In recent years, a number of studies have been carried out, employing both experimental and computational methods to identify regulatory or small RNAs (sRNAs) in *Staphylococcus aureus* (1–12). Hundreds of sRNAs have been identified, and many, in addition to the *agr* effector RNAIII, have been shown to play a role in gene regulation (while these molecules go by a variety of names such as regulatory RNAs or noncoding RNAs, we will use sRNAs to refer to them all, as previously recommended [13]). Despite advancements in sRNA identification, the roles of most of these molecules remain unknown, because in many cases, limited functional information can be gathered from analysis of their sequence alone.

One additional factor that has hampered the study of sRNAs in *S. aureus* has been the lack of a clear nomenclature and annotation system. This absence of a systematic identification and annotation process has led to the repeated discovery of the same sRNAs on multiple occasions, the reidentification of already known sRNAs (e.g., RNAIII), and even to important protein-coding genes being ascribed as sRNAs (e.g., the α-PSM transcript, which is not annotated in most *S. aureus* genome files). Recent work by Sassi et al. (14) established an online database for staphylococcal sRNAs; however, most sRNAs, including the well-studied RNAIII, are still not included in annotated *S. aureus* GenBank genome files. This is a marked oversight, as annotated genome files serve as the reference for global genomic and transcriptomic studies; thus, the absence of sRNAs from these files severely impedes their study and prevents us from gaining an overarching picture of regulatory circuits.
In *S. aureus*, most sRNA identification studies have been performed in a single background, the hospital-acquired methicillin-resistant *S. aureus* (MRSA) strain N315. The existence of many of these sRNAs has been demonstrated experimentally in strain N315 (1, 3, 4, 6, 7); however, very few of them have been investigated in other *S. aureus* strains, including the epidemic community-associated MRSA (CA-MRSA) strain USA300 (15). As such, their existence, location, and copy number in most *S. aureus* isolates is unknown, preventing us from gaining a sense of their role in the physiological and pathogenic differences between strains.

To better understand the sRNA content of multiple *S. aureus* strains, we explored the genomes of three well-studied *S. aureus* strains (USA300, MRSA252, and NCTC 8325). We identified the location(s) of previously discovered sRNAs and created new GenBank genome annotation files for each strain, inserting ~260 sRNAs. These newly annotated files serve as a valuable resource allowing us to do the following: (i) search the genome of each strain for as yet unidentified sRNAs without mistakenly reidentifying known species, and (ii) calculate expression values for these genes using transcriptome sequencing (RNA-seq) data.

To demonstrate the application of these new files, we performed RNA-seq on CA-MRSA strain USA300 growing in laboratory media and human serum and aligned the data to our newly created sRNA annotated genome files. Examining the data, we identified 39 novel putative sRNAs that had not been previously reported. These novel sRNAs were annotated, cross-referenced in the genomes of strains NCTC 8325 and MRSA252, and added to the newly created genome files as well. During the cross-referencing process, we observed numerous examples of inconsistent genome annotation in different *S. aureus* strains. We highlight examples clearly demonstrating genome misannotation and demonstrate how this phenomenon is adversely affecting the identification and characterization of sRNAs. Finally, we calculate expression values and examine the global sRNA expression profile of strain USA300, uncovering a wealth of molecules that display differential expression in human serum. The latter point is of significant importance, as it gives us a unique look into the sRNA transcriptome, not only during growth of *S. aureus* but also in a pathophysiologically relevant growth environment. The new genome annotation files described in this work have been deposited in the figshare depository and are freely available for download. We suggest that these newly reannotated genomes will be a valuable resource to the *S. aureus* research community for sRNA identification and analysis hereafter, as they can be incrementally added to as new sRNAs are discovered.

RESULTS

Annotation of sRNAs on the *S. aureus* genome. Previous studies in our lab have utilized RNA-seq to determine the global transcriptomic profile of *S. aureus* in the community-associated MRSA strain USA300 (16, 17). Analysis of these data sets revealed a large number of *S. aureus* transcripts that map to intergenic regions where no protein-coding genes have been annotated. We hypothesized that many of these transcripts represent sRNAs because of the following. (i) Most *S. aureus* genome annotation files do not contain annotations for sRNA genes. (ii) Recent studies carried out in the *S. aureus* N315 background have demonstrated that there are several hundred sRNAs encoded in the *S. aureus* genome (12, 14). To facilitate improved global transcriptomic analysis of *S. aureus* by RNA-seq, we created new GenBank genome annotation files for three commonly used *S. aureus* strains, NCTC 8325, MRSA252, and USA300. To do this, we elected to expand the sRNA annotation and nomenclature system already present for 25 sRNAs in strain MRSA252 (see Table S1 in the supplemental material) and apply a similar annotation/nomenclature system to strains USA300 and NCTC 8325 (for details, see supplemental material). To include annotations for known sRNAs, we performed a literature search to identify studies in which sRNAs in *S. aureus* were reported. A total of 12 papers that employed a variety of methods to identify sRNAs, including computational approaches, microarray studies, cDNA cloning, and high-throughput sequencing, were investigated (1–12). Using the information provided in these publications, a list of 928 potential sRNAs was assembled (Table S2) (1–12). In order to ensure accurate annotation of each sRNA from this list, RNA-seq experiments were performed for each of the three strains growing under standard laboratory conditions. Reads generated from these works were aligned to the respective genomes and used as a guide to identify the specific location of each sRNA for each of the three strains (see Fig. S1 in the supplemental material).

This work condensed the 928 putative sRNAs to a total of 248 annotations for strain MRSA252, 254 annotations for strain NCTC 8325, and 264 annotations for strain USA300 (for a more-comprehensive explanation of how sRNAs were identified and annotated, see Text S1 in the supplemental material). Such a dramatic reduction in the number of sRNAs points to the scale of overlapping identification, reidentification, and duplicate naming that was extant in the literature for these elements and the poor state of sRNA curation in *S. aureus* genomes. Our newly generated GenBank files for all three strains represent the first comprehensive list of sRNAs annotated directly in the *S. aureus* genome, which will serve as a valuable reference point for future sRNA discovery, and more broadly, global transcriptomic analyses of *S. aureus* by RNA-seq.

Detection of novel sRNAs in strain USA300. The creation of GenBank files containing annotations for previously identified sRNAs provided us with a unique opportunity to examine RNA-seq data for novel forms of these elements, without mistakenly reidentifying those that are already known. Accordingly, we set out to perform such an exploration using the community-associated MRSA isolate USA300, an undeniably relevant clinical strain for which no sRNA identification studies have yet been performed. To maximize the probability of identifying novel sRNAs, we performed RNA-seq with USA300 grown under both laboratory conditions (trypsic soy broth [TSB]), and in media that was more pathophysiologically relevant to its infectious lifestyle (human serum) (18).

