Analysis of the Small RNA Transcriptional Response in Multidrug-Resistant *Staphylococcus aureus* after Antimicrobial Exposure

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The critical role of noncoding small RNAs (sRNAs) in the bacterial response to changing conditions is increasingly recognized. However, a specific role for sRNAs during antibiotic exposure has not been investigated in *Staphylococcus aureus*. Here, we used Illumina RNA-Seq to examine the sRNA response of multiresistant sequence type 239 (ST239) *S. aureus* after exposure to four antibiotics (vancomycin, linezolid, ceftobiprole, and tigecycline) representing the major classes of antimicrobials used to treat methicillin-resistant *S. aureus* (MRSA) infections. We identified 409 potential sRNAs and then compared global sRNA and mRNA expression profiles at 2 and 6 h, without antibiotic exposure and after exposure to each antibiotic, for a vancomycin-susceptible strain (JKD6009) and a vancomycin-intermediate strain (JKD6008). Exploration of this data set by multivariate analysis using a novel implementation of nonnegative matrix factorization (NMF) revealed very different responses for mRNA and sRNA. Where mRNA responses clustered with strain or growth phase conditions, the sRNA responses were predominantly linked to antibiotic exposure, including sRNA responses that were specific for particular antibiotics. A remarkable feature of the antimicrobial response was the prominence of antisense sRNAs to genes encoding proteins involved in protein synthesis and ribosomal function. This study has defined a large sRNA repertoire in epidemic ST239 MRSA and shown for the first time that a subset of sRNAs are part of a coordinated transcriptional response to specific antimicrobial exposures in *S. aureus*. These data provide a framework for interrogating the role of staphylococcal sRNAs in antimicrobial resistance and exploring new avenues for sRNA-based antimicrobial therapies.

Over the past 50 years, *Staphylococcus aureus* has evolved resistance to almost all classes of antibiotics, highlighted by the evolution of methicillin-resistant *S. aureus* (MRSA) (1) and, more recently, vancomycin resistance (2). A limited range of antibiotics are available to treat serious MRSA infections, including vancomycin, and a number of new agents, such as linezolid (an oxazolidinone), ceftobiprole (a new β -lactam active against MRSA), and tigecycline (glycylcycline family). Globally, multilocus sequence type 239 (ST239) *S. aureus* has been a dominant multidrug-resistant clone of MRSA, including in Australasia for more than 30 years (3–5). We have recently reported the complete DNA sequence of an Australasian ST239 *S. aureus* strain (6), providing a basis to employ omic approaches and understand the success of this clone, including its tendency toward multidrug resistance.

The significance of small regulatory RNAs (sRNAs) in the context of bacterial regulation is becoming increasingly apparent. These molecules (~50 to 500 nucleotides [nt] in length) are involved in the regulation of a wide range of physiological responses, reacting to environmental signals, such as pH or temperature shifts (7), and can act by modulating transcription, translation, mRNA stability, DNA maintenance, or silencing. They can help modulate changes in cellular metabolism to optimize utilization of available nutrients and improve the probability for survival, as well as contributing to virulence (8–10). While initial discovery studies of bacterial sRNAs relied heavily on bioinformatic predictive searches of intergenic regions (11–14), more recently deep sequencing technologies have allowed direct detection of putative regulatory sRNAs within bacterial transcriptomes (15, 16). The full extent of sRNAs in bacterial chro-

mosomes is unclear, especially in *S. aureus*, where the study of sRNAs is relatively recent.

The best studied sRNA in *S. aureus* is RNAIII, a key quorum-sensing regulator of virulence that acts by an RNA-RNA binding mechanism (17). Recent studies of sRNAs in *S. aureus* have defined 10 to 20 sRNAs with limited exploration of their functional roles and regulatory profiles (8, 9, 11, 14, 18). These studies have, however, demonstrated the significant roles played by selected sRNAs in *S. aureus* metabolism and virulence. Recently, using high-throughput transcriptomics, 195 putative sRNAs encoded on the chromosome and plasmid of the Japanese MRSA strain N315 were described, demonstrating for the first time that the number of sRNAs present in *S. aureus* is much higher than previously defined (16).

Despite the important role played by sRNAs in adaption to environmental conditions, no studies have examined the sRNA transcriptional responses to antibiotic exposure in *S. aureus*. Re-

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cently, Yu and Schneiders identified four sRNAs that were up-regulated after tigecycline or tetracycline exposure in *Salmonella enterica*, suggesting that sRNAs may play an important role in the bacterial response to antimicrobials altering ribosome metabolism (19). Here, we hypothesized that selected sRNAs constitute a critical component of the *S. aureus* response to antimicrobial exposure. To evaluate that hypothesis, we used Illumina RNA-Seq analysis and performed an extensive analysis of sRNA transcriptional profiles in a vancomycin-susceptible clone of MRSA (JKD6009) and its *in vivo*-derived vancomycin-intermediate derivative (JKD6008) (20, 21) after exposure to sub-MIC amounts of molecules from four major classes of antibiotic. The complex nature of the data generated provided challenges for comprehensive and meaningful analysis, and so, in addition to traditional transcriptional expression analysis, we utilized a type of multivariate analysis that employs nonnegative matrix factorization (NMF). This is a clustering algorithm that operates on nonnegative data. NMF was initially introduced by Lee et al. as an unsupervised, parts-based learning paradigm in which a nonnegative matrix is decomposed into two nonnegative matrices by a multiplicative updates algorithm (22). Recently, NMF has been introduced to a range of fields in computational biology, including molecular pattern discovery, functional characterization of genes, and biomedical informatics, including transcriptional data (23–25). This approach allows reduction of dimensions of complex expression data from thousands of genes to a small number of metagenes and allows identification of distinct molecular patterns, or “molecular logic” (23).

