SNP in CC30 PSM $\alpha$ 3 contributes to an attenuated proinflammatory potential in this lineage. The variance can also be attributed use of the mouse as a surrogate for human infections. The cardiovascular physiology of mice is distinct from humans, which compromises their use as models to assess pathogenesis and mechanisms of immune protection against *S aureus* infection. Future studies will use a rabbit model, whose immune and cardiovascular systems are more similar to humans [32].

The JOC analysis demonstrated that the CC30 isolates possess a distinct set of core proteins, with 25 proteins uniquely associated with CC30 isolates. Examples include a nitric oxide reductase (Nor) and fatty acid desaturase (Fad), which likely contribute to protection against production of radical nitric oxide (NO) by activated phagocytes and changes in membrane fluidity, respectively. CC30 Nor is a homolog of *Neisseria meningitidis* MC58 *norB*, which is essential for persistence in the nasopharynx and evasion of macrophage response [33]. All lineages of *S aureus* are capable of metabolically adjusting to nitrosative stress by expressing an NO-inducible L-lactate dehydrogenase (*ldh1*) and a NO-detoxifying flavohemoglobin (*hmp*) and do not require Nor for this protective response [34]. However, recent work by Lewis et al [35] on CC30 Nor suggests that it contributes to NO-dependent respiration during nitrosative stress, potentially enhancing protection from NO in the CC30 lineage. The *S aureus* Fad is a homolog of a fatty acid desaturase from *Bacillus subtilis*, Des, which is involved in membrane adaptation during cold shock, decreased daptomycin susceptibility, and contributes to long-term survival in vitro [36]. In a similar manner, the *S aureus* Fad may play a role in maintaining membrane functions and contribute to the development of persistent infections. It is noteworthy that homologs of Fad are found in isolates of *S epidermidis* and

*Staphylococcus capitis*, suggesting that *fad* was acquired through lateral gene transfer from these commensal staphylococcal species associated with endocarditis and bloodstream infections [37, 38]. Functional characterization of CC30 strains with targeted deletions of *fad* is needed to determine its role in multiple staphylococcal species.

The potential association between the transcriptome of CIG CC30 isolates to virulence and fitness were assessed by RNA-Seq transcriptome analysis of the 29 CIG isolates and reference strains MRSA252, N315, and UAMS-1. The CC30 isolates segregated into a transcriptome cluster that is distinct from all other CCs (Figure 5), which suggests that the transcriptome is a distinguishing feature of the CC30 that contributes to the attenuated virulence and persistence of this lineage. It is noteworthy that UAMS-1, a commonly used laboratory strain of CC30, has transcriptional features distinct from the CIG CC30 clinical isolates. A significant feature of the CC30 transcriptome is increased expression of protein A, a consequence of *agr*C<sub>G55R</sub> and defective Agr global regulatory system. Binding of protein A to different subclasses of immunoglobulins inhibits the B-cell responses during infection, interferes with the development of protective immunity [39–41], and leads to the overall immune suppression against staphylococcal infections [42]. A diminished immune response in healthcare-associated *S aureus* infections may contribute to persistence and survival of CC30, leading to chronic complicated infections [17].

Down-regulation of genes in the pyrimidine biosynthetic pathway (*carB*, *pyrR*, *pyrE*, and *pyrF*) (Figure 4) and subsequent decreases in levels of thymidine in the CC30 lineage are suggestive of thymidine-dependent small colony variants (SCVs) [43]. Other aspects of CC30 that are similar to thymidine-dependent SCVs include an Agr negative phenotype and increased levels of protein A. Thymidine-dependent SCVs also have decreased levels of *hla* transcription, whereas the *hla* in CC30 has a stop codon at amino acid (Glutamine 113: Q113Stop), making it nonfunctional. Our analysis suggests that the CC30 lineage shares some of the key metabolic features of SCVs and may be able to more quickly transition to a SCV-like bacterium.

## CONCLUSIONS

In summary, we have previously demonstrated that the CC30 lineage is distinguished by its ability to persist in the human host where it can initiate complicated infections such as infectious endocarditis and osteomyelitis. The inability of CC30 to cause sepsis-induced mortality in animal models suggests that this lineage has acquired genetic features including increased expression of protein A, which, in concert with the accumulation of SNPs in several virulence genes and regulators (eg, *agrC*, *hla*, *psm $\alpha$ 3*), may be of strategic significance for CC30 isolates to better persist in and colonize the human host by escaping the immune response and attenuating its virulence until the

conditions become favorable for bacteremia and subsequent hematogenous seeding. In addition, genes unique to CC30 such as *nor* and *fad* and decreased expression of metabolic genes responsible for production of SCVs may contribute to immune evasion and long-term survival of these strains in the endocardial and osteoarticular host environments. Further analysis, including transcriptome profiling using in vivo models and functional analysis of the genes unique to CC30, is needed to validate the molecular mechanisms responsible for the persistent phenotype.

## Supplementary Material

Supplementary material is available online at *Open Forum Infectious Diseases* (<http://OpenForumInfectiousDiseases.oxfordjournals.org/>).

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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