RNA-seq reads from bacteria grown under these conditions were mapped to the newly created USA300 GenBank file (containing 264 sRNA annotations), followed by a thorough examination for the presence of novel sRNA transcripts. A total of 39 potential sRNAs were identified from both conditions, which we named *tsr1* to *tsr39* for Tampa small RNA (Table 1) (for details regarding the criteria used for our determination of novel sRNAs, see Text S1 in the supplemental material). Although the majority of the *tsr* genes were located in intergenic regions, a number were identified as being antisense to annotated genes or as partially overlapping annotated coding DNA sequence (CDS) genes. The novel sRNAs were also added to our newly created USA300 genome file, using
the nomenclature system described in Text S1, resulting in a GenBank file with a total of 303 sRNAs in the USA300 genome. This brings the total number of genes annotated on the USA300 genome from 2,850 to 3,153, representing an approximately 10% increase in annotated genes.

To investigate the conservation of tsr genes in S. aureus, we analyzed their corresponding chromosomal loci on the S. aureus NCTC 8325 and MRSA252 genomes by performing a BLAST search (Table 1). Of the 39 tsr genes, 29 were found in strain MRSA252, while 32 were found in strain NCTC 8325. Annotations were again added to the MRSA252 and NCTC 8325 genome files for each of the tsr genes identified using the nomenclature outlined (see Text S1 in the supplemental material). Importantly, five of the tsr genes appear to be unique to USA300 (tsr1, tsr2, tsr3, tsr5, and tsr30), with no homologues found in either MRSA252 or NCTC 8325. In addition, five tsr genes were present in USA300 and NCTC 8325 but absent in MRSA252 (tsr7, tsr8, tsr20, tsr24, and tsr29), while two were present in USA300 and MRSA252 but absent in NCTC 8325 (tsr26 and tsr27) (see Fig. S2 in the supplemental material).

**Northern blot analysis of tsr transcripts.** To validate our RNA-seq-based approach for sRNA discovery, five putative tsr transcripts were examined by Northern blot analysis. The transcripts investigated had different expression patterns in TSB compared to human serum. Three of the transcripts investigated (tsr8, tsr26, and tsr31) showed a decrease in expression in TSB compared to human serum, one (tsr25) demonstrated an increase in expression, while another (tsr33) showed a decrease (see Table S3 in the supplemental material). Northern blot analysis confirmed the predicted size and orientation of tsr25, tsr26, and tsr31 (Fig. 1). In the case of tsr8 and tsr33, bands were identified; however, they were considerably larger than those predicted by RNA-seq analysis (Fig. 1). For example, the predicted size of tsr33 was 85 nucleotides (nt); however, the size observed by Northern blotting was

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**TABLE 1** Novel sRNAs identified in strain USA300

| sRNA designation | sRNA gene | Strand | Location | Size (nt) | Chromosomal location of tsr gene in strain: |
|------------------|-----------|--------|----------|----------|------------------------------------------|
| SAUSA300s265     | tsr1      | >      | 52438–53094 | 656       | USA300b – M RSA252b – N CT C 8325b |
| SAUSA300s266     | tsr2      | <      | 57712–57804 | 92        | IG – – – |
| SAUSA300s267     | tsr3      | <      | 61388–61550 | 162       | IG – – – |
| SAUSA300s268     | tsr4      | >      | 73511–74139 | 628       | AS AS AS |
| SAUSA300s269     | tsr5      | <      | 79346–79425 | 79        | AS – – – |
| SAUSA300s270     | tsr6      | >      | 120785–120897 | 112      | IG IG IG |
| SAUSA300s271     | tsr7      | >      | 169903–170079 | 176      | IG – – – |
| SAUSA300s272     | tsr8      | >      | 170013–170214 | 201      | IG – – IG |
| SAUSA300s273     | tsr9      | <      | 228412–228796 | 384      | IG CDS CDS |
| SAUSA300s274     | tsr10     | <      | 349895–350058 | 163      | IG OL OL |
| SAUSA300s275     | tsr11     | <      | 336689–356782 | 93       | OL OL OL |
| SAUSA300s276     | tsr12     | >      | 457272–457333 | 61       | IG OL OL AS |
| SAUSA300s277     | tsr13     | >      | 484942–485025 | 83       | OL OL OL OL |
| SAUSA300s278     | tsr14     | >      | 833430–834885 | 545      | IG IG AS |
| SAUSA300s279     | tsr15     | >      | 896563–897379 | 816      | AS AS AS AS |
| SAUSA300s280     | tsr16     | >      | 911264–911364 | 118      | AS AS OL OL |
| SAUSA300s281     | tsr17     | >      | 973559–973971 | 412      | IG CDS CDS |
| SAUSA300s282     | tsr18     | >      | 1074292–1074484 | 192   | IG CDS CDS |
| SAUSA300s283     | tsr19     | >      | 1080302–1080394 | 92      | IG IG IG |
| SAUSA300s284     | tsr20     | >      | 1154300–1154753 | 453     | IG IG IG |
| SAUSA300s285     | tsr21     | >      | 1154827–1156001 | 1174    | CDS CDS CDS |
| SAUSA300s286     | tsr22     | >      | 1165484–1165865 | 381     | IG IG CDS |
| SAUSA300s287     | tsr23     | >      | 1256521–1256545 | 24      | IG IG IG |
| SAUSA300s288     | tsr24     | >      | 1429517–1429754 | 237     | IG IG IG |
| SAUSA300s289     | tsr25     | >      | 1442862–1443042 | 180     | IG AS AS AS |
| SAUSA300s290     | tsr26     | >      | 1641611–1641732 | 121     | IG IG IG |
| SAUSA300s291     | tsr27     | >      | 1642820–1642923 | 103     | IG IG IG |
| SAUSA300s292     | tsr28     | >      | 1715900–1715975 | 75      | OL OL OL OL |
| SAUSA300s293     | tsr29     | >      | 1954961–1955091 | 130     | IG IG IG |
| SAUSA300s294     | tsr30     | >      | 2126434–2126545 | 111     | IG IG IG |
| SAUSA300s295     | tsr31     | >      | 2244964–2245035 | 71      | IG IG IG |
| SAUSA300s296     | tsr32     | >      | 2337922–2338072 | 150     | IG IG CDS |
| SAUSA300s297     | tsr33     | >      | 2410564–2410648 | 84      | IG IG IG |
| SAUSA300s298     | tsr34     | >      | 2591032–2591131 | 99      | IG IG IG |
| SAUSA300s299     | tsr35     | >      | 2680847–2680894 | 547     | IG CDS CDS |
| SAUSA300s300     | tsr36     | >      | 2680812–2680845 | 525     | IG AS AS AS |
| SAUSA300s301     | tsr37     | >      | 2620285–2620621 | 336     | IG CDS CDS |
| SAUSA300s302     | tsr38     | >      | 2664856–2664945 | 89      | IG IG IG |
| SAUSA300s303     | tsr39     | >      | 2811278–2811330 | 52      | IG IG IG |