In this study, we have identified over 400 potential sRNAs in ST239 *S. aureus* and, importantly, used NMF to uncover a previously unknown coordinated and specific sRNA transcriptional response to antimicrobial exposures. These data provide evidence for the role of sRNAs in the *S. aureus* response to antimicrobials and provide opportunities for exploring new avenues for sRNA-based therapies.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and antibiotic susceptibility. Staphylococcal strains JKD6009 (vancomycin-susceptible *S. aureus* [VSSA]) and JKD6008 (vancomycin-intermediate *S. aureus* [VISA]; derived *in vivo* from JKD6009) were stored in glycerol broth at -80°C and subcultured twice onto horse blood agar (Oxoid) before being used for any experiment. The MICs for vancomycin, ceftobiprole, linezolid, and tigecycline were evaluated by performing microbroth liquid serial dilution of antibiotics. *Staphylococcus aureus* strains were grown in Mueller-Hinton broth (MHB) (Difco) at 37°C , after a 1:100 dilution of overnight broth culture.

RNA preparation. Twenty individual RNA preparations were performed using methods previously described (16). Briefly, total RNA was extracted from strains JKD6009 and JKD6008 after cultures in MHB (Difco) for 2 h and 6 h with shaking, after no antibiotic exposure, or after exposure to $0.5\times$ the MIC of ceftobiprole, vancomycin, linezolid, or tigecycline for 10 min, using the RNeasy kit (Qiagen). Before sequencing experiments, growth profiles of each strain were monitored with and without antibiotic exposure to confirm that the treatments did not affect the bacterial growth. In these experiments, exposure times of greater than 10 min significantly impacted bacterial growth. In previous transcriptional studies assessing the impact of antibiotics on *S. aureus*, exposure times of 5 min (26) and 10 min (27, 28) have been successfully used; therefore, a short exposure time of 10 min was employed in this study. RNAs were then treated with the MICROBExpress kit (Ambion) to limit the contamination by rRNAs, following the manufacturer's recommen-

dations. At each step, RNA quality was controlled by Bioanalyzer (Agilent) using the RNA Nano Chips, and quantity was assessed using the ND-8000 (NanoDrop Technologies).

Library construction and Illumina RNA-Seq. Extracted RNA was prepared to enrich for mRNA or sRNA as follows. For mRNA preparations, initial fragmentation using zinc was followed by treatment with first Antarctic phosphatase and then with PNK enzyme. Specific tags were ligated before reverse transcription (RT) to conserve strand orientation. Single-stranded ligation of the 3' Illumina v1.5 adapter was followed by single-stranded ligation of 5' bar-coded adapter. Reverse transcription and PCR amplification were performed to generate a cDNA colony template library, and gel purification with selection of fragments of 20 to 300 nt was performed. Quality control of the DNA colony template library was performed by cloning an aliquot into a TOPO plasmid and sequencing 8 clones. The same process was used for sRNA preparation, without the zinc fragmentation and using a size selection of 20 to 200 nt. The 40 cDNA libraries were sequenced twice using a whole flow cell (8 lanes) in an Illumina genome analyzer GAII for 36 cycles.

Read mapping and visualization, identification of noncoding RNA transcripts, and comparative expression analysis. The reads were mapped onto the annotated JKD6008 reference genome (6) (GenBank accession no. CP002120.1) in a strand-specific manner using MAQ software (29) and SHRIMP 2.2.2 (30). The reference genome was searched for characterized noncoding RNAs using Infernal 1.2 (31) against the Rfam 10.0 database (32). A detailed manual examination was performed by viewing jointly the strand-specific aligned reads, the existing JKD6008 gene annotations, and the predicted Rfam features in Artemis 12.0 (33). The read mapping allowed computation of a sequencing coverage that reflects the number of reads covering each nucleotide of the genome. Coverage profiles were computed separately relating to each condition. For visualization and analysis of coverages, we used the Artemis genome viewer (34) and the IGV software (35), allowing us to study expression of sRNAs and mRNAs in their genomic context. Detection of sRNA (cutoff value a minimum of 15 reads overlapped in one defined position) was performed visually from a merged coverage of all conditions (time point, strain, and treatment) specific to sRNA sequencing runs. *trans*-encoded small RNAs were annotated when expression signal was detected in an intergenic region and was independent of the expression of the flanking annotated genes. Antisense RNA molecules corresponded to a transcription signal encoded from the opposite strand of the annotated gene (coding sequence [CDS]). 5'- and 3'-untranslated regions (UTRs) displaying a substantial size (more than 30 to 50 nt longer than the gene annotation) were also annotated. Riboswitches were identified by comparison with the Rfam database and literature.

Alignment of RNA-Seq reads from each condition provided counts (reads) for each sRNA and mRNA that were proportional to their transcription level. Counts were normalized using the trimmed-mean normalization function in EdgeR, part of the BioConductor package (36). For the mRNA samples, this was based on counts of reads aligning to CDS features. For the sRNA, it was based on counts of reads aligning to sRNA features. Reads that aligned equally well to several locations were assigned at random to one location. Statistically significant differences in sRNA or mRNA transcription between conditions were determined using Voom and Limma (37). Voom log transforms RNA-Seq gene counts into microarray-like intensities which can be robustly analyzed using moderated *t* tests and *F* tests in Limma. These tests estimate the within-group variation as an optimized blend of variation observed for each gene and variation averaged over all genes. Linear models were fitted to test the impact of time, strain, or antibiotic exposure on expression. Moderated *t* tests and *F* tests were used to obtain the significance levels for different terms (and sets of terms) in the model for each feature. The false discovery rate (FDR) was used to correct for multiple testing within each of these. Differential expression was considered statistically significant if the FDR was <0.05 .

Heat maps were created based on $\log_2$ -transformed expression levels. To be included in the heat map, genes were required to have at least 10

alignments totaled over all samples and the standard deviation (SD) of the $\log_2$ expression levels had to exceed 2.