a >, forward strand; <, reverse strand.
b Characteristics of the chromosomal location of the tsr gene. IG, located in the intergenic region; –, absent, deleted, or no homologue; AS, antisense to the annotated gene; CDS, located within an existing annotated CDS; OL, partially overlaps CDS gene; CDS, the corresponding locus contains three annotated CDSs.
approximately 600 nt. When looking at the tsr33 transcript, one observes that it is located at the 3' end of the sarR gene and is transcribed in the same orientation. The combined size of sarR and tsr33 would be around 600 nt, therefore suggesting that tsr33 represents a large 3' untranslated region (UTR) for the sarR gene. However, RNA-seq alignment from strain USA300 growing in human serum demonstrates a much greater depth of coverage (and hence abundance) of tsr33 than of sarR, suggesting that a tsr33-specific RNA may exist under these conditions (see Fig. S3 in the supplemental material). On the basis of the above information, we predict that tsr33 is cotranscribed as a 3' UTR of sarR, but under certain conditions (e.g., growth in human serum), it is possible that a tsr33 RNA may exist independently of sarR.

The second tsr demonstrating a size difference was tsr8, which was predicted by RNA-seq to be 201 nt; however, two bands were observed by Northern blotting with approximate sizes between 300 and 400 nucleotides. A possible explanation for this may come from the fact that tsr8 and tsr7 are convergently transcribed, that the two transcripts are complementary at the 3' ends, and that these regions overlap. The density of reads, in both directions, mapping to this region of complementarity make it difficult to predict the precise location of each transcript based on RNA-seq data, resulting in an underestimation of tsr8 size. Given our experimental findings, the size of tsr8 was amended to ~350 nt in the GenBank files. It is interesting to note that this type of genetic organization (convergent transcripts overlapping at the 3' end) is common among toxin-antitoxin (TA) systems in S. aureus (19, 20), hence tsr7 and tsr8 could potentially represent a novel serum-induced TA system.

For some of the tsr elements expressed at low levels, Northern blot detection proved unsuccessful (data not shown); therefore, we employed a reverse transcriptase PCR (RT-PCR)-based approach, which is inherently more sensitive. Using this methodology, we were able to validate the presence of an additional six transcripts, tsr1, tsr2, tsr18, tsr24, tsr29, and tsr32 (see Fig. S4 in the supplemental material), suggesting that our RNA-seq-based identification approach is effective at identifying legitimate sRNA molecules.

Inconsistent genome annotation in strains USA300, MRSA252, and NCTC 8325. Twenty-seven tsr genes were found in the genomes of all three strains (USA300, MRSA252, and NCTC 8325). For 14 of these genes, the corresponding genomic loci were similarly annotated in all three strains, e.g., tsr6 is located in an intergenic region in all three strains, while tsr15 is located antisense to an annotated CDS. Interestingly, for 13 tsr genes, the genomic loci in the three strains studied are differentially annotated (Table 1). In many cases, the NCTC 8325 and MRSA252 genomes contain annotations for CDS genes, while the USA300 genome specifies these loci as being intergenic (e.g., tsr9, tsr17, tsr18, etc.). An open reading frame (ORF) search reveals that 11 tsr genes have the potential to encode proteins (of 30 amino acids or larger in size). Seven of these genes are annotated as CDS in strains MRSA252 and NCTC 8325. This raises the possibility that some tsr genes may in fact be protein-coding genes that were omitted from the USA300 genome annotation. Conversely, it is also possible that the NCTC 8325 and MRSA252 genomes may be incorrect and that these annotated genes do not encode proteins. Upon close examination, our data clearly demonstrate that incorrect genome annotation accounts for at least some of the discrepancies observed. For example, the tsr12 locus is annotated differently in all three
strains (Fig. 2). In strain USA300, \textit{tsr12} is located in an intergenic region (between \textit{SAUSA300}_0404 and \textit{hsdM}). In strain MRSA252, \textit{tsr12} incompletely overlaps with a CDS gene annotated in the same orientation, while in strain NCTC 8325, it partially overlaps with a CDS gene annotated in the opposite direction. Bioinformatic analysis of these CDS genes reveals that they both specify very small proteins (43 amino acids in MRSA252 and 33 amino acids in NCTC 8325) that possess no known structural motifs and have no homology to any protein in the database beyond counterparts in a handful of other \textit{S. aureus} strains. Furthermore, in USA300 and NCTC 8325, the \textit{tsr12} locus is 100\% identical at the nucleotide level, making large-scale differences in coding sequences (e.g., inverse open reading frames) unlikely. Collectively, this demonstrates that there is likely misannotation in the genomes of MRSA252 and NCTC 8325 and that our suggested annotation of \textit{tsr12} as an sRNA is likely the correct one.

\textit{tsr12} is an example where incorrect genome annotation is rather clear and raises important questions regarding how commonplace this type of genome misannotation may be. To investigate the issue of inconsistent genome annotation in \textit{S. aureus} further, we selected another sRNA gene for additional study. Teg23 (SAUSA300s148, SARs145, SAOUHSCs144) was originally identified by Beaume et al. (7) as a potential 5’ UTR of the \textit{SAS083} gene in \textit{S. aureus} strain N315. In strains NCTC 8325 and MRSA252, a gene is annotated in the position corresponding to \textit{SAS083} (SAOUHSC_02572 and SAR2384, respectively), but no gene is annotated in strain USA300 (Fig. 3A). Upon analysis, SAS083, SAOUHSC_02572, and SAR2384 once again encode small, hypothetical proteins with no known structural features, functional domains, or apparent homologues and are therefore likely misannotated genes (as noted above for the \textit{tsr12} locus).