NMF. The NMF analysis was performed using a tool which we make available as the “nmf” tool in the Nesoni tool suite (<http://www.vicbioinformatics.com/software.nesoni.shtml>), which makes use of the Lee algorithm (38) as implemented in the R+ package NMF (39).

The starting point is a matrix of normalized counts as described above. We discard any gene with fewer than 10 reads in total. This gives a matrix, V , of normalized counts with genes as rows and samples as columns, with size $n_{\text{genes}} \times n_{\text{samples}}$. We will approximate this matrix with two smaller matrices, W and H , such that $V \approx WH$.

Given a desired number of classes (the rank of the approximation), the size of W is $n_{\text{genes}} \times n_{\text{classes}}$ and the size of H is $n_{\text{classes}} \times n_{\text{samples}}$. W and H are constrained to only contain nonnegative values, and the columns of W are constrained to sum to 1. They are chosen to minimize a row-weighted sum of squares e of the values in the error matrix

$$E = V - WH$$

$$e = \sum_{ij} \left(\frac{E_{ij}}{w_i} \right)^2$$

Weights are chosen for each row as a smooth function of the row average so that each gene contributes roughly equally to the weighted sum of squares. This is achieved using an iterative algorithm. The initial weighting, w , is the row averages of V . An NMF factorization is found using this weighting, and a new weighting is chosen as a loess approximation to the root mean square of the rows of E as a function of the row averages of V . This process is then iterated several further times to allow the weights to stabilize.

The matrices W and H can be interpreted as n_{classes} classes, with the strength of membership of each gene in each class represented by W and the strength of membership of each sample in each class represented by H .

A spreadsheet containing information about the genes in the each rank 8 class for sRNA and rank 6 class for mRNA was generated. The “weight” column shows the degree of membership of each gene in this class, with weight totaling to 1. Also shown is the degree of membership in the other classes. For those elements of the gene weight matrix that were nonzero, we assessed whether they could be said to be significantly greater than zero, as follows. For simplicity we assume the elements that were assigned zero by the NMF algorithm are correctly assigned as such. For each gene, a linear model was fitted by least squares using the rows of the sample weight matrix H corresponding to nonzero elements in the row corresponding to the gene in the gene weight matrix. The fitted coefficients exactly reconstruct that row of the gene weight matrix W . t tests were then used to calculate P values for each coefficient being greater than zero. A false discovery rate correction was applied within each class.

RNA-sequencing data accession number. The sequence reads have been deposited in the GEO database under study accession no. [GSE40864](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE40864).

RESULTS AND DISCUSSION

***S. aureus* strain characteristics.** The reference genome for accurate read mapping of the RNA-Seq data was *S. aureus* JKD6008, which is a vancomycin-intermediate *S. aureus* (VISA) derivative of ST239 vancomycin-susceptible *S. aureus* (VSSA) strain JKD6009 (6). We have previously investigated the mutations present in JKD6008 compared to JKD6009 and found only 10 single nucleotide substitutions (40). The MICs of JKD6008 and JKD6009 were determined for the antibiotics used in this study and were as follows: ceftobiprole, 2 $\mu\text{g/ml}$ for both; linezolid, 4 $\mu\text{g/ml}$ for both; vancomycin, 4 $\mu\text{g/ml}$ for JKD6008 and 1 $\mu\text{g/ml}$ for JKD6009; and tigecycline, 0.5 $\mu\text{g/ml}$ for both.

Transcriptional profile of ST239 MRSA using RNA-Seq to define sRNAs. There are three main categories of sRNAs in bacteria, including antisense RNAs (*cis*-encoded RNAs) and *trans*-

encoded RNAs that act as independent transcripts and riboswitches, which are the 5' part of the mRNA they regulate. The mRNA and sRNA transcriptional profiles of JKD6008 and JKD6009 was determined using Illumina RNA-Seq during *in vitro* growth after no antibiotic exposure and after exposure to cell wall active antibiotics (ceftobiprole and vancomycin) and antibiotics where the ribosome is the target (linezolid and tigecycline). These antibiotics were selected, as they are “last-line” agents for treatment of multiresistant *S. aureus* infections. In addition, because expression of small RNAs is generally regulated during bacterial growth, we measured transcriptional profiles at the beginning (2 h) and the end (6 h) of exponential growth. We therefore performed 40 independent RNA-Seq experiments comprising two strains (JKD6009 and JKD6008), two time points (2 h and 6 h), 5 antibiotic treatments (no antibiotics or vancomycin, ceftobiprole, linezolid, or tigecycline), and 2 independent sample preparations (sRNA and mRNA).

Read mapping against the JKD6008 chromosome was performed with between 7.1×10^5 and 6.9×10^6 reads aligned to the reference for each of the 40 samples. We validated the method by controlling the adequate orientation of the protein-coding transcripts on annotated CDS. The profiles of normalized, strand-specific, mapped reads were visually inspected along the JKD6008 chromosome, including nonannotated regions, and 409 putative sRNAs were identified. The sRNA content of JKD6008 defined in this study is presented in full in Data Set S1 in the supplemental material and is named in sequential order. Among these 409 potential molecules, 46% were transcribed from the positive strand of the JKD6008 genome, while 54% were encoded from the negative strand. The distribution of these transcripts was homogeneous along the chromosome.