Northern blot analysis using \textit{S. aureus} USA300 grown in human serum (where Teg23 is strongly upregulated) detected a band of around 310 nt (as predicted by RNA-seq), alongside an additional band of approximately 215 nt (Fig. 3B). The smaller band (which we designated Teg23.1) was more abundant than the larger band (designated Teg23.2) and was repeatedly detected in multiple Northern blots (data not shown), suggesting that two forms of the transcript may exist within the cell. The CDS annotated in the NCTC 8325 genome at the Teg23 position (SAOUHSC_02572) potentially encodes a protein of 80 amino acids (Fig. 3C, red), while the corresponding locus in strain MRSA252 (SAR2384) potentially encodes a protein of 64 amino acids (Fig. 3C, blue). The difference is due to a 4-bp insertion in the MRSA252 genome that results in the creation of a stop codon (Fig. 3C). The nucleotide sequence of the 432-bp intergenic region from strain USA300 is almost identical with the corresponding locus in strain NCTC 8325 (431/432 identical), and it does not contain the 4-bp insertion found in MRSA252 (Fig. 3C). Therefore, an 80-amino-acid protein could potentially be generated from a transcript originating from this locus in strain USA300 (Fig. 3C, green), similar to NCTC 8325 (for simplicity, this potential protein will be referred to as Teg23P for Teg23 protein).

To test whether Teg23P is produced in strain USA300, we cloned a His\textsubscript{6}-tagged variant of its putative coding sequence, along with its native promoter, into an \textit{S. aureus} shuttle vector.

FIG 2 Variation in genome annotation of the \textit{tsr12} locus. RNA-seq read alignment data are shown for strains USA300 (A), MRSA252 (B), and NCTC 8325 (C). Annotations for CDS genes are shown by black arrows, and the depth of coverage is shown by the blue histograms. The location of \textit{tsr12} is shown by a red arrow. (A) There is no CDS annotation at the \textit{tsr12} locus in strain USA300. (B) In strain MRSA252, a gene (SAR0432) is annotated in the forward direction at the \textit{tsr12} locus. (C) In strain NCTC 8325, a gene (SAOUHSC_00396) is annotated in the reverse direction at the \textit{tsr12} locus.
Blots of USA300 strain bearing this construct revealed no detectable band using an antihistidine antibody, while the positive control (histidine-tagged RpoE) was detected (Fig. 3D). Quantitative RT-PCR (qRT-PCR) to confirm that the construct was being expressed demonstrated a 2.92-fold-higher expression of Teg23 in S. aureus containing the Teg23P-his6 plasmid (Fig. 3E). These data confirm that the plasmid gene-encoded Teg23 is expressed; however, no protein corresponding to Teg23P-his6 is generated. This strongly suggests that no protein corresponding to Teg23P is produced in strain USA300.
shown). To test this further, we performed PCR using cDNA generated from strain USA300 containing the Teg23P-his\_6 plasmid (Fig. 3G). In contrast, no product was generated in PCRs using one primer that binds within the Teg23 sequence (primers 1 and 3 [Fig. 3F]). These data show that the Teg23 RNA is being generated but that it terminates prior to the 3' end of Teg23P, confirming the results from the Western blot analysis that Teg23 does not encode an 80-amino-acid protein.

While it is impossible to completely rule out the existence of a putative protein corresponding to the SAOUHSC\_02572 gene, its expression is unlikely to be significant, as the transcript generated at this locus terminates before the annotated 3' end of the gene (Fig. 3F). Consequently, transcript generated at this locus terminates before the end of the annotated gene, and therefore, an 80-amino-acid protein corresponding to the SAOUHSC\_02572 gene is unlikely to be made. A similar pattern of transcript termination is observed in the S. aureus USA300 RNA-seq data set, further suggesting that the Teg23P protein could not be produced in this strain (data not shown). To test this further, we performed PCR using cDNA generated from total RNA from strain USA300. PCR performed using primers that bind within the Teg23 sequence (primers 2 and 3 [Fig. 4F]) generated products using template cDNA from USA300 and USA300 containing the Teg23P-his\_6 plasmid (Fig. 3G). In contrast, no product was generated in PCRs using one primer within the Teg23 sequence and a second primer located at the 3' end of the Teg23P sequence (primers 1 and 3 [Fig. 3F]).

The RNA-seq read alignment data demonstrate that >99% of the Teg23 reads in strain NCTC 8325 terminate 88 nucleotides upstream of the annotated 3' end of the gene (Fig. 3F). Consequently, transcript generated at this locus terminates before the end of the annotated gene, and therefore, an 80-amino-acid protein corresponding to the SAOUHSC\_02572 gene is unlikely to be made. A similar pattern of transcript termination is observed in the S. aureus USA300 RNA-seq data set, further suggesting that the Teg23P protein could not be produced in this strain (data not shown). To test this further, we performed PCR using cDNA generated from total RNA from strain USA300. PCR performed using primers that bind within the Teg23 sequence (primers 2 and 3 [Fig. 4F]) generated products using template cDNA from USA300 and USA300 containing the Teg23P-his\_6 plasmid (Fig. 3G). In contrast, no product was generated in PCRs using one primer within the Teg23 sequence and a second primer located at the 3' end of the Teg23P sequence (primers 1 and 3 [Fig. 3F]). These data show that the Teg23 RNA is being generated but that it terminates prior to the 3' end of Teg23P, confirming the results from the Western blot analysis that Teg23 does not encode an 80-amino-acid protein.

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protein corresponding to Teg23P, these results strongly suggest that the CDS annotations for Teg23P in strains N315, MRSA252, and NCTC 8325 (i.e., SAS083, SAR2384, and SAOUHSC_02572, respectively) are incorrect. Teg23 was originally identified as a 5′ UTR for the SAS083 gene (i.e., the gene encoding Teg23P in N315) (7); however, the data presented herein demonstrates that this is incorrect and Teg23 likely represents a nontranslated sRNA. This comprehensively highlights how overannotation of genomes with CDS genes may be masking the identification of transcripts that encode sRNAs.