Comparisons among 19 published genome sequences allowed us to define a 2.3-Mbp *S. aureus* core genome and a 604-kb accessory genome for JKD6008. We then used read mapping to assign the 409 sRNAs as core or accessory features (see Data Set S1 in the supplemental material) and found 79 of the 409 sRNAs encoded within the JKD6008 accessory genome. A total of 171 sRNAs in JKD6008 were antisense (42%) to coding RNAs, a much higher percentage than previously identified in the N315 genome (17.9%) (16). However, this is in keeping with recent data demonstrating abundant antisense sRNAs in *S. aureus*, where 75% of detected sense RNAs were subjected to antisense transcription (41). Similar findings have been reported in other bacterial species, including *Helicobacter pylori* (42). Ninety-four of the identified sRNAs were predicted as *cis*-acting, while 116 were *trans*-encoded. In 94 sRNAs, we identified a UCCC motif reported as a consensus sequence involved in the initial binding of sRNAs and mRNA targets (11). Most transcripts showed a high degree of conservation among the species (91%) and sometimes among the genera (17%), suggesting that these molecules are likely of major importance for the cell. A small fraction (9%) of the discovered transcripts appeared to be specific to tested strains or to particular mobile genetic elements. This observation notably concerns RNA molecules located in pathogenicity islands, transposons, or an integrated plasmid (see Data Set S1 in the supplemental material). Decreasing the stringency of the homology cutoff allowed us to evaluate the partial conservation of the detected RNA molecules among bacteria. BLAST (Basic Local Alignment Search Tool) searches using these criteria revealed that some of these sequences are conserved in *Bacillales*, *Clostridia*, *Bacilli*, or *Proteobacteria*,

and moreover, in bacteria known to be highly virulent, such as *Bacillus anthracis* or *Clostridium difficile*. In addition, more than 25% of the discovered transcripts are located in the vicinity of previously annotated virulence genes. Finally, for 10% of these small transcripts, the GC content was significantly different ($\leq 22\%$ or $\geq 42\%$) from that of the average bacterial chromosome (32%), suggesting potential acquisition during horizontal transfer. A total of 112 of the JKD6008 sRNAs were identified in our previous study of sRNAs in *S. aureus* strain N315 (annotated as Tegs) (16), meaning that about 70% of the sequences of Tegs are strictly conserved in the JKD6008 strain. Many of these sRNAs have been experimentally confirmed by Northern blotting or reverse transcription (RT)-PCR previously (52 in total); these are described in Data Set S1 in the supplemental material. Forty-five of the sRNAs were predicted using Rfam analysis of the JKD6008 genome, with 18 of these previously validated using other methods.

Nonnegative matrix factorization revealed condition-specific sRNA and mRNA class responses in *S. aureus*. Initially, global sRNA and mRNA expression profiles for the data sets were explored using heat maps that included sRNAs or genes that demonstrated a standard deviation of $\log_2$ expression level across the samples of greater than 2 (Fig. 1 and 2). These heat maps demonstrated clear patterns of response to different conditions, and while in the mRNA data set, the parameters “strain” and “time” were associated with response patterns, in the sRNA heat map, antimicrobial exposure was associated with clear patterns of response. These data are summarized in Data Set S2 (sRNA) and Data Set S3 (mRNA) in the supplemental material and reveal 80 sRNAs and 124 mRNAs where >2 standard deviations (SD) of $\log_2$ expression was demonstrated across the feature. Notably, the sRNA data set includes multicopy sRNAs antisense to 23S rRNA repeated sequence. These results suggest that the determinants of differential regulation in the sRNA and mRNA data sets are unique, with antimicrobial exposure significantly contributing to the sRNA transcriptional profile.

To gain further comprehensive and meaningful insights from these complex expression data and explore the data in a nonsubjective manner, we employed nonnegative matrix factorization (NMF) to look for global expression patterns. The aim of NMF in this project was to determine whether condition-specific sRNA and mRNA class responses occur in *S. aureus*. This type of algorithm has previously been successfully used for microarray data analysis as well as in pattern recognition studies. The sRNA and mRNA factorizations are presented separately as their expression patterns were very different. For both data sets, NMF factorizations into 2 to 15 classes were examined manually. This means that the NMF was examined visually using different class numbers from 2 to 15. Each class corresponds to a subcluster of genes with a similar pattern of response, and the number of classes that provided the best description of the data was further utilized. For further analysis of the sRNA data, the 8-class factorization was chosen as demonstrating clear condition-specific responses (Fig. 3), while for the mRNA analysis, the 6-class factorization was chosen (see Fig. S1 in the supplemental material). For the sRNA data, this means that 8 different classes (or sRNA transcriptional responses) were explored and the contribution of each of the conditions (sRNA samples, including strain, time, and antibiotic exposure) to each of these classes was determined.

sRNA NMF. The membership and relative contribution of

FIG 1 Heat map of sRNA responses. This heat map includes 80 sRNAs where a standard deviation (SD) of $\log_2$ expression levels across the samples of greater than 2 was detected across the individual sRNA feature. The heat map demonstrates clear antimicrobial-responsive sRNA transcriptional patterns. The details are presented in Data Set S2 in the supplemental material. Abbreviations: none, no antibiotic; cefto, ceftobiprole; linez, linezolid; tige, tigecycline; vanco, vancomycin.

each sample condition to each of the eight sRNA classes are summarized in the heat map in Fig. 3. Here, increased intensity indicates enhanced membership of the relevant class. For example, the sample condition of JKD6008 at 2 h, with no antibiotic exposure, was a member of only class 2. Similarly, JKD6008, at 2 h after ceftobiprole exposure, demonstrated membership in only class 4. This shows that each sample condition (dependent on strain, time, and antibiotic exposure) was a member of predominately one or two classes. Interestingly, antibiotic-specific classes were demonstrated, in particular for classes 4, 5, 6, and 7, where antimicrobial exposure appeared to be the dominant condition leading to the class membership (Fig. 3). Strong membership in class 4 was demonstrated for all ceftobiprole-exposed samples, while vancomycin exposure was dominant in class 5. Linezolid exposure was linked to a very class-specific membership (class 6), while a moderate class-specific membership was demonstrated for samples exposed to tigecycline (class 7). A more generic antimicrobial-related class membership was demonstrated for all antibiotics (class 8).