Global analysis of sRNA gene expression in strain USA300. The inclusion of 303 sRNA gene annotations in the GenBank file of strain USA300 allowed us to calculate global gene expression values for sRNAs using our RNA-seq data sets. To examine variation in sRNA gene expression, we calculated and compared expression values for S. aureus USA300 growing in TSB and human serum, two conditions known to result in widespread changes in gene expression (18). The location, relative expression, and fold change for each of the 303 sRNA genes in strain USA300 are shown in Fig. 4 and Table S3 in the supplemental material. To identify sRNA genes with meaningful differences in expression, we applied cutoffs to eliminate genes expressed at low levels and those displaying fold changes that are less than 3-fold (see Text S1 in the supplemental material for details). This resulted in a total of 83 sRNAs displaying alterations in gene expression under the two conditions tested. Forty-two were upregulated in human serum (Table 2), while 41 were downregulated (Table 3). Of the newly identified tsr genes, 19 displayed differential regulation, with 16 downregulated in serum, while 3 (tsr18, tsr25, and tsr30) were upregulated. Interestingly, the newly identified tsr25 sRNA demonstrated the largest upregulation in serum of any sRNA (583-fold). To validate these findings, we performed Northern

| sRNA designation       | sRNA gene name or feature | TSB expression value (RPKM)b | Serum expression value (RPKM) | Fold change |
|------------------------|---------------------------|------------------------------|-------------------------------|-------------|
| USAUSA300s148          | Teg23                     | 256.6                        | 2,950.59                      | 11.5        |
| USAUSA300s050          | RsaD                      | 2,737.91                     | 30,473.59                     | 11.13       |
| USAUSA300s226          | JKD6008sRNA173            | 50.05                        | 539.79                        | 10.78       |
| USAUSA300s053          | SsrC                      | 365.2                        | 6,917.84                      | 10.48       |
| USAUSA300s260          | JKD6008sRNA396            | 16.13                        | 150.26                        | 9.31        |
| USAUSA300s294          | tss                      | 470.64                       | 4,383.00                      | 9.31        |
| USAUSA300s027          | sprA1                     | 1,097.34                     | 9,306.35                      | 8.48        |
| USAUSA300s233          | JKD6008sRNA205            | 96.96                        | 717.53                        | 7.4         |
| USAUSA300s003          | T-box riboswitch          | 158.5                        | 1,156.13                      | 7.29        |
| USAUSA300s094          | Teg108                    | 19.39                        | 132.54                        | 6.83        |
| USAUSA300s099          | Teg124                    | 37.1                         | 245.19                        | 6.61        |
| USAUSA300s012          | sprA3                     | 36.05                        | 233.31                        | 6.47        |
| USAUSA300s082          | tsr18                     | 105.4                        | 653.5                         | 6.2         |
| USAUSA300s024          | GlnS ribozyme             | 185.53                       | 1,064.95                      | 5.74        |
| USAUSA300s117          | srr8                      | 12.13                        | 67.9                          | 5.6         |
| USAUSA300s038          | sprG3                     | 191.04                       | 1,049.33                      | 5.49        |
| USAUSA300s120          | srr28                     | 188.03                       | 1,012.53                      | 5.39        |
| USAUSA300s028          | sprA2                     | 99.08                        | 504.62                        | 5.09        |
| USAUSA300s210          | JKD6008sRNA071            | 136.39                       | 691.13                        | 5.07        |
| USAUSA300s100          | Teg124                    | 469.39                       | 2,313.98                      | 4.93        |
| USAUSA300s002          | SAM+ riboswitch           | 56.06                        | 268.4                         | 4.79        |
| USAUSA300s031          | sprD                      | 804.11                       | 3,810.91                      | 4.74        |
| USAUSA300s021          | SAM riboswitch            | 239.09                       | 941.21                        | 3.94        |
| USAUSA300s075          | rsaOL                     | 273.5                        | 1,010.42                      | 3.69        |
| USAUSA300s091          | Teg60                     | 95.9                         | 350.09                        | 3.65        |
| USAUSA300s034          | sprF3                     | 1,227.97                     | 4,261.30                      | 3.47        |
| USAUSA300s016          | T-box riboswitch          | 316.76                       | 1,059.28                      | 3.34        |
| USAUSA300s084          | Teg27                     | 15,788.22                    | 52,571.03                     | 3.33        |
| USAUSA300s042          | rsaOC                     | 26.75                        | 88.89                         | 3.32        |
| USAUSA300s135          | srr100                    | 322.65                       | 1,050.22                      | 3.26        |
| USAUSA300s163          | Teg28as                   | 46.74                        | 149.53                        | 3.2         |
| USAUSA300s079          | rsaOU                     | 0                            | 85.01                         | 3           |
| USAUSA300s151          | Teg134                    | 0                            | 204.13                        | 3           |

* SAM, S-adenosylmethionine.

b RPKM, reads per kilobase of transcript per million mapped reads.

protein corresponding to Teg23P, these results strongly suggest that the CDS annotations for Teg23P in strains N315, MRSA252, and NCTC 8325 (i.e., SAS083, SAR2384, and SAOUHSC_02572, respectively) are incorrect. Teg23 was originally identified as a 5′ UTR for the SAS083 gene (i.e., the gene encoding Teg23P in N315) (7); however, the data presented herein demonstrates that this is incorrect and Teg23 likely represents a nontranslated sRNA. This comprehensively highlights how overannotation of genomes with CDS genes may be masking the identification of transcripts that encode sRNAs.

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TABLE 3 41 sRNAs that are downregulated in human serum versus TSB