A more detailed analysis of the transcriptional response within each antibiotic class shows that the sRNA classes are predominately condition specific (Fig. 4). In this figure, the miniature heat

FIG 2 Heat map of mRNA responses. This heat map includes 124 mRNAs where a standard deviation of $\log_2$ expression levels across the samples of greater than 2 was detected across the individual mRNA feature. The heat map demonstrates clear time- and strain-specific mRNA transcriptional patterns. The details are presented in Data Set S3 in the supplemental material. Abbreviations: none, no antibiotic; cefto, ceftobiprole; linez, linezolid; tige, tigecycline; vanco, vancomycin.

map indicates the relative membership of samples in the class, while the larger heat map describes the membership of specific sRNAs statistically associated with the relevant class. The number of sRNAs significantly associated with each class ($FDR < 0.05$) ranged from 23 (class 5) to 98 (class 8). The full list of sRNAs significantly linked to each of the 8 classes is presented in Data Set S4 in the supplemental material. The table in Data Set S4 also indicates the relative contribution (weight) of each of the sRNAs listed to each of the classes. There are several class-specific sRNAs within each of the classes shown in Fig. 4, especially for the linezolid-associated class (class 6). For example, a number of class 6-specific sRNAs were identified, including sRNA121, sRNA108, sRNA160, and sRNA127. In the vancomycin-responsive class, sRNA356 (previously described as RsaI [11], Teg24 [16], and RsaOG [18]) was specific to that class. In class 4 (ceftobiprole related), sRNA116 and sRNA259 did not contribute to any other class responses.

FIG 3 Nonnegative matrix factorization of the sRNA data set. The results of the 8-class NMF are presented. The 20 different experimental conditions are represented, and their relative membership to each of the 8 classes is demonstrated. Black indicates no membership in the relevant class, while increasing color intensity indicates greater membership. Each experimental condition contributed predominately to one class; however, membership of other classes also occurred. Abbreviations: none, no antibiotic; cefto, ceftobiprole; linez, linezolid; tige, tigecycline; vanco, vancomycin.

mRNA NMF. The NMF of the mRNA samples demonstrated a less-well-defined class membership, but a 6-class analysis provided the best description of the data. There was a clear difference between the mRNA and sRNA analyses. The mRNA class membership was more clearly influenced by strain and time (see Fig. S1 and S2 in the supplemental material). For example, mRNA from JKD6008 at 2 h was highly associated with class 1, independent of antibiotic exposure, while JKD6009 samples at 2 h were class 2 specific. A more detailed analysis of these responses (see Fig. S2D) appeared to demonstrate a linezolid-responsive mRNA class (class 4); however, the genes in this class also showed membership in many other classes. Despite previous descriptions of specific transcriptional profiles linked to exposure to cell wall active antimicrobials (such as vancomycin and ceftobiprole) (43, 44), no clear cell wall active antibiotic transcriptional profile was demonstrated in this NMF. The summary of gene membership and statistical significance for the mRNA NMF is presented in Data Set S5 in the supplemental material.

Specific sRNA transcriptional responses associated with antibiotic exposure. A previous study using *Salmonella enterica* demonstrated an sRNA transcriptional response after exposure to tigecycline; however, the number of sRNAs detected was small (19), which is probably due to the different technical approach used in this work. In our study, NMF strongly supported initial observations from heat map analysis of normalized expression profiles that antibiotic exposure was significantly influencing sRNA expression. So, we next investigated the transcriptional responses of all sRNAs upon individual antibiotic exposure and “any antibiotic” exposure using statistical analysis of the sRNA expression by Voom and Limma (see Data Set S6 in the supplemental material). This direct analysis of the sRNA transcriptional response to antibiotic exposure is different from the NMF analysis, where patterns in response to different conditions were explored. Hence, the collection of sRNAs that were statistically

FIG 4 sRNA membership in class 4, 5, 6, and 7 responses. The membership of each of the 20 sample conditions is demonstrated in the miniature heat maps at the top of each class. In the larger heat maps for each class, the degree of membership for the top 25 statistically significant sRNAs is demonstrated for classes 4, 5, 6, and 7. Black indicates no membership of the relevant class, while increasing color intensity indicates greater membership. Note, only 23 sRNAs were statistically significant in class 5. (A) The class 4 response, primarily related to ceftobiprole exposure, demonstrates a small number of class-specific sRNAs, including sRNA116 and sRNA259. (B) Class 5 was primarily related to vancomycin exposure and demonstrated 2 highly class-specific sRNAs, sRNA97 and sRNA356. (C) Class 6 was clearly linked to linezolid exposure, and a large number of class-specific sRNAs were identified. (D) Class 7 appeared to be tigecycline responsive and demonstrated a small number of class-specific sRNAs, including sRNA144. Abbreviations: C, ceftobiprole; L, linezolid; T, tigecycline; V, vancomycin. The first column of the miniature heat maps is the no-antibiotic sample.

linked with antibiotic exposure by Voom may not be exactly the same as those found in the NMF analysis.

Using individual antibiotic exposure as the major determinant, we have identified 39 sRNAs that were significantly differentially regulated after at least one antibiotic exposure ($P < 0.05$ after FDR correction). These sRNAs and their characteristics are summarized in Table 1. Four of the sRNAs are repeated antisense RNAs to the 23S ribosome, and therefore the analysis indicated identical transcriptional responses for these sRNAs (sRNAs 101, 117, 322, and 339). In some cases, all four antimicrobials induced the sRNA transcriptional response (for example, consistent downregulation of sRNA241 and sRNA242 was demonstrated after exposure to all antimicrobials). In other cases, one or two antibiotic exposure conditions resulted in significant differential regulation. These included 15 sRNAs for which antimicrobial exposure resulted in significant upregulation of sRNA expression. Miniature heat maps of these sRNAs are presented in Fig. 5 and demonstrate highly antibiotic-specific responses in these cases. Linezolid exposure was responsible for most of the antibiotic-specific sRNA transcriptional responses; however, all antimicrobials resulted in at least one specific sRNA response.

Using a pooled analysis of “any antibiotic” versus no antibiotic, 47 sRNAs (~11% of all sRNAs defined in these strains) were differentially regulated at an FDR of <0.05 (see Data Set S6 in the supplemental material). In these analyses, the linear model tests whether the hypothesis that log expression is a linear function of the different antibiotics, the strain, and the time fits the data significantly better than the null hypothesis that it is a linear function of the strain and the time alone. Examples of the eight sRNAs that were detected in this analysis, but not in the analysis of individual antibiotics, include sRNA356. This sRNA was strongly and specifically associated with the class 5 sRNA profile in the NMF—the class associated with vancomycin exposure. sRNA356 (also called Teg24, RsaI, or RsaOG) is a *trans*-encoded sRNA that has been previously well studied and been demonstrated to increase in expression upon oxidative stress and during stationary growth (11). Further analysis of sRNA356 in this study (see Fig. S3 in the supplemental material) confirms that time of culture (6 h versus 2 h) is a significant determinant of sRNA356 expression; however, enhanced expression was noted after vancomycin exposure at 6 h.

mRNA transcriptional response to antibiotic exposure. In the mRNA NMF analysis, distinct antibiotic transcriptional responses were not prominent; however, exposure to cell wall active antibiotics is known to induce a specific transcriptome response, such as upregulation of the bacterial cell wall stimulon (44). The normalized transcriptional data were also investigated to determine mRNA transcriptional effects of antibiotic exposure, including effects of “any antibiotic” compared to no exposure. Twelve CDS were differentially regulated based on an FDR of <0.05 and demonstrated antibiotic-specific mRNA responses to each of the antibiotics (see Fig. S4 in the supplemental material). This clearly demonstrates an mRNA response was occurring to the antibiotic exposure in this study, despite the relatively short exposure time of only 10 min. More profound mRNA responses might be expected with prolonged antibiotic exposure.

Potential role of sRNAs in *S. aureus* antimicrobial responses. Based on the NMF and the individual antibiotic-induced transcriptional analysis, it is clear that an antibiotic-induced sRNA transcriptional response exists in *S. aureus*. The majority of differentially expressed sRNAs were antisense (35 of 39 sRNAs

TABLE 1 List of sRNAs differentially expressed after antibiotic exposure

JKD6008 sRNA	JKD6008 coordinates	Predicted size (nt) ^a	No. of antibiotics ^b	Differential expression (log ₂) ^c				Type of sRNA	Overlap	Gene product
				Vanco	Cefto	Linez	Tige			
sRNA1	7899–8070	172	1		–4.55			Antisense	<i>gyrA</i>	DNA topoisomerase (ATP-hydrolyzing) subunit A
sRNA10	46391–46424	34	2	–5.05	–6.20			Antisense	<i>mecA</i>	Penicillin-binding protein 2'
sRNA33	228497–228707	211	1			–4.33		Antisense	SAA6008_00192	2-component sensor histidine kinase
sRNA58	352262–352379	118	2		–6.24	–3.96		Antisense	SAA6008_00305	3-β-Hydroxysteroid dehydrogenase/isomerase
sRNA62	388145–388244	100	2	–5.19	–6.56			Antisense	<i>ychF</i>	GTP-dependent nucleic acid-binding protein, EngD
sRNA72	413630–413660	31	1			9.22		Antisense	<i>ahpF</i>	Alkyl hydroperoxide reductase, large subunit
sRNA75	425821–425872	52	1			–4.02		Antisense	<i>guaA</i>	Bifunctional GMP synthase/glutamine amidotransferase protein
sRNA97	519471–519525	55	1		–4.16			Antisense	<i>trcC</i>	α-Phosphotrehalase
sRNA99	521007–521088	82	1			–4.99		<i>trans</i> -encoded		
sRNA100	526093–526120	28	1			–3.73		Antisense	SAA6008_r01	16S rRNA
sRNA101, –117, –322, –339	528069–528123	55	4	–6.60	–10.27	–10.96	–14.05	Antisense	SAA6008_r02 (–r06, –r12, –r15)	23S rRNA (repeated sequence)
sRNA105	543082–543115	34	3	–10.05	–8.43		–9.82	Antisense	SAA6008_00498	Conserved hypothetical protein
sRNA106	543203–543407	205	1		–4.17			Antisense	SAA6008_00498	Conserved hypothetical protein
sRNA108	547233–547423	191	3	–6.52	–5.83		–7.62	Antisense	<i>gcdD</i>	Putative UDP-N-acetylglucosamine pyrophosphorylase
sRNA113	568141–568168	28	1			6.16		Antisense	<i>lysS</i>	Asparaginyl-tRNA synthetase
sRNA115	570375–570405	31	1			–3.56		Antisense	SAA6008_r05	16S rRNA
sRNA116	571261–571291	31	1		7.90			Antisense	SAA6008_r05	16S rRNA
sRNA121	594290–594320	31	1			7.38		<i>trans</i> -encoded		
sRNA124	595877–595920	44	2	4.77		4.37		Antisense	<i>rplK</i>	50S ribosomal protein L11
sRNA126	596651–599945	295	3	–7.14	–6.56	–5.61		Antisense	<i>rpoB</i>	DNA-directed RNA polymerase subunit β
sRNA127	608434–608465	32	1			7.64		Antisense	<i>fus</i>	Translational elongation factor G
sRNA128	610805–610851	47	2	5.61		6.60		Antisense	<i>tuf</i>	Translational elongation factor Tu
sRNA144	753739–753771	33	1				5.18	Antisense	<i>rimL</i>	GNAT family acetyltransferase
sRNA148	782301–782329	29	3	–7.57	–6.03		–5.95	Antisense	SAA6008_00723	Putative membrane protein
sRNA160	840969–841025	57	1			5.48		Antisense	<i>uvrB</i>	Excision endonuclease subunit UvrB
sRNA182	977447–977519	73	3		–4.87	–4.58	–5.71	Antisense	<i>addA</i>	ATP-dependent nuclease subunit A
sRNA197	1133289–1133320	32	1			6.12		<i>trans</i> -encoded		
sRNA213	1228954–1229181	228	1		4.00			Antisense	<i>pyrF</i>	Putative orotidine 5'-phosphate decarboxylase
sRNA215	1262175–1262237	63	1			4.30		Antisense	<i>smc</i>	SMC superfamily ATP-binding chromosome segregation protein
sRNA217	1287334–1287419	86	1		7.00			Antisense	<i>rpsB</i>	30S ribosomal protein S2
sRNA241	1533523–1533604	82	4	–7.20	–9.11	–3.44	–9.97	<i>trans</i> -encoded		
sRNA242	1564459–1564621	163	4	–7.31	–6.75	–7.14	–8.96	Antisense	<i>rpsA</i>	Putative 30S ribosomal protein S1
sRNA323	2210781–2210964	184	1			–3.42		Antisense	SAA6008_r13	16S rRNA
sRNA338	2326017–2326084	68	1		5.10			Antisense	SAA6008_r15	23S rRNA
sRNA342	2352336–2352594	259	1	6.27				Antisense	SAA6008_02225	Conserved hypothetical protein
sRNA404	2850424–2850460	37	1		7.74			Antisense	SAA6008_02706	α-Glucosyltransferase