| sRNA designation    | sRNA gene name or feature | TSB expression value (RPKM)* | Serum expression value (RPKM) | Fold change |
|---------------------|---------------------------|-----------------------------|-------------------------------|-------------|
| SAUSA300s277        | tsr13                     | 509.65                      | 0.43                          | -1,187.67   |
| SAUSA300s113        | sbrC                      | 1,834.12                    | 6.3                           | -291.07     |
| SAUSA300s266        | tsr2                      | 122.44                      | 0.42                          | -290.6      |
| SAUSA300s298        | tsr32                     | 92.33                       | 0.44                          | -210.65     |
| SAUSA300s171        | Sau-6569                  | 41,077.46                   | 250.83                        | -163.77     |
| SAUSA300s049        | RsaC                      | 8,079.72                    | 77.42                         | -104.37     |
| SAUSA300s004        | Purine riboswitch         | 2,277.90                    | 45.36                         | -50.22      |
| SAUSA300s303        | tsr39                     | 3,132.51                    | 64.48                         | -48.58      |
| SAUSA300s162        | Teg25as                   | 6,668.30                    | 191.51                        | -34.82      |
| SAUSA300s125        | ssr47                     | 6,272.84                    | 256.03                        | -24.5       |
| SAUSA300s052        | RsaF                      | 35,374.06                   | 1,713.79                      | -20.64      |
| SAUSA300s275        | tsr11                     | 398.93                      | 20.01                         | -19.94      |
| SAUSA300s301        | tsr37                     | 390.39                      | 23.36                         | -16.72      |
| SAUSA300s292        | tsr28                     | 513.06                      | 31.91                         | -16.08      |
| SAUSA300s297        | tsr33                     | 8,987.32                    | 592.2                         | -15.18      |
| SAUSA300s062        | Sau-31                    | 141.32                      | 9.7                           | -14.57      |
| SAUSA300s022        | RNAIII                    | 66,968.70                   | 5,178.15                      | -12.93      |
| SAUSA300s283        | tsr19                     | 162.04                      | 13.93                         | -11.63      |
| SAUSA300s118        | ssr16                     | 478.47                      | 49.19                         | -9.73       |
| SAUSA300s302        | tsr38                     | 251.17                      | 27.55                         | -9.14       |
| SAUSA300s280        | tsr16                     | 254.42                      | 30.88                         | -8.24       |
| SAUSA300s095        | Teg116                    | 1,149.05                    | 155.21                        | -7.4        |
| SAUSA300s211        | JKD6008sRNA073            | 59.06                       | 8                             | -7.38       |
| SAUSA300s287        | tsr21                     | 970.48                      | 133.71                        | -7.26       |
| SAUSA300s054        | RsaH                      | 4,110.36                    | 629.99                        | -6.52       |
| SAUSA300s087        | Teg41                     | 481.01                      | 74.55                         | -6.45       |
| SAUSA300s078        | rsaOT                     | 17,890.11                   | 2,790.22                      | -6.41       |
| SAUSA300s127        | ssr54                     | 3,245.19                    | 584.15                        | -5.56       |
| SAUSA300s051        | RsaE                      | 2,929.20                    | 534.76                        | -5.48       |
| SAUSA300s074        | rsaOl                     | 721.93                      | 135.52                        | -5.33       |
| SAUSA300s237        | JKD6008sRNA258            | 100.89                      | 20.87                         | -4.83       |
| SAUSA300s298        | tsr34                     | 200.17                      | 41.54                         | -4.82       |
| SAUSA300s267        | tsr3                      | 85.01                       | 19.21                         | -4.43       |
| SAUSA300s114        | sbrE                      | 424.58                      | 102.21                        | -4.15       |
| SAUSA300s073        | Sau-6072                  | 265.82                      | 64.27                         | -4.14       |
| SAUSA300s276        | tsr12                     | 295.29                      | 73.2                          | -4.03       |
| SAUSA300s291        | tsr27                     | 135.22                      | 34.48                         | -3.92       |
| SAUSA300s204        | RsaX04                    | 265.58                      | 70.09                         | -3.79       |
| SAUSA300s086        | Teg39                     | 50.39                       | 13.67                         | -3.69       |
| SAUSA300s141        | ssr153                    | 74.69                       | 21.06                         | -3.54       |
| SAUSA300s010        | T-box riboswitch          | 487.82                      | 149.37                        | -3.27       |

* RPKM, reads per kilobase of transcript per million mapped reads.

The data presented above represent the first global transcriptional analysis of sRNA gene expression in the CA-MRSA strain USA300 and demonstrate the utility of the sRNA annotation files constructed in this study. The biological functions of most sRNAs are unknown, making it difficult to ascertain the impact on the bacterial cell of their differential regulation. Studying the variation in sRNA expression in response to changing environmental conditions may provide insight into which sRNAs play a role in the adaptive nature of S. aureus. tsr25 and rsaOG, for example, are upregulated >300-fold in human serum, suggesting a role for these molecules under these conditions. In addition, a number of conserved, well-studied sRNAs were among those differentially regulated in human serum. 4.5S RNA (SAUSA300s005) and ssrS (SAUSA300s026) demonstrated increased expression in human serum (12-fold increase for both). The increased expression of these RNA species may reflect physiological changes to the bacterial cell in this environmental niche (see Discussion).

DISCUSSION

In 1995, the release of the first fully sequenced bacterial genome heralded a new era of bacterial genomic research (21). Over the past 20 years, the number of sequenced bacterial genomes has risen exponentially, and new research strategies, techniques, and applications have emerged to exploit the opportunities that these resources provide. While raw genomic sequence data are valuable, the availability of fully annotated genome sequences, outlining the positions of known genes and genomic features, dramatically increases their utility. Global expression analysis techniques such as...
microarrays and RNA-seq depend heavily on annotated genome sequences as a reference source for genes in the bacterial cell. These techniques have proved extremely useful; however, recently, certain limitations to their application are becoming apparent. A major concern in this regard is that they do not provide expression data for genes that are not included in genome annotation files. Bacterial sRNAs represent a class of genes that are frequently absent from genome annotation files; consequently, their expression is rarely studied on a global level. In this work, we added annotations for 303 sRNA genes to the S. aureus USA300 genome, increasing the number of annotated genes from 2,850 to 3,153 (a 10% increase). Including sRNA gene annotations in S. aureus GenBank files facilitates global expression analysis of these understudied molecules. The 303 newly added annotations undoubtedly do not represent an exhaustive list of the complete S. aureus sRNA repertoire, as it is likely that subsequent studies, using different techniques and environmental conditions, will continue to add to this number. It is also likely that among the 303 annotations, there may be false positives as only 92 of the 303 sRNAs reported and annotated here (30%) have been confirmed by independent experimental methods other than high-throughput sequencing and microarray hybridization. Therefore, while the sRNA annotated GenBank files generated herein may not be definitive, they nonetheless represent a significant step forward and pave the way for future studies that can validate the existence, elucidate the role, and demonstrate the biological impact of these molecules on S. aureus physiology and virulence.

The 303 sRNA genes annotated in this study (representing 10% of all known genes on the S. aureus genome) were identified in S. aureus growing under a limited number of environmental conditions. How many sRNAs remain unidentified is unknown; however, on the basis of the data presented in this study and by others (14), it seems likely that sRNA genes may account for >15 to 20% of all transcripts in the S. aureus cell. Importantly, a benefit of the nomenclature system used in this study to annotate sRNAs (in addition to keeping original names intact) is that it can be expanded upon to accommodate new sRNAs as they are identified.