^a Estimated length is based on predicted sRNA start and end using read mapping.

^b Number of antibiotics leading to differential expression.

^c Abbreviations: vanco, vancomycin; cefto, ceftriaxone; linez, linezolid; tige, tigecycline.

FIG 5 Heat maps of antibiotic-responsive sRNAs. Shown are miniature heat maps of 15 sRNAs that were upregulated after antimicrobial exposure. These are colored based on the $\log_2$ expression level compared to the gene average, but scaled so that the sample furthest from the mean is fully red or fully blue. Very clear and consistent antimicrobial responses are demonstrated, especially for linezolid-responsive sRNAs. Abbreviations: C, ceftibiprole; L, linezolid; T, tigecycline; V, vancomycin.

presented in Table 1). Dramatic up- and downregulation of selected RNAs occurred after antimicrobial exposure, and in some cases, all antibiotics resulted in differential expression (invariably in the same direction), while in other cases, antibiotic-specific responses were demonstrated. A remarkable feature of the results is the prominence of antisense sRNAs to genes encoding proteins involved in protein synthesis and ribosomal function. For example, all antibiotics resulted in significant downregulation of a repeated sRNA encoded antisense to 23S rRNA (sRNA101/sRNA117/sRNA322/sRNA339). The identical nature of these sequences led to ambiguous alignment of reads to these regions; however, even when this was accounted for by random allocation of reads, these antisense sRNAs were significantly downregulated. Other protein synthesis-related antisense sRNAs included downregulation of sRNA242 (antisense to *rpsA*, coding for 30S ribosomal protein S1) in all antibiotic exposure groups, while ceftibiprole uniquely upregulated sRNA217 expression (antisense to *rpsB*, coding for 30S ribosomal protein S2), and vancomycin and linezolid resulted in upregulation of sRNA124 (antisense to *rplK*, coding for 50S ribosomal protein L11). Three antibiotics resulted in downregulation of sRNA126 (antisense to *rpoB*, coding for RNA polymerase subunit beta). Two of the four sRNAs detected in *S. enterica* after antibiotic exposure were mapped to regions between the 16S and 23S rRNA-encoding regions, further highlighting the potential role of sRNAs in ribosomal metabolism modification in bacteria after antibiotic exposure (19).

Notably, linezolid was the antibiotic that demonstrated the most profound sRNA response in both the NMF and individual transcriptional analysis. In fact, 24 of the 39 sRNAs listed in Table 1 were differentially regulated upon linezolid exposure. These included upregulation of sRNA121, a small (predicted 31-base) *trans*-encoded RNA, sRNA127 a small (32-base) sRNA encoded antisense to *fus*, which encodes the translational elongation factor G, and sRNA128, an antisense RNA encoded on the complementary strand to *tuf*, the translational elongation factor Tu.

The cell wall active antibiotics vancomycin and ceftobiprole induced a number of sRNA transcriptional changes, including downregulation of sRNA10 that is antisense to *mecA*, suggesting a possible role for this sRNA in penicillin-binding protein 2a (PBP2a) production. No additional antisense sRNAs to other cell wall stimulon genes were differentially regulated.

Antisense sRNAs have traditionally been thought to act via base pairing with RNA expressed from the complementary strand (45), suggesting that the transcriptional responses we have demonstrated here in *S. aureus* after antibiotic exposure are leading to changes in protein production capacity within the cell, by interacting with mRNA encoding key ribosomal proteins and RNAP. This is especially true for linezolid exposure, which may be a consequence of linezolid's mechanism of action, because it acts by binding to 23S rRNA to inhibit initiation of protein synthesis. At this stage, it is not clear if the profound sRNA changes observed after linezolid exposure represent an adaptive response by the organism to attempt to overcome the inhibition of ribosomal function or are in fact a response that further disrupts protein synthesis and inhibits the cell-enhancing antimicrobial activity. Recently it has been demonstrated that *cis*-antisense RNAs may act in *trans* in *S. aureus* (9), meaning that some of these antisense sRNAs may have alternate functions and are not solely interacting by full base pairing with their complementary region on the opposite strand.