An unexpected observation arising from this study is the lack of consistency in genome annotation across multiple strains of S. aureus. The examples highlighted in this study (specifically tsr12 and Teg23) demonstrate that this phenomenon occurs even at genomic loci with a high degree of homology (100% identical in the case of tsr12 in strains USA300 and NCTC 8325). Genome annotation is typically performed bioinformatically and is rarely validated and curated (22); therefore, this variation in annotation likely arises because different annotation pipelines have been used to annotate different genomes. This raises the important question of whether certain genomes have been under- and/or overannotated. It is likely that examples of both situations occur; however, on the basis of our data, it appears that the overannotation of genomes (i.e., the inclusion of CDS annotations that are not legitimate mRNA transcripts) is common and could have deleterious consequences for sRNA identification and study. sRNA transcripts that map to genomic regions containing CDS annotations will mistakenly be assumed to encode proteins. Highlighting this point is the data presented in Fig. 3, which strongly suggest that Teg23 is an sRNA and not a protein. Nonetheless, we acknowledge that this type of data, by its very nature, cannot exclude the possibility that such a gene/protein exists, and hence, once a CDS has been annotated, it is difficult or impossible to conclusively prove that it does not exist. Careful attention must be paid to the location of transcript start/stop sites, the existence of ribosome binding sites and the predicted function of annotated genes. We observed many instances of inconsistent genome annotation where a given gene encoded a protein of unknown function with no homologues and no functional domains. This lack of homology may indicate that while sequence analysis alone may suggest that a gene is possible at this locus, it is not certain to exist. The results presented herein are a timely reminder that, although genome annotation files are valuable resources that are increasingly relied upon by next-generation DNA sequencing technology, these annotations should be treated with a reasonable degree of caution and not seen as an infallible reference. Confirmation of the existence of a gene/transcript/protein by traditional biochemical methods (such as 5′ and 3′ rapid amplification of cDNA ends [RACE], primer extension, and in vivo translation) remains essential.

Including sRNA annotations in GenBank files allowed us to perform global sRNA expression analysis by RNA-seq for the first time in the CA-MRSA isolate USA300. The data generated dem-

**FIG 5** Northern blot analysis of serum-regulated sRNAs in strain USA300. (A) Analysis of sRNAs demonstrating upregulation in human serum. RNA-seq read alignment data and Northern blot analysis are shown for SAUSA300s289 (tsr25) and SAUSA300s046 (rsaOG) during growth in TSB (T) or human serum (S). (B) Analysis of RNAIII expression in human serum and TSB. RNA-seq read alignment data are shown for the entire agr locus. Northern blot analysis was performed using an oligonucleotide probe specific for the RNAIII transcript. Annotations for CDS genes are shown by black arrows, annotations for sRNAs are shown by red arrows, and depth of read coverage on the genome is shown by the blue histograms.
onstrated that 83 sRNAs are differentially expressed in TSB versus human serum (Tables 2 and 3). This represents 27% of the known sRNAs in strain USA300. It is not easy to interpret how their differential regulation affects the bacterial cell (because the biological functions of most of them are unknown); however, certain inferences can be made. The newly identified tsr25 sRNA demonstrated a 582-fold increase in expression in human serum. It is tempting to speculate that increased expression of tsr25 in serum suggests that it plays an important role during S. aureus bloodstream infections. A small number of conserved, well-studied sRNAs were also among the differentially regulated sRNAs in serum. 4.55 RNA, a component of the signal recognition particle, was upregulated 12-fold in human serum, perhaps reflecting altered protein secretion and/or protein composition in the cell membrane in this environment. Another important cellular RNA that has been well explored is 6S RNA (ssrS), which we demonstrate also has a 12-fold increase in expression during growth in serum. In Escherichia coli, 6S RNA binds to the housekeeping sigma factor σ^70 in Escherichia coli, and inhibits transcription from σ^70-dependent promoters. It is thought that this diverts RNA polymerase to alternative sigma factors (such as the stress response sigma factor), resulting in increased expression of adaptive and stress-circumventing genes (23). The upregulation of ssrS in human serum suggests that a similar situation occurs in S. aureus, whereby σ^3-dependent genes are downregulated and σ^A-dependent genes are upregulated by the action of 6S RNA.

Examining the global transcriptome can provide valuable insights into bacterial physiology and adaptation to environmental conditions. In the past, global transcriptomic analysis has focused on protein-coding genes, but here, we conduct global transcriptomic analysis and include newly annotated sRNA genes. Like protein-coding genes, sRNA genes display differential regulation that allow bacteria to adapt to environmental changes. The annotation files presented herein, which facilitate this kind of global analysis will prove to be a valuable resource for the future study of sRNAs in S. aureus and will more generally broaden our understanding of regulatory circuits.

**MATERIALS AND METHODS**

**Strains, plasmids, and primers.** Bacterial strains, plasmids, and primers used in this study are listed in Table 4. *S. aureus* and *E. coli* were grown routinely at 37°C with shaking in tryptic soy broth (TSB) and lysogeny broth (LB), respectively. Pooled human serum from anonymous donors, used in this study are listed in Table 4.

**MATERIALS AND METHODS**

**Materials.** Broth (LB), respectively. Pooled human serum from anonymous donors, prepared as follows for analysis will prove to be a valuable resource for the future study of sRNAs in S. aureus and will more generally broaden our understanding of regulatory circuits.

**Northern blots.** Northern blots to identify the size and abundance of sRNAs were performed by the method of Caswell et al. (25). Briefly, 10 μg of total RNA isolated from a 3 h USA300 culture in TSB were loaded on a 10% polyacrylamide gel (7 M urea, 1× Tris-borate-EDTA [TBE]) and separated by gel electrophoresis. The samples were then transferred via electroblotting to an Amersham Hybond N+ membrane (GE Healthcare). The membrane was exposed to UV light to cross-link samples to the membrane. Subsequently, membranes were prehybridized (1 h, 45°C) in ULTRAhyb-Oligo buffer (Ambion) and then incubated (16 h, 45°C) with sRNA-specific oligonucleotide probes end labeled with γ −32P[ATP and T4 polynucleotide kinase (Thermo Scientific). After incubation, membranes were washed with 2×, 1×, and 0.5× SSC buffer (1× SSC is 300 mM sodium chloride and 30 mM sodium citrate) at 45°C for 30 min each to remove unspecific bound probes. Finally, X-ray film was exposed to membranes for sRNA detection.