A short antibiotic exposure time of 10 min was used in this study; however, despite this, significant transcriptional effects were detected. This is consistent with previous studies that have demonstrated transcriptional effects of very short antibiotic exposures in *S. aureus* (26–28), as well as exposure to other bioactive molecules, such as antimicrobial peptides (46). The advantage of the short exposure time is that the dominant transcriptional effects associated with bacterial killing and growth inhibition were avoided. These rapid changes in sRNA expression perhaps reflect a mechanism for bacteria to implement a swift protective response to the presence of antibiotics and other bioactive small molecules in the environment. Future studies that explore the impact of longer antibiotic exposures on the sRNA transcriptional response are also warranted, while being mindful of the secondary effects associated with longer exposure.

Strain-specific sRNA profiles. In this study, we used a vancomycin-susceptible *S. aureus* strain, JKD6009, and the vancomycin-intermediate strain JKD6008, which evolved *in vivo* during clinical vancomycin exposure (20, 21). These strains provided an opportunity to explore the sRNA transcriptional profile of vancomycin-intermediate *S. aureus* compared to vancomycin-susceptible *S. aureus*. Only five sRNAs in total were differentially regulated between the two strains (Fig. 6), including the effector molecule of the accessory gene regulator system, RNAIII. We have previously demonstrated downregulation of RNAIII expression in JKD6008 compared to JKD6009 using real-time PCR from mid-exponential-stage cultures (20), and the results from this RNA-Seq analysis

FIG 6 Heat maps of strain-specific sRNAs. Shown are miniature heat maps of differentially regulated sRNAs in JKD6009 (VSSA) compared to those of JKD6008 (VISA). These are colored based on log₂ expression level compared to the gene average, but scaled so that the sample furthest from the mean is fully red or fully blue. Abbreviations: C, ceftobiprole; L, linezolid; T, tigecycline; V, vancomycin.

are consistent with this (Fig. 6). Two sRNAs demonstrated enhanced expression in the VISA strain JKD6008 at 2 h, compared to JKD6009: sRNA131 and sRNA260 (Fig. 6). It is interesting to note the same pattern of expression in these two sRNAs; however, it is not clear if these changes represent a cause or a consequence of the VISA phenotype. sRNA131 is located in the vicinity of the *vraA* gene (encoding long-chain fatty acid-coenzyme A [CoA] ligase), which is part of the *vra* operon. Upregulation of the *vra* operon and enhanced VraX protein expression have previously been documented in glycopeptide-intermediate *S. aureus* (47), potentially implying a role for the sRNA131 molecule in the glycopeptide resistance phenotype.

Distinct strain-specific mRNA transcriptional responses were found in the initial heat map analysis (Fig. 2). An analysis of mRNA transcriptional profiles based on strain revealed 107 features that were differentially regulated with an FDR of <0.05 (see Data Set S7 in the supplemental material). These findings were consistent with those previously described in this strain pair using microarray analysis, including upregulation of genes encoding aspartate and pyruvate biosynthesis pathways (*ilvD*, *ilvC*, *dapA*, and *dapD*), the transglycosylase encoded by *sceD*, and downregulation of virulence-associated genes, including the accessory gene regu-

lator locus *sbj*, encoding an IgG-binding protein, and fibronectin binding proteins (*fnbA*) (48).

Concluding comments. Here we have used state-of-the-art molecular technologies and bioinformatic analysis to explore the role of sRNAs in the response of *S. aureus* to antimicrobial exposure. Hospital clones of *S. aureus* have evolved resistance to most classes of antimicrobials since the first introduction of penicillin in the 1940s. In this study, we chose a successful hospital-associated clone of *S. aureus* (ST239) because it has evolved resistance to almost all classes of antibiotics, including vancomycin (represented by strain JKD6008). We explored the effect of exposure to “last-line” antimicrobials and demonstrated a very clear antimicrobial-responsive sRNA profile in this strain, suggesting that the regulatory effects of sRNAs in ST239 *S. aureus* contribute to the antimicrobial exposure response. This was in contrast to the mRNA profiles under the same conditions, where the time of culture and the strain were the major determinants leading to differential regulation, with little consistent impact of antimicrobial exposure. These results were confirmed using traditional differential expression analysis; however, because of the complexity of the data set generated in this study, we also applied a nonnegative matrix factorization analysis. Both types of analysis led to the same conclusions regarding the impact of antimicrobials on sRNA transcription.

The use of high-throughput RNA sequencing has led to the discovery of large numbers of sRNAs in bacterial species. Despite this, we were surprised by the number of sRNAs detected in this ST239 clone of *S. aureus* (409 in total). This is a significant increase from the 195 sRNAs detected in our previous study of the Japanese MRSA strain N315 (16), predominantly related to the impressive number of antisense sRNAs detected in this study. The difference in detection is potentially due to a difference in sequence coverage between the two studies, as well as the large number of exposure conditions used here. Fifty-two of the sRNAs detected in this study have been previously experimentally confirmed (see Data Set S1 in the supplemental material), predominantly using Northern blotting, confirming the validity of our RNA-Seq results. The significant number of sRNAs detected in ST239 *S. aureus* underscores a previously underappreciated complexity of regulatory networks within *S. aureus* to control important cellular functions, over and above those previously focused on understanding the role of protein-coding sequences. While the role in controlling virulence of a small number of sRNAs has been demonstrated in *S. aureus* (49), this is the first study to address the issue of sRNAs and antimicrobial resistance in *S. aureus*. New antibacterial targets are needed to combat the spread of multi-drug-resistant bacteria, none more so than *S. aureus*. Antibacterial agents that target riboswitches have demonstrated activity in some bacterial species (50–52), and a small molecule that alters mRNA turnover has also recently been shown to have antimicrobial activity against *S. aureus* (53). Given the apparent role of sRNAs in the adaptation of *S. aureus* to antimicrobial exposure we have demonstrated in this study, especially to linezolid, these results provide an exciting opportunity to explore the potential therapeutic benefits of manipulating individual sRNA responses in combination with selected antimicrobial therapy in *S. aureus*.

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