**Cloning of histidine-tagged Teg23P.** The genomic region containing Teg23P (including its native promoter) was amplified using USA300 genomic DNA and primers OL3222 and OL3223. The reverse primer OL3223, in addition to the gene-specific region, carries a sequence that encodes a hexahistidine (His) tag, which allows the detection of a possible encoded protein via Western blotting. The amplified 761-bp product was cloned into shuttle vector pMK4, and the plasmid was transformed into chemically competent E. coli DH5α. The resulting colonies were screened for correct constructs employing a colony PCR approach using identical primers to those used for the amplification of the initial fragment. After identification of positive clones, the plasmid was verified via Sanger sequencing with the plasmid-specific standard primers M13F (including its native promoter) was amplified using USA300 genomic DNA and primers OL3222 and OL3223. The reverse primer OL3223, in addition to the gene-specific region, carries a sequence that encodes a hexahistidine (His) tag, which allows the detection of a possible encoded protein via Western blotting. The amplified 761-bp product was cloned into shuttle vector pMK4, and the plasmid was transformed into chemically competent E. coli DH5α. The resulting colonies were screened for correct constructs employing a colony PCR approach using identical primers to those used for the amplification of the initial fragment. After identification of positive clones, the plasmid was verified via Sanger sequencing with the plasmid-specific standard primers M13Fw (Fw stands for forward) and M13Rv (Rv stands for reverse) (Eurofins MWG Operon). This construct was then transformed into S. aureus RN4220 and confirmed via PCR. Finally, the plasmid was transduced into S. aureus USA300 using a ph1 plage lysate, and after final confirmation of the construct using PCR, the strain was utilized for subsequent analysis.

**qRT-PCR.** Quantitative reverse transcriptase PCR (qRT-PCR) was conducted as described by our research group previously (26), employing the teg23-specific primers OL3281, OL3282, OL3232, and OL3282 and RNA isolated from S. aureus USA300 cultures grown in TSB as described above for RNA-seq experiments. As a reference, 16S rRNA was amplified using primers OL1184 and OL1185 (27). All experiments were performed in triplicate.

**Western blots.** The evaluation of Teg23 protein abundance was performed by Western immunoblotting as described previously (28). USA300 cells harboring the His^-tagged version of Teg23P were grown for 3 h in TSB, before they were pelleted and their cytoplasmic proteins were isolated. Following SDS-polyacrylamide gel electrophoresis and transfer of the separated proteins to a polyvinylidene difluoride (PVDF) membrane, detection was performed using anti-His monoclonal mouse antibody (Covance) and horseradish peroxidase (HRP)-conjugated anti-
TABLE 4  Bacterial strains, plasmids, and primers used in this study

| Bacterial strain, plasmid, or primer | Characteristic(s) or sequence | Reference or source | Comment |
|-------------------------------------|-----------------------------|-------------------|---------|
| **S. aureus** strains               |                             |                   |         |
| USA300 Houston                     | Community-associated MRSA clinical isolate | 26                |         |
| SH1000                              | Laboratory strain; rsbU functional | 29                |         |
| UAMS-1                              | Osteomyelitis clinical isolate | 30                |         |
| RN4220                              | Restriction-deficient transformation recipient | 31                |         |
| AW2192                              | USA300 pMK4::teg23P-his6<sup>6</sup> | This study        |         |
|                                        |                             |                   |         |
| **E. coli** strains                 |                             |                   |         |
| DH5α                                | Routine cloning strain      | Invitrogen        |         |
| **Plasmids**                        |                             |                   |         |
| pMK4                                 | Shuttle vector; Cm<sup>R</sup> | 32                |         |
| pAW105                               | pMK4::teg23P-his6<sup>6</sup> | This study        |         |
| **Primers**                          |                             |                   |         |
| OL1184                               | 5′ TGTGAGCGGTTCCTCAGTTCC 3′  | 27                |         |
| OL1185                               | 5′ AGCCGACCTGAGGTTGA 3′      | 27                |         |
| OL2701                               | 5′ CCAATTAGGACATGTCATCGG 3′  |                   |         |
| OL3201                               | 5′ GTACTCCATTTCTACGACAATTGCA 3′ |                   |         |
| OL3208                               | 5′ ACCGGGTATAAAAAGGGGAAATTTG 3′ |                   |         |
| OL0121                               | 5′ CTCAACAATTTGCTAGGGG 3′    |                   |         |
| OL3216                               | 5′ TCAATAATCTGAAGGGGACGCTAT 3′ |                   |         |
| OL3217                               | 5′ TTAGCTCAATGCTGATATTAAAAT 3′ |                   |         |
| OL3222                               | 5′ ACGGCACGACCCTGTCTGACGCG 3′ |                   |         |
| OL3223                               | 5′ CGGGATCTTGGTGTTGGTGTTGGC 3′ |                   |         |
| OL3224                               | 5′ CGCCCAAGGTTCACAGGC 3′     |                   |         |
| OL3281                               | 5′ TAAACAACATACAGGCCATTG 3′  |                   |         |
| OL3282                               | 5′ GAGATTTGAGGACGATAT 3′     |                   |         |
| OL3880                               | 5′ GCCAGGATAATGTATCTTAA 3′   |                   |         |
| OL3882                               | 5′ CCATTAATTTACTACAAAGGG 3′  |                   |         |
| OL3883                               | 5′ GCTTCGTGTTGATCTC 3′       |                   |         |
| OL3886                               | 5′ CAGCTCTCTGTGATACAC 3′     |                   |         |
| OL3916                               | 5′ CATAACCTCTTTAACAACAG 3′   |                   |         |
| OL3917                               | 5′ GGAGGATAATCTCATGCTC 3′    |                   |         |
| OL3935                               | 5′ GGAGGAATCAAACACA 3′       |                   |         |
| OL3937                               | 5′ GTCTCGCCATTAAAAATAC 3′    |                   |         |
| OL3946                               | 5′ GTCCTTTACAAACGCAACG 3′    |                   |         |
| OL3948                               | 5′ GGTTTTATCTTTGGAAGAAGG 3′  |                   |         |
| OL3956                               | 5′ GATCCGGGAAAATTTTG 3′      |                   |         |
| OL3957                               | 5′ GTGCCGAAATATATTG 3′       |                   |         |

<sup>a</sup> F, forward; R, reverse.

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Accession numbers. The GenBank files generated have been deposited in figshare (https://dx.doi.org/10.6084/m9.figshare.2061132.v1). The files are provided in .gkb format and can be viewed using a variety of genome browser software (examples of freely available genome browsers include Artemis, Genome Compiler, and CLC Sequence Viewer). The RNA-seq data files have been deposited in GEO under accession number GSE74936. Newly identified sRNAs (i.e., the tsr sRNAs) have been deposited in Genbank under accession numbers KU639719-KU639757 for SAUSA300s265-USAUSA300s303, KU639758-KU639789 for USAUHSCs255-USAUHSCs286, and KU639790-KU639818 for SARs249-SARs277.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01990-15/-/DCSupplemental.

Text S1, DOCX file, 0.2 MB.
Figure S1, TIF file, 2 MB.
Figure S2, TIF file, 0.5 MB.
Figure S3, TIF file, 0.4 MB.

Figure S4, TIF file, 0.5 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, XLSX file, 0.1 MB.
Table S3, XLSX file, 0.1 MB.